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PhD Thesis

iPSC-based strategy to correct the bleeding phenotype in Hemophilia A

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Summary

Hemophilia A (HA) is an X-linked bleeding disorder caused by mutations in the coagulation factor VIII (FVIII) gene. The current therapy is based on the administration of recombinant or plasma-derived FVIII. However, this treatment is not a definitive cure and the 20-40% of patients develops neutralizing antibodies. In this context, gene and cell therapy represent attractive alternative therapeutic strategies. Reprogramming of somatic cells can be used to generate induced Pluripotent Stem Cells (iPSCs), which can be differentiated into progenitor cells relevant for gene and cell therapy applications. iPSCs offer the potential to generate patient-specific cells, reducing the risk of immune rejection by offering an autologous source of cells for transplantation. Moreover, for monogenic disease treatment iPSCs technology could be associated with gene therapy allowing the genetic correction of the disease defect. Since Yamanaka's pivotal work, iPSCs were generated starting from several cell types. Human iPSCs are most frequently derived from dermal fibroblasts because of their accessibility and relatively high reprogramming efficiency. However, in hemophilic patients, to harvest fibroblasts from skin biopsies is dangerous because of the risk of bleeding. In this context, we utilized patient-derived peripheral blood cells as an easy-to-access source of cells to generate iPSCs. Then we genetically corrected and differentiated them into functional endothelial cells (ECs) secreting FVIII. Reprogrammed healthy and hemophilic CD34+ cells gave rise to *bona fide* iPSCs colonies showing embryonic stem cells-like morphology. iPSCs were positive at alkaline phosphates staining, expressed both nuclear and surface pluripotency markers at RNA and protein levels. Moreover, the CpG islands at iPSCs NANOG core promoter showed a demethylated profile, confirming that obtained colonies were well reprogrammed at morphological, transcriptional and epigenetic levels. iPSCs-derived embryoid bodies expressed the three germ layers markers and were able to differentiate in adipose, osteogenic and chondrogenic cells. Then, both healthy and hemophilic iPSCs were differentiated into ECs. The obtained ECs expressed endothelial specific markers (KDR, Tie2, CD31, VEC) and formed tubules when cultured in matrigel. HA-iPSCs corrected by transduction with a lentiviral vector carrying the human B domain deleted form of FVIII (BDD-FVIII) under the endothelial specific promoter of VE-cadherin efficiently expressed FVIII after differentiation. Moreover, when transplanted by portal vein injection in the liver of our immunocompromised mouse model of HA, corrected HA-ECs were able to engrafted, proliferate and functionally secreted FVIII up to 12 weeks post transplantation. Therapeutic correction was also reached when corrected ECs were injected in association with microcarrier beads in the peritoneum of the mice. Together these data showed the possibility to obtained functionally FVIII secreting endothelial cells, starting from patient specific gene corrected iPSCs confirming the suitability of this approach for HA gene-cell-therapy.

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Introduction

Hemophilia A and coagulation factor VIII

Hemophilia A (HA) is a recessive X-linked bleeding disorder that occurs in 1:5000 male live births. It is due to the lack or reduced activity of coagulation factor VIII (FVIII) [1-3], 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 generating clot formation (Fig. 1).

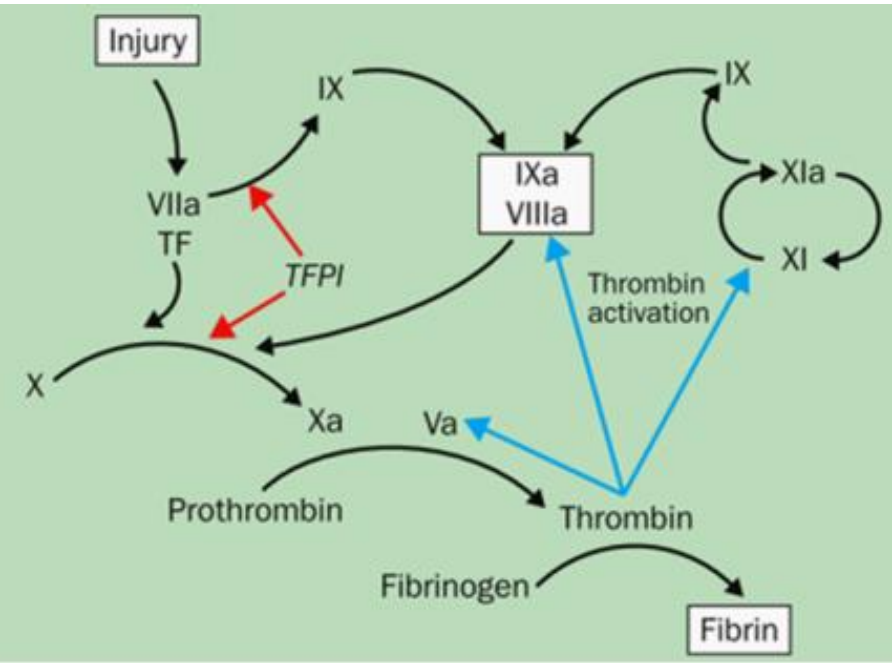

Figure 1. Schematic representation of coagulation cascade. Activated FVIII binds FIX forming a complex activating FX and converging in the common pathway of coagulation cascade.

The reduction of FVIII in the plasma causes hemophilic patients to have a lifelong bleeding tendency of clinical severity proportional to the degree of FVIII reduction. Indeed, based on the residual FVIII activity, there are three forms of HA: 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 [1]. Several mutations in coagulation FVIII gene (F8) cause hemophilia A. 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) [4]. Other mutations causing the severe form are small deletions or insertions, missense and non-sense mutations that taken together occur in 45% of severe HA patients. Less frequent

mutations are large deletions, splice site mutations and intron 1 inversion [5]. In contrast, missense mutations are mainly associated with moderate and mild form [2]. Disease clinical manifestations range from spontaneous bleeding, with frequent haemarthroses in the most severe form, to secondary bleeding with rare haemarthroses in milder form [4]. Diagnosis is made based on 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) [1]. The main diagnostic technique used in laboratory is the evaluation of activated partial thromboplastin time (aPTT), which estimates the ability of patients 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 assay, chromogenic assay and genetic investigation may be more informative [6]. Moreover, the analysis of F8 mutation is useful to further characterize the gene defect and the risk of inhibitor development, one of the major concern of hemophilia A actual treatment [7]. It was reported that patients who had severe hemophilia A and mutations predicting a null allele developed inhibitors more frequently (22% to 67%) than patients with missense mutations (5%) [8]. The current therapeutic practice consists in the administration of recombinant FVIII or blood products and is carried out based on the disease severity. Patients with severe hemophilia receive prophylaxis 2-3 times a week, while patients with mild and moderate form receive replacement therapy on demand [9]. 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 prophylaxis means the intention of treating for 52 weeks/year and at least 45 weeks/year (85%) [10]. Patients receive 25-40 IU kg-1 per dose administered intravenously three times a week [10].

The main complication 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 and expose patients to an increased risk of morbidity and mortality [11]. The treatment of patients with inhibitors is more problematic and the therapeutic strategies are mainly focused on the treatment or prevention of bleeding episodes using agents that bypass the inhibitor (e.g. plasma-derived activated prothrombin complex concentrates and recombinant activated FVII). Eradication of the inhibitor through long-term intensive treatment with large doses of FVIII (immune tolerance induction; ITI) is effective in approximately two third of cases. Despite the good outcomes the high costs of ITI reduced its feasibility to few patients

requiring the development of other strategies such as blockade of costimulation, oral tolerance, immunosuppressive treatment and antigen-specific regulatory T cell [12].

F8 was firstly characterized and cloned in 1984 [13]. F8 maps to band Zq28 at the tip of the long arm of the X chromosome. 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 [14]. FVIII is synthesized as an inactive single chain and it 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 [15] (Fig. 2).

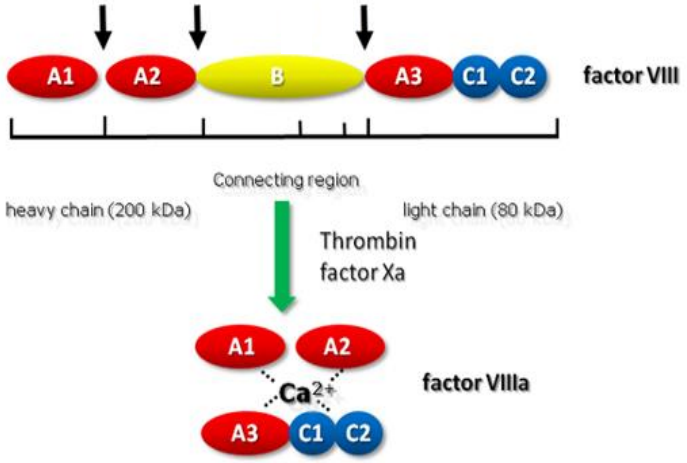

Figure 2. Domain structure and processing of FVIII.FVIII is organized in 6 domains: A1, A2, B, A3, C1, C2. After cleavage at the C-terminal region of B domain, FVIII consists in a heterodimer formed by the heavy chain and the light chain linked by a divalent metal ion between the A1 and A3 domains.

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 [16]. Moreover C domain share some homologies between FVIII, FV and proteins that bind negatively charged phospholipids (e.g., fat globular protein and the lipid-binding lectin discoidin I) [17, 18]. In contrast, FVIII B domain is unique and do not show significant similarity with FV or other proteins [19]. 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 asparagines 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 [20]. 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) [19]. Defects in these molecules cause the combined deficiency of circulating FVIII and FV, which share in part the same intracellular processing of FVIII [21]. 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 [22]. 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 [23]. 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 [24]. 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 [24]. The FVIII B domain (40% of the protein) is not essential for hemostatic cofactor activity. Indeed, early biochemical studies demonstrated that deletion of the B domain resulted in a functional molecule [25]. This observation was used to develop a shorter version of FVIII, called B-domain-deleted (BDD)-FVIII, characterized by the removal of most of the B domain, with only 14 residual amino acids. The BDD-FVIII was expressed more efficiently compared with wild-type FVIII resulting in a 17-fold increase in mRNA levels over full-length FVIII and in a concomitant increase in the amount of synthesized primary translation product [25]. Moreover, no immunologic differences were detected between BDD- and wild-type FVIII [26, 27]. The first generation of these recombinant FVIII products was synthesized by gene-transfection in mammalian cells (Chinese Hamster Ovary cells) [27]. Currently, it is produced in absence of any animal or human proteins reaching the highest level of safety and it is extensively used in clinics [26]. Although FVIII mRNA is detected in different human and mouse organs such as liver, spleen, lymph nodes, kidney [28-31] and hematopoietic cells [32, 33], transplantation studies in hemophilic animal models and patients demonstrated that liver is the primary source of FVIII [34, 35]. However, the identity of liver cells expressing FVIII was controversial. Both hepatocytes and liver sinusoidal endothelial cells (LSECs)

were proposed to be the main sources of FVIII but their role in FVIII production is still debated. 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 [28, 36-38]. Although in early years the presence of FVIII was reported mainly in LSECs rather than hepatocytes. Several studies reported the presence of the FVIII antigen in LSECs, but not in the parenchyma of adult human liver [39-41]. Subsequently, Kumaran et al. showed that hemophilia A mice transplanted with unfractionated liver cells, (mixture of hepatocytes, LSECs, Kupffer cells, and hepatic stellate cells) or of the cell fraction enriched in LSEC survived a bleeding injury challenge, whereas purified hepatocytes transplantation did not prevent deadly bleeding[42]. Then, it was reported that the only cells producing FVIII mRNA and protein in the fetal and adult human liver are LSECs [43, 44]. Recent works 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 produced in hepatocytes but mainly secreted by EC [45, 46]. Fahs et al. used promoters to target defects in endothelial cells and hepatocytes and demonstrated that a severe hemophilic phenotype was associated with lack of FVIII expression in endothelial cells [46]. Likewise, Everett et al. reached the same conclusion knocking out, in hepatocytes or LSEC, Lman1, a cargo receptor in the early secretory pathway that is responsible for the efficient secretion of FVIII to the plasma [45].All together these studies pointed out LSECs as the main source of FVIII in the liver, bringing them to the forefront in the design of alternative therapeutic approach for hemophilia A, such as gene and cell therapy. Gene and cell therapy approaches could allow the continuous FVIII production and secretion in the blood stream achieving sustained levels of FVIII rather than the peaks in circulation of the replacement therapy due to the short half-life of infused FVIII protein [43].

Gene and cell therapy for hemophilia A

Gene and cell therapy could constitute a powerful therapeutic approach for many pathologies, in particular for monogenic diseases. They can be applied alone or combined depending on therapeutic needed (Fig.3).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.

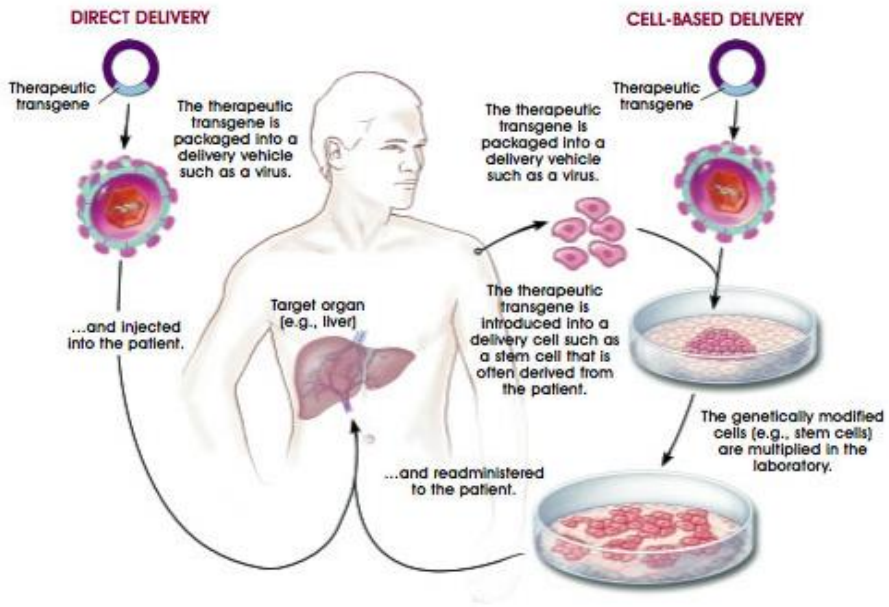

Figure 3: Strategy of delivery of a therapeutic transgene in "pure" gene therapy (direct delivery) or in a combined cell-gene therapy strategy. In gene therapy the therapeutic transgene packaged into a delivery vehicle was directly injected into the patient. When gene therapy was combined to cell therapy, the transgene in inserted into delivery cells expanded in culture and then re-infused into the patient. (Terese Winslow2001)

Hemophilia A represent an ideal target for gene therapy since it is a monogenic disease and to restore FVIII activity al level superior than 2% is sufficient to ameliorate the bleeding phenotypes of patients with an overall increase of quality of life. Good clinical trials results were already obtained from Hemophilia B gene therapy using adeno associated-viral vector (AAV) to deliver FIX into the patients [47]. In particular, the best results were obtained in a clinical trial using an AAV pseudotyped with AAV8 capsid protein. It has been reported that the AAV8 capsid has stronger liver tropism and lower seroprevalence in human, in comparison with the AAV2 used in the previous trial. Moreover, it provide less virus uptake by antigen presenting cells and is able to mediate effective transduction in animals with pre-existing immunity to AAV2 [48]. Regarding the expression cassette, an artificial liver specific promoter (LSP), based on the hAAT promoter and an ApoE enhancer, was used to drive the expression of a codon optimized FIX cDNA [49]. 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 [47]. Nevertheless, this clinical trial showed the feasibility of gene therapy for hemophilia B, increasing the quality of patients' life 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 [50];iii) FVIII is naturally 5-6 fold more immunogenic than FIX, making the transgene mediated immune response a big concern. Moreover, the AAV-FIX clinical trials detected an anti-AAV capsid immune response at high vector doses suggesting a limit to the administered vector dose [47]. This limit is very challenging for FVIII gene therapy. Indeed, it is established that the necessary dose to achieve therapeutic levels of FVIII are higher than for FIX [51]. However, 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 [52, 53]. Nevertheless, 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, reducing the efficiency of the strategy and increasing the overall dose of vector to use. Despite these difficulties other studies reported that delivering the heavy and the light chains in two separate AAV resulted in a dose-response with sustained expression of FVIII at therapeutic levels both in dog and mouse models of hemophilia A [54, 55]. Another option to overcome the size limit of AAV vectors was to use a B domain deleted FVIII (BDD-FVIII) that reduced by one third the final size of cDNA without compromising the coagulation biological activity of the protein. Even though a minimum promoter is required to not exceed the package capacity of AAV. By this attempt, several studies have showed sustained FVIII levels in mice [56, 57]. However, they reported in most of treated mice anti-FVIII antibodies formation that was overcome by using the serotype AAV8 instead the AAV1 [57]. AAV vectors containing the BDD-FVIII have demonstrated to be suitable to induce FVIII expression also in hemophilic dogs [58-61]. 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 and given the dose-dependence of immune response to capsid, the use of this vector could be not feasible in humans [51]. 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 [62]. However, the macaques were injected with a non-species specific transgene developing anti-FVIII antibodies requiring transient immunosuppression to reduce anti-FVIII antibodies titer, thus the long term follow up was not possible [62]. Among the other viral vectors lentiviral and retroviral were more feasible to be 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 [63]. This approach was also successful in a canine model of hemophilia A by targeting transgene expression in the liver without antibodies formation [64]. However, when retrovirus was used for human gene therapy only low circulating FVIII was detected in patients [65, 66]. In 2000, Park and colleagues demonstrated that also LV could be use *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 (EF1α) 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 [67]. 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 [68]. Notably, the ubiquitous transgene expression mediated by LV may be detrimental because of the adverse reaction given by proteins ectopically expressed [69] or the innate or adaptive immune response against the transgene once it is expressed by antigen presenting cells (APC) [70, 71]. To overcome this issues the use of cell specific promoter to de-targeted transgene expression in APC allowed the sustained expression of therapeutic gene in the selected cells by reducing immune response [72, 73]. Over the years many authors have used several tissue specific promoters to drive transgene expression in cells of interest including endothelial cells, hepatocytes, dendritic cells, hematopoietic stem cells, megakaryocytes, B cells [74-76]. Moreover, another degree of cell targeting is represented by the post-transcriptional regulation based on micro RNAs (miRNAs). 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. For example, in 2007, Brown and colleagues showed that the presence of miRT142-3p,complementary to miRNA 142-3p which is selectively expressed in hematopoietic cells avoided transgene expression (FIX) in APC limiting immune response against FIX by preventing transgene expression in those cells allowing FIX expression only in hepatocytes using a liver specific promoter [77].Despite its advantages, “pure” gene therapy approaches face some challenges in hemophilia A treatment. In light of these findings, an approach combining gene and cell therapy deserves further consideration to determine if an effective and safe cell-based treatment can be developed. Cell therapy consists in

the transplantation in a patient of cells from external sources in order to achieve the treatment of a specific disease. For genetic disease, cell therapy approaches involving the gene transfer of the correcting gene into the cells to be transplant can represent an alternative strategy to gene therapy alone. To design a cell-based therapy approach it is fundamental to identify the best cell sources to treat the disease (Fig. 4). To cure hemophilia A cell sources could include cells competent for FVIII production, such as liver sinusoidal endothelial cells (LSECs), stem cells derived from bone marrow, blood-outgrowth endothelial cells (BOECs), endothelial progenitor cells derived from the differentiation of induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). Transplantation of LSECs was demonstrated in several animal models to be able to correct hemophilia A bleeding phenotype [42, 43, 78]. Several liver-derived cells were studied to be applied in the therapy of hemophilia A.

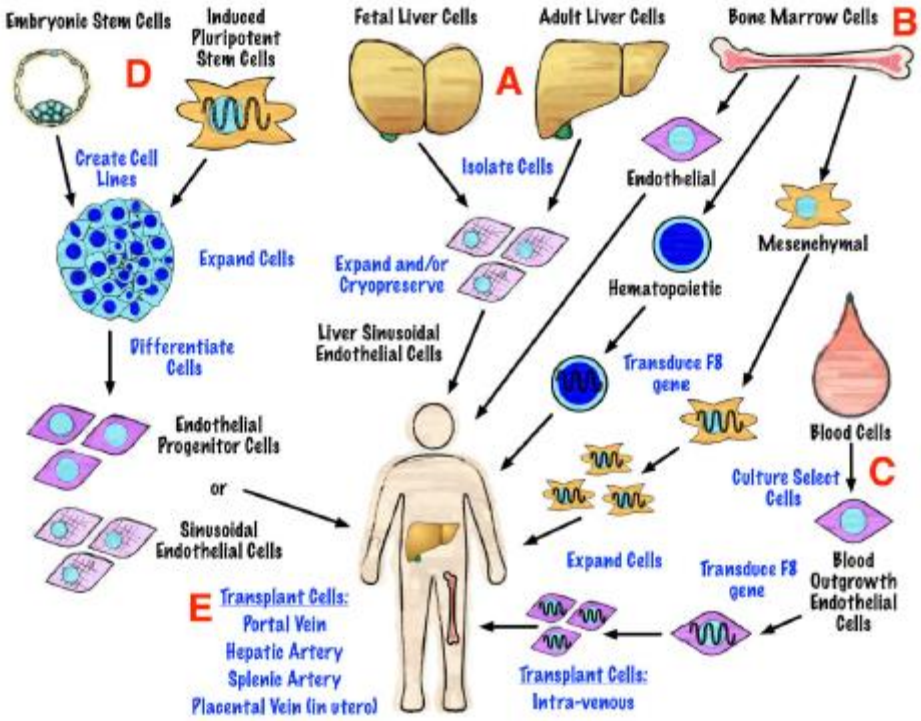

Figure 4. Potential cell sources for hemophilia A treatment [79].

In mice, in 2005, it was demonstrated that the presence of endothelial cells in different liver-cell mixtures was fundamental to correct clotting dysfunction in hemophilic transplanted mice [42]. Subsequently, Follenzi et al. transplanted purified mature LSECs into the portal vein of hemophilic mouse model and demonstrated the engraftment in the liver and the therapeutic correction of hemophilia A with plasma FVIII activity more than 10% of normal plasma levels [78]. Additionally, successful transplantation of human LSECs isolated from adult liver was recently reported [43, 80]. Filali et al. isolated liver endothelial cells and transplant them into monocrotaline-

treated immunodeficient mice yielding LSEC engraftment. Interestingly, although culture conditions caused loss of LSEC fenestration, normal phenotype was re-acquired after transplantation, pointing out the role of microenvironmental signals in supporting some of the unique features of the LSEC phenotype. Another important observation was that macrovascular endothelial cells obtained from human umbilical vein and microvascular endothelial cells obtained from adult adipose tissue failed to repopulate liver. These findings indicate irreversible differentiation of these endothelial cell compartments that cannot be overcome by the signals provided by the liver environment [80]. Accordingly, cellular therapy for hemophilia A could need to rely on LSECs or immature endothelial progenitors as sources of transplantable cells. Cell therapy with adult LSECs is limited by access to living donor tissue or cadaverous livers. Cryopreservation could be used to bank adult liver cells as LSECs were shown to engraft into uPA-NOG mice liver after thawing [43]. *Ex vivo* expansion of LSECs could be used to increase the number of adult LSECs available for transplantation, but the effects of *in vitro* culture on the viability, proliferation and function of these cells needs to be further evaluated. Due to the restricted proliferative capacity of adult cells, an immortalized cell line was recently developed from adult human LSECs using lentiviral transduction with Htert [81]. These cells expressed some LSEC specific markers and demonstrated endocytic properties. However, the safety and potential of this cell line to engraft and produce FVIII remains to be tested.

An alternative cell source is represented by BOECs, clinically-promising peripheral blood-derived cell type. Most of the freshly isolated BOECs originate from vascular walls, however 5% are estimated to come from the bone marrow [82]. These bone marrow precursors have a much higher proliferative potential and are responsible for the majority of cell outgrowth in culture and are, thus, referred to as BOECs. When intravenously injected, BOECs primarily engrafted the spleen and bone marrow. Moreover, BOECs transfected with FVIII and transplanted into NOD/SCID mice were able to secrete human FVIII into the plasma [83]. FVIII-transduced BOECs have also been grown into sheets and then transplanted subcutaneously resulting in partial correction of the disease phenotype in HA mice [84].However, BOECs are present at a low frequency in adult peripheral blood and can be limited expanded in culture (PD=20;[85]). Thus, other stem cell and progenitor populations were evaluated for their potential for *in vivo* FVIII production either by acting as carrier cells for ectopic FVIII expression or differentiation into LSECs after transplantation. For instance, rat bone marrow-derived CD133+CD45+CD31+ cells have been shown to differentiate into LSECs after transplantation [86-88], but the potential of these cells as a source of FVIII still remains to be evaluated. Even though LSECs and stem cells derived from various adult and fetal sources may play a great role in the therapy of hemophilia A, there are

potential limitations that may hinder their clinical utility such as: i) lack of availability of a suitable donor tissue; ii) low yield of LSECs from liver homogenates, iii) limited expansion potential of stem cells or LSECs due to loss of functional or proliferative capacity *in vitro*. The extent of these potential limitations is not fully known and requires further studies. Nonetheless, to provide a sufficient number of cells to treat hemophilia A, investigation into alternative LSECs sources is prudent. The discovery of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) has revolutionized the study of regenerative medicine due to their extensive potential for self-renewal, growth and pluripotency allowing creation of cells belonging to all three primary germ layers and, thus, including LSECs. Wang et al. showed that mouse chimeras created by injecting ESCs into FVIII-deficient mouse blastocysts were able to correct the bleeding disorder as FVIII was stably expressed in chimeras [89]. Although this study offers a basic proof of principle that ESCs can differentiate into FVIII-producing cells, much work is required to optimize the culture conditions required to generate transplantable and functional FVIII-producing cells such as LSECs or their precursors from pluripotent stem cells. There are also safety concerns that must be addressed, such as the risk of teratoma formation or genetic mutation of the extensively cultured and selected cells, before pluripotent stem cells can be considered for human transplantation. Although ESCs are viewed as the gold-standard of pluripotent stem cells, many current studies focus on iPSCs as they offer several advantages over ESCs. In particular, iPSCs offer the potential to generate patient-specific cells, reducing the risk of immune rejection by offering an autologous source of cells for transplantation.

Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells are ESC-like cells obtained by the reprogramming of adult somatic cells. In 2006, Takahashi and Yamanaka demonstrated that the introduction into mature cells of transcription factors relevant for the maintenance of ESCs identity could induce pluripotency. In particular, they demonstrated that the ectopic expression of the transcription factors Oct4, Sox2, Klf4 and c-Myc was sufficient to revert the phenotype of mouse and human somatic cells to a pluripotent state [90, 91]. Oct4, also known as Pou5f1, belongs to the Octamer binding protein family. In humans, the OCT4 gene generate three isoforms but in most reports Oct4 mainly refers to OCT4A that has been found to maintain stemness in pluripotent stem cells [92]. Oct4 null embryos die in uterus during the peri-implantation stages of development [93, 94]. Although these embryos are able to reach the blastocyststage, in vitro culture of the inner cells mass (ICM) of homozygous mutantblastocysts produces only trophoblast lineages. ESCs cannot be derived from Oct4 null blastocysts. Suppression of Oct4 resulted in spontaneous differentiation into the

trophoblast lineages in both mouse [95] and human ESCs [96]. Oct4 acts in concert with other regulatory factors as Sox2 and Nanog (Fig. 5). Sox2 is a part of family DNA binding protein known as sex-determining region Y (SRY) related high mobility group (HMG-box) proteins expressed in ESCs [97]. Like Oct4, Sox2 marks the pluripotent lineage of the early mouse embryo; it is expressed in the ICM, epiblast, and germ cells. Sox2 null embryos die at the time of implantation due to a failure of epiblast (primitive ectoderm) development [98]. Homozygous mutant blastocysts appear morphologically normal, but undifferentiated cells fail to proliferate when blastocysts are cultured *in vitro*, and only trophectoderm and primitive endoderm-like cells are produced. Sox2 forms a heterodimer with Oct4 and synergistically regulates Fgf4 [97], UTF1 [99], and Fbx15 [100]. In addition, similar co-regulation by Sox2 and Oct4 has been reported in the regulation of the two transcription factors themselves [99, 101, 102], as well as Nanog[103, 104]. Genome-wide chromatin immunoprecipitation analyses demonstrated that Oct4, Sox2, and Nanog share many target genes in both mouse and human ESCs [105, 106]. Surprisingly,Sox2 deletion in mouse ESCs is rescued by the cDNA introduction of not only Sox2 but also Oct4, suggesting that one of the primary functions of Sox2 might be to maintain Oct4 expression [107]. Klf4 or Krüppel like factor 4 is a zinc finger transcription factor that regulates cell proliferation and differentiation [108]. Klf4 is highly expressed in undifferentiated mouse ESCs [109]. Klf4 can function both as a tumor suppressor and an oncogene. In cultured cells, the forced expression ofKlf4 results in the inhibition of DNA synthesis and cell cycle progression [110, 111].Klf4 null embryos develop normally, but newborn mice die within 15 hours and show an impaired differentiation in the skin [112] and in the colon [113], thus indicating that it plays a crucial role as a switch from proliferation to differentiation. KLF4 interacts as transactivator with Oct4-Sox2 in the synergistic activation of Nanog [114, 115]. Nanog is a homeodomain transcription factor expressed in pluripotent cell lines and in the ICM and maintain undifferentiated state by inhibiting and regulating the activity of pro-differentiation Bone Morphogenic Protein (BMP) [116, 117]. Nanog have been shown to be dispensable for reprogramming, indeed its expression can be triggered by the exogenous introduction of Sox2 and Oct4 that tightly regulate Nanog transcription. However, since 2006, several transcription factors cocktail were used to reprogramming somatic cells involving also Nanog and Lin 28 [118, 119], to avoid the use of c-Myc. It is a proto-oncogene required for cell growth and proliferation and in stem cell maintenance [120]. Although it improves the efficiency of the reprogramming process, the removal of c-Myc from the reprogramming factors paved the way for the development of a safer system [121] to generate iPSCs.

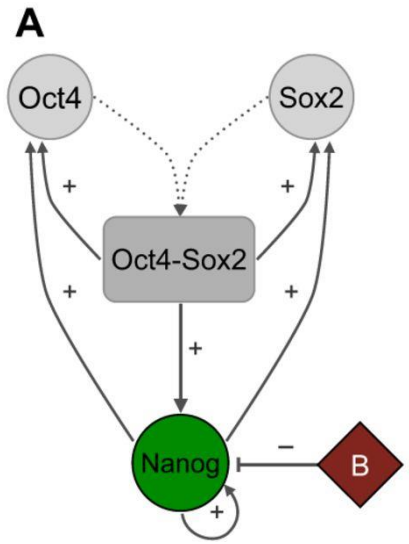

Figure 5: Core network of pluripotency regulation. Oct4 and Sox2 form a heterodimer (dotted lines) that activates Oct4, Sox2 and Nanog transcription. Nanog regulates its own transcription and activates Oct4 and Sox2. An external signal, termed B, suppresses Nanog expression[122].

The established iPSCs showed ESCs-like phenotype, could differentiate into cell types of all three germ layers and possessed the ability of unlimited self-renewal in all cell types (Fig. 6). iPSCs grow on feeders, exhibit high nucleus to cytoplasm ratio, large nucleoli and form compact and uniform colonies with well-defined borders [123]. They show the same proliferation rate and feeder dependence of ESCs. Colonies stain positive for alkaline phosphatase (AP). iPSCs express pluripotency markers such as surface markers (SSEA-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81) as well transcription factors Oct4, Sox2, Klf4 and Nanog at comparable levels to those of ESCs. iPSCs have high telomerase activities, that is typical of pluripotent cells and fundamental to prevent cells aging [124]. Moreover, iPSCs remodel their epigenome during the reversion of differentiated cells into a stem-like state [125]. For example, they show unmethylated state of CpG islands at core promoter of pluripotent cells specific transcription factors, such as Oct4 and Nanog. Finally, iPSCs could differentiate into cell types of all three germ layers (ectoderm, mesoderm and endoderm). This ability can be assessed in vivo by teratomas formation assay [126] and *in vitro* evaluating the embryoid bodies (EB) formation capability and then the expression of the three germ layers markers. Although teratoma formations is one of the assay to be done for a complete characterization, iPSCs that form teratomas might not be the best choice for *in vivo* use [127, 128].

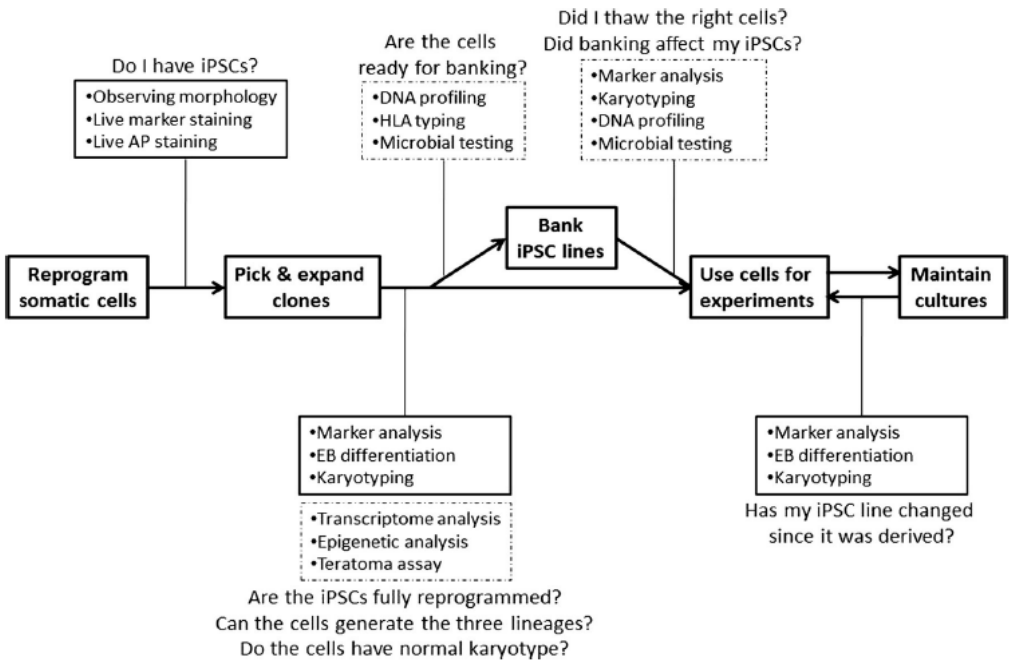

Figure 6: Scheme of iPSCs characterization to assess they acquired ESC-like phenotype, gene expression and epigenetic patterns. Common characterization studies are found in solid boxes, while less common in dotted boxes [129].

Generation of iPSCs

The generation of patient-specific iPSCs is a critical step in cell therapy and other clinical applications. Since Yamanaka pivotal work, scientists investigated several strategies to generate iPSCs, considering several cells sources for the reprogramming and a variety of methods for the reprogramming factors delivery in cells. Started from fibroblasts iPSCs were obtained from several cell types: keratinocytes [130], melanocytes [119], hepatocytes [131], gastric epithelial cells [132], neural stem cells [133], adipose stem cells [134], pancreatic cells [135], cord blood cells [136], blood progenitor cells [137, 138], peripheral blood cells [139, 140] (Table 1).

Human iPSCs are most frequently derived from dermal fibroblasts because of their accessibility and relatively high reprogramming efficiency. However, to obtain fibroblasts to be reprogrammed skin biopsy and a prolonged period of cells expansion in culture are required. It cannot be ignored that patients would experience the pain and the risk of infection when obtaining dermal fibroblasts. These issues limit the application of iPSCs, in particular for patients with coagulopathies that risk uncontrolled bleeding in consequence of biopsy. In this perspective, blood cells are most easily accessible source of patient's tissues for reprogramming because it is not need to maintain cell cultures extensively prior to reprogramming experiments. Furthermore, the venipuncture is safer than skin biopsy. In 2008, Hanna et al. used four retroviral vectors carrying

Species	Cell type	Factor or chemical	Vector	Reference
Mouse	Fibroblast	OKSM or OKS	Retrovirus	Takahashi and Yamanaka, 2006; Nakagawa *et al*., 2008; Wernig *et al*., 2008
	Fibroblast	OSE or KSNr	Retrovirus	Feng *et al*., 2009; Heng *et al*., 2010
	Fibroblast	mir302/367 cluster	Lentivirus	Anokye-Danso *et al*., 2011
	Fibroblast	OKSM	PB transposon and 2A peptides	Kaji *et al*., 2009
	Fibroblast	Proteins (OKSM)	Poly-arginine	Zhou *et al*., 2009
	Fibroblast	OKSM	Plasmid or adenovirus	Okita *et al*., 2008; Stadtfeld *et al*., 2008a
	Dermal papilla	OKM or OK	Retrovirus	Tsai *et al*., 2010
	Melanocyte	OKM	Drug-inducible lentivirus	Utikal *et al*., 2009
	Mature B and T cell	OKSM	Retrovirus	Eminli *et al*., 2009
	Myeloid progenitor	OKSM	Retrovirus	Eminli *et al*., 2009
	Hematopoietic stem cell	OKSM	Retrovirus	Eminli *et al*., 2009
	Pancreatic β cell	OKSM	Drug-inducible lentivirus	Stadtfeld *et al*., 2008b
	Intestinal epithelial cell	OKSM	Drug-inducible lentivirus	Wernig *et al*., 2008
	Hepatocyte	OKS	Retrovirus	Aoi *et al*., 2008
	Gastric epithelial cell	OKSM	Retrovirus	Aoi *et al*., 2008
	Adipose stem cell	OKSM	Retrovirus	Sugii *et al*., 2010
	Neural stem cell	OK or O	Retrovirus	Kim *et al*., 2008; 2009b
Human	Fibroblast	OKSM or OKS	Retrovirus	Takahashi *et al*., 2007; Nakagawa *et al*., 2008
	Fibroblast	OSLN	Lentivirus	Yu *et al*., 2007
	Fibroblast	OKSM or OKS	Floxed lentivirus	Soldner *et al*., 2009
	Fibroblast	OS and valproic acid	Retrovirus	Huangfu *et al*., 2008
	Fibroblast	Proteins (OKSM)	Poly-arginine	Kim D. *et al*., 2009
	Fibroblast	OKSM	Adenovirus	Zhou and Freed, 2009
	HUVEC	OKSM	Retrovirus	Lagarkova *et al*., 2010
	Peripheral blood cell	OKSM	Drug-inducible lentivirus	Loh *et al*., 2010; Staerk *et al*., 2010
	Cord blood endothelial cell	OSLN	Lentivirus	Haase *et al*., 2009
	Cord blood stem cell	OKSM or OS	Retrovirus	Eminli *et al*., 2009; Giorgetti *et al*., 2009
	Adipose stem cell	OKSM	Lentivirus	Sun *et al*., 2009
	Adipose stem cell	OKS	Retrovirus	Aoki *et al*., 2010
	Amniotic cell	OKSM	Retrovirus	Li C. *et al*., 2009
	Amniotic cell	OSN	Lentivirus	Zhao *et al*., 2010
	Neural stem cell	O	Retrovirus	Kim J.B. *et al*., 2009a
	Marrow mesenchymal cell	OKSM or OK	Retrovirus	Park *et al*., 2008
	Adipose stem cell	OSLN	Nonviral minicircle DNA	Park *et al*., 2008
	Hepatocyte	OKSM	Retrovirus	Liu *et al*., 2010
	Astrocyte	OKSM	Retrovirus	Ruiz *et al*., 2010
	Keratinocyte	OKSM or OKS	Retrovirus	Aasen *et al*., 2008
Pig	Fibroblast	OKSM	Drug-inducible lentivirus	Wu *et al*., 2009
Rabbit	Hepatocyte and stomach cell	OKSM	Lentivirus	Honda *et al*., 2010
Rat	Fibroblast	OKS	Retrovirus	Chang *et al*., 2010
	Fibroblast	OKSM	Lentivirus	Liao *et al*., 2009
	Neural progenitor cell	OKS	Retrovirus	Chang *et al*., 2010
	Liver progenitor cell	OKS	Retrovirus	Li W. *et al*., 2009
Marmoset	Fibroblast	OKSM	Retrovirus	Wu *et al*., 2010
Rhesus monkey	Fibroblast	OKSM	Retrovirus	Liu *et al*., 2008

HUVEC: human umbilical vein endothelial cell; O: *Oct3/4*; S: *Sox2*; K: *Klf4*; M: *c-Myc*; E: *Esrrb*; L: *Lin28*; N: *Nanog*; Nr: *Nr52a*

Table 1. iPSCs derived from different species and somatic cell types[141].

Oct3/4, Klf4, Sox2, and c-Myc to reprogram mouse B lymphocytes [142]. Then, one year later, iPSCs where generated from mouse T lymphocytes by the introduction of Oct4, Sox2, Klf4, and c-Myc in a p53-null background [143]. In the same year, Haase et al. generated human iPSCs from cord blood (CB) [136]. It is an advantage that CB can be obtained from public and commercial banks without any risk to donors and iPSCs were efficiently derived from frozen sample [144]. However, the use of CB is still limited in the perspective of therapeutic applications because it is not an autologous source of cells. Interestingly, Loh et al. [139], Seki et al. [145], and Staerk et al. [140] independently derived iPSCs from human peripheral blood cells. Loh et al. separated mononuclear cells (MNCs) and granulocyte colony stimulating factor (G-CSF)-mobilized CD34+ cells from peripheral blood samples, which were collected through venipuncture and Ficoll density centrifugation[139]. After infection with lentiviruses expressing Klf4, Sox2, Oct4, and c-Myc, CD34+ cells showed a reprogramming efficiency of 0.002%, whereas MNCs showed relatively low values of 0.0008% to 0.001%. Staerk et al. utilized a doxycycline-inducible lentivirus construct to derive iPSCs from T lymphocytes and myeloid cells [140]. This lentivirus construct encoded four the reprogramming factors into a polycistronic expression cassette (pHAGE2-TetOminiCMV-hSTEMCCA). Their results showed that the reprogramming efficiency of T lymphocytes was higher than that of myeloid cells. Because T lymphocytes exhibited a higher proliferation rate and had a better long-term growth potential in vitro than myeloid cells, Seki et al. induced T lymphocytes into iPSCs by a temperature-sensitive mutant SeV vector encoding human Oct4, Sox2, Klf4, and c-Myc with an efficiency of 0.1%. This SeV vector is a non-integrating type, and it could not proliferate at standard culture temperatures [145]. So these characteristics significantly increase the safety for the generation of iPSCs. Interestingly, in 2013 iPSCs were efficiently generated from non-mobilized CD34+ cells reprogrammed using a policystronic lentiviral vector carrying the four factors (STEMCCA vector). A mean of 5.3±2.8 iPSC colonies per 20 mL of non-mobilized peripheral blood were obtained [146]. All together these works provided the evidence that iPSCs from peripheral blood cells can be considered reliable and safe. Therefore, methods to generate iPSCs from human peripheral blood cells improving the efficiency of the reprogramming process could accelerate research and promote clinical applications of iPSCs in the future.

Other than the identification of the best source of cells to be reprogrammed, a key point in iPSCs generation is the choice of the strategy to reprogram somatic cells (Fig. 7). Actually, gene-delivery of transcription factors crucial for the induction and the maintenance of the pluripotency is the preferred method to induce cell reprogramming [147]. Scientists have used either viral or non-

viral methods to deliver reprogramming factors into cells [148, 149]. Lately, new reprogramming methods using RNA, protein and microRNA delivery have been used to generate iPSCs [150-152].

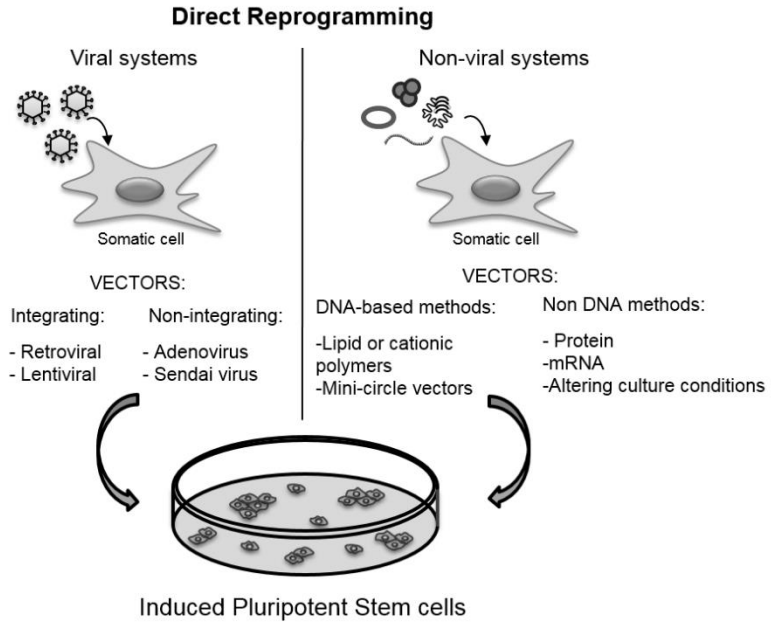

Figure 7. Schematic illustration of the reprogramming methods. iPSCs can be obtained both by viral or non-viral systems. Viral methods include the use of integrating (Retroviral and Lentiviral) and non-integrating (Adenovirus and Sendai virus) vectors. Non-viral methods consist of the use of DNA-based (lipid or cationic polymers and mini-circle vectors) and non-DNA-based methods (protein, mRNA and the alteration of culture conditions). (Talmon et al., Submitted).

Viral delivery systems. Viral reprogramming methods can be summed up in the use of retroviral, lentiviral and non-integrating viral vectors. Initially, iPSCs were obtained by retroviral vector transduction to successfully introduce the four Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) into mouse or human somatic cells [90, 91]. Four different retroviral vectors were used with each one carrying a transcription factor. Mouse iPSCs created chimeric mice that were competent for germline transmission [153]. However, both the chimeras and progeny derived from mouse iPSCs had an increased incidence of tumor formation, due primarily to reactivation of the endogenous c-Myc [153]. The removal of c-Myc from Yamanaka's transcription factor cocktail has been a pivotal action in the generation of safer iPSCs, reducing the tumorigenicity of these cells [121]. Despite a reduced efficiency, murine and human iPSCs were successfully generated with only Oct4, Sox2 and Klf4.Moreover, chimeric mice generated from iPSCs obtained omitting c-Myc, did not form tumors after 100 days; when c-Myc was included, 20% of chimeric mice developed tumors [121]. An advantage of retroviral vectors is their capability to be spontaneously silenced after reprogramming induction and activation of the endogenous transcription factors. However,

these vectors transduce only dividing cells and the use of high doses of retroviral particles from integrating viruses increased the risk for insertional mutagenesis [154].

An alternative approach is the use of lentiviral vectors (LVs). LVs have several advantages: the high transduction efficiency to a wide variety of dividing and non-dividing cells, the stable and reproducible transgene expression and the possibility to generate a single polycistronic LV carrying all the reprogramming factors reducing the insertional mutagenesis risk [155]. Moreover, temporal expression control of the reprogramming factors was permitted by the use of doxycycline-inducible LVs [156]. However, given that the reprogramming cassettes integrate into the host genome, the risk of eventual reactivation exists. Thus excision strategies to remove LV expression cassette, including Cre-loxP recombination system and PiggyBac (PB) transposition, have been used. When the endogenous reprogramming machinery is activated, the exogenous sequences may be removed for safer reprogrammed cells [157]. In Cre-excisable LV the reprogramming cassette is flanked by two loxP sites in the LTR sequences. After the induction of pluripotent state, the recombinase Cre could be transiently expressed resulting with removal of the expression cassette. PB transposition is host-factor independent, and has been demonstrated to be functional in various human and mouse cell lines [157]. The PB transposon/transposase system requires only the inverted terminal repeats flanking a transgene and transient expression of the transposase enzyme to catalyze insertion or excision events. Murine and human embryonic fibroblasts were efficiently reprogrammed using doxycycline-inducible transcription factors delivered by PB transposition [157]. Importantly, PB transposons are completely removable from their integration site without any modification of the original DNA sequence, making this reprogramming system the ideal method to generate non-genetically modified human iPSCs for regenerative medicine.

Moreover, to obtain more clinically applicable iPSCs, non-integrating vectors were used [158, 159]. These vectors have a mutated integrase coding sequence, so they do not integrate in the host genome, remaining present in an episomal form in the nucleus [160] without losing the transduction efficiency of the integrating counterpart [161]. For these reasons, adenoviral and Sendai viral vectors, derived from non-integrating viruses, were used to reprogram somatic cells. Stadfeldtet al. [159] reprogrammed mouse fetal liver cells and finally adult hepatocytes with adenoviral vectors containing the four reprogramming factors. Although the efficiency was lower than integrating vectors, they obtained *bona fide* iPSCs. Nevertheless, they were not able to generate iPSCs from adult fibroblasts, probably because hepatocytes required lower expression of reprogramming factors than fibroblasts. One year later, adenoviral vectors were used to generate iPSCs from human embryonic fibroblasts using a higher multiplicity of infection. The obtained cells were pluripotent, able to differentiate and, most important, free from integrated viral DNA in

the host chromosomes [162]. This work demonstrated that reprogramming with adenoviral vectors generated *bona fide* iPSCs, but with a low rate comparing the number of transduced to reprogrammed cells. Conversely, using Sendai Virus (SeV)-based vectors, an RNA virus that replicates in the cytoplasm and does not integrate in the host genome, iPSCs were originated with an higher efficiency compared to the other methods [158]. One advantage of SeV is that RNA viruses are diluted during cell passage, reducing the number of viral particles at each passage; however, some residual viruses can still be present after several passages. Regardless, it is possible to eliminate the cells still containing the virus by a negative selection using a specific antibody that recognizes the hemagglutinin-neuraminidase (HN), the major protein expressed in SeV-infected cells, resulting in the maintenance of only virus-free reprogrammed cells [158]. Integration of reprogramming transcription factors into the cell genome is not necessary for maintenance of pluripotency [163]. The silencing of reprogramming factors expressed from a retroviral vector, the possibility to excise transgenes in LVs, and the efficient use of non-integrating vectors indicate that sustained expression of exogenous factors is not required. Indeed, the exogenous transgenes reactivate the endogenous transcriptional machinery of stem cells factors that are able to self-maintain the pluripotent status.

Non-viral delivery systems. The alternative safe and cost-effective approaches are non-viral delivery systems of reprogramming factors, which can be divided into DNA-based and non-DNA-based methods.

DNA-based methods. Plasmids carrying reprogramming factors are encapsulated into lipid or cationic polymers and subsequently transfected into cells. Plasmids remain in an episomal form and are allowed a short-term transgene expression [164, 165]. In 2008, Okita *et al.*[164]described the successful reprogramming of mouse embryonic fibroblast to iPSCs obtained by lipofectamine transfection of two plasmids: one containing c-Myc, and the other containing the three factors: Oct4, Sox2 and Klf4. Later, it was demonstrated that the nucleofection of a polycistronic plasmid co-expressing Oct4, Sox2, Klf4, and c-Myc was equally efficient [166]. iPSCs from human foreskin fibroblasts were successfully generated by sequential transfections of non-episomal plasmids that independently encoded the four factors Oct4, Nanog, Sox2, and Lin28 [167]. iPSCs obtained by these methods did not show any plasmid integration but were able to give rise to teratomas when injected into mice and contributed to adult chimeras. However, cells were reprogrammed with 100-1000x lower efficiency compared with viral methods probably due to lower transgene expression levels. Thus, further studies are required to optimize these systems [158]. To this purpose, a reprogramming strategy with the use of mini-circle DNA was developed. Mini-circle DNA lacks

plasmid backbone sequences and confers higher levels of sustained transgene expression upon delivery. Transgene-free iPSCs were generated from human adipose stem cells [168]using a mini-circle vector carrying a single cassette containing Oct4, Sox2, Lin28, and Nanog. Mini-circle DNA provides higher transfection efficiency and longer ectopic expression compared with regular plasmids and could be useful in translational studies because adult cells can be reprogrammed without genomic modification. Therefore, the production of these virus-free iPSCs addressed a critical safety concern for potential use of iPSCs in regenerative medicine.

Non-DNA methods. Up to now, the methods described involved the use of genetic materials, which could cause unexpected genetic alterations. Thus, alternative strategies have been investigated, such as the delivery of reprogramming proteins or mRNA directly into the cells and the manipulation of cell culture conditions parameters. In 2009, the first successful generation of protein-induced iPSCs (piPSCs) was described. In this system, the purified Oct4, Sox2, Klf4 and c-Myc proteins were fused to polyarginine peptide tags, which allowed the recombinant proteins to cross the plasma membrane. The first colonies appeared after four rounds of protein delivery and subsequent 30-35 days of culture. However, this reprogramming method is not as efficient as gene-delivery systems; indeed, multiple protein transductions are required [152]. An alternative strategy is the generation of iPSCs by direct mRNA transfection [169, 170]. Synthetic mRNA of the classic reprogramming factors and LIN28 were manufactured and modified to overcome an antiviral response [169]. Daily transfection gave rise to colonies after only 18 days, showing a higher efficiency and kinetic rate. This method eliminates the risk of genomic integration and insertional mutagenesis and allows the regulation of protein stoichiometry in culture [169]. Another level of reprogramming regulation can be through the manipulation of cell culture conditions [171]. The Oct4, Sox2 and Nanog genes are not completely dormant so they can be activated by altering culture conditions through exposing the cells to a lower amount of atmospheric oxygen or altering the culture media by adding different chemical compounds, such as valproic acid or histone deacetylase, which modifies the chromatin state [172, 173]. For example, histone deacetylase (HDAC) inhibitors such as Valproic Acid and sodium butyrate, improved reprogramming efficiency, resulting in upregulation of epigenetic remodeling of pluripotency-associated genes [172, 174, 175]. Moreover, it was reported that cocktails containing inhibitors of the MAPK/ERK kinase, GSK3β, transforming growth factor β (TGF-β)/Activin/Nodal receptor in addition to the human leukemia inhibitory factor (hLIF), facilitates iPSC generation [176, 177]. Furthermore, work from Ding's laboratory showed the additional use of the histone methyltransferase (HMT) inhibitor, BIX-01294, activating calcium channels in the plasma membrane improved the reprogramming efficiency using the four Yamanaka factors [178, 179]. Very recently, Li *et al.*, [180] reported that a

compound cocktail containing cyclic pifithrin-a (a P53 inhibitor), A-83-01, CHIR99021, thiazovivin, NaB and PD0325901 significantly improves the reprogramming efficiency (170-fold more) for human urine-derived cells. However, this short-term induction is not always self-sufficient to induce and maintain a genuine pluripotency state, but it can help the reprogramming, preventing the use of c-Myc, Klf4 or other potential oncogenes [172].

Molecular challenges of reprogramming

Reprogramming is a stochastic event with variable efficiency [181]. This characteristic is due to the molecular barriers that must be overcome to reach pluripotency. Somatic cells, to return to ESC-like state, have to remodel their gene expression, their transcriptome, their miRNAs pattern and their epigenome (Fig. 8).

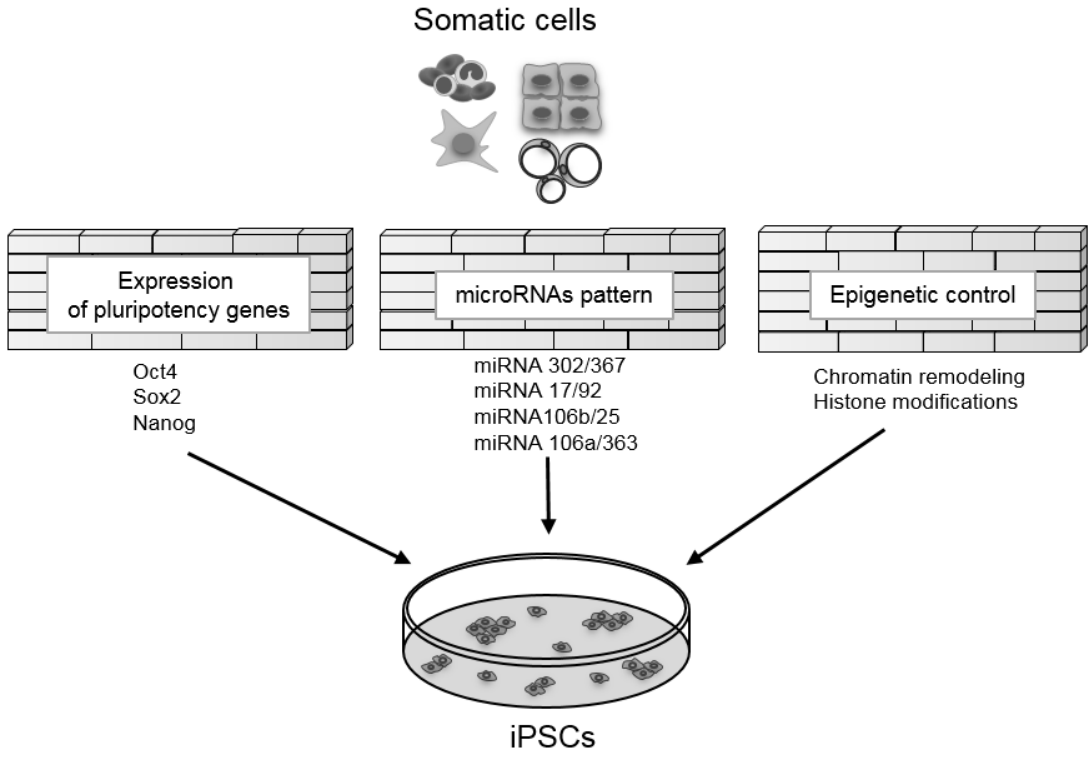

Figure 8. Molecular challenges for reprogramming. Somatic cells reprogrammed to iPSCs will remodel their transcriptome, miRNA pattern and epigenome to return to a stem cell state (Talmon et al., Submitted).

Genome-wide expression analysis identified a large set of ESC-specific or enriched genes. Among them there were included he extensively studied pluripotency-promoting transcription factors (TFs) Oct4, Sox2, and Nanog [90, 107]. Genome-wide profiling of Oct4, Sox2 and Nanog binding sites, showed that they bind the promoters of several hundred genes [105, 106], acting both as transcriptional activators and repressors. Indeed, they enhance gene expression to maintain

pluripotency and, at the same time, they downregulate lineage-specific genes to prevent differentiation. Oct4, Sox2 and Nanog are involved in a complex network of gene regulation that also includes positive/negative feedback-loops that balance the expression level of the pluripotency TFs. Moreover, many of the target genes are transcriptional factors and chromatin modifiers acting in early embryogenesis to maintain the pluripotent state *in vivo* or *in vitro* [182, 183].

In addition to TF regulation, miRNAs have emerged as a novel class of gene expression regulators. The stemness of pluripotent cells is also sustained by specific miRNAs that are enriched in ESCs, which regulate genes involved in cell cycle, cell signaling and epigenetics. In ESCs and iPSCs, several stem cell-specific miRNAs were identified as being highly related to each other as they are grouped in clusters on the same chromosome and are transcribed in a single primary transcript. These miRNAs maintain ESCs properties by promoting the G1-S transition of the cell cycle and that aberrant miRNA biogenesis impairs the proliferation of ESCs, which accumulate in the G1 phase. The key miRNAs for these functions are the miR-290 cluster in mouse and miR-302/367 cluster in humans, both of which are abundantly expressed in pluripotent cells and absent in somatic cells [184, 185]. These two clusters have similar targets and/or regulatory networks and are master regulators of the ESC cell cycle, which promotes self-renewal and pluripotency. For example, in human ESCs, Oct4/Sox2 regulates the miR-302 cluster to post-transcriptionally modulate cyclin D1, a key controller of cell cycle progression [186]. In mouse ESCs, the miR-290 cluster controls *de novo* methylation through Retinoblastoma-like Protein 2 (Rbl2)-dependent regulation of DNA methyltransferase (Dnmts) [187] . Based on these findings, it appears that the main function of the miR-290/302 seed family is to shorten the G1 phase of cell cycle to support self-renewal, and to secure the epigenetic status to maintain ESCs pluripotency. Moreover, Oct4, which binds to the miR302 promoter in a vitamin C-dependent manner, transcriptionally regulates miR-302/367 expression [188]. Thus, there is a tight interplay between ESC-specific TFs and miRNAs. The biogenesis of miRNAs is critical to achieving efficient reprogramming [189]. Indeed, depletion of the miR-302/367 family reduces reprogramming efficiency in response to transduction with Oct4, Sox2, Klf4 with or without c-Myc [190, 191], suggesting that the miR-302/367 family plays essential roles in the process. On the contrary, the ectopic expression of the miR-290 or miR-302 clusters has been shown to improve the reprogramming efficiency [192]. Other miRNAs also have been identified to favor the achievement of a pluripotent state. For example, miR-17/92, miR-106b/25, and miR-106a/363 clusters enhance reprogramming by targeting and inhibiting the transforming growth factor beta-receptor 2 (TGFBR2), which is strictly involved in the regulation of cell proliferation. Notably, miR-17,miR-29, miR-93, and miR-106a have also been highly induced in the early stages of reprogramming [189, 193]. Moreover, the miR-130/301/721 family

downregulate the homeobox transcription factor Meox2 to achieve ~2-fold increase in reprogramming [194]. All these miRNAs share a similar seed region with the miR-290/302 family, suggesting that an abundance of miRNAs containing the miR-290/302 seed region play significant roles in various biological functions regulation of cell cycle, cell proliferation and self renewal and intrinsically act as positive regulator of reprogramming [195].

Finally, another important level of regulation for iPSC generation is the epigenome. Chromatin status and histone modifications are crucial in the regulation of transcription mechanisms. Currently, for the improvement of iPSCs generation and differentiation, it is very important to understand the epigenetic marks and mechanisms that are involved in the induction and maintenance of the pluripotent state, and if epigenetic memory can influence these processes. It is well known that cells of the early mammalian embryo, including pluripotent ESCs and primordial germ cells (PGCs), are epigenetically dynamic and heterogeneous. The histological analysis of the nuclei of stem cells, progenitors and differentiated progeny show that several cellular types, like neoblast cells in planaria and hematopoietic stem cells in mammals, are characterized by chromatin open state [196]. This particular state implements the transcription program and allows a rapid switch upon induction of differentiation [196]. During development and differentiation, regulation of gene transcription is governed at an epigenetic level by the balance between activating and repressing modifications, such as the trimethylation of lysine 4 of histone 3 (H3K4me3) and the trimethylation of lysine 27 of histone 3 (H3K27me3), respectively, in the nucleosomes of the chromatin [197]. H3K27 methylation is mediated by polycomb group (PcG) proteins [105] (Fig. 9). It is interesting to note that some targets of PcG proteins tend to be co-occupied by the TFs Nanog, Oct4 and Sox2 [198].

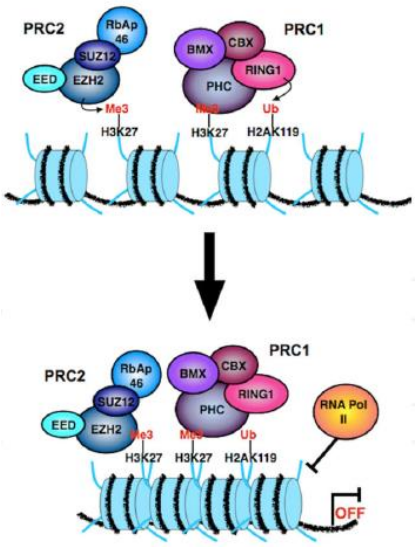

Figure 9: Polycomb group (PcG) proteins mediated H3K27 methylation. PRC2 induces EZH2-mediated H3K27me3. H3K27me3 recruitsPRC1 promotes chromatin compaction and gene silencing [199].

Another histone mark commonly associated with gene repression is histone 3 lysine methylation (H3K9). One enzyme associated with this mark is the euchromatic histone-lysine N-methyltransferase 2 (EHMT2), which is notably required to silence Oct4 during differentiation [183]. The low level of H3K9 methylation in undifferentiated ESCs is maintained by H3K9 histone demethylases (HDMs),jumonji domain-containing 1A (jMjD1A) and jumonji domain-containing 2C (jMjD2C). These enzymes regulate global levels of the repressive marks H3K9 and maintain the pluripotent state by directly demethylating H3K9 in the promoter regions of ESC TFs, allowing their expression. Interestingly, Oct4regulates the genes encoding jMjD1A and jMjD2C,which represents a positive feedback-loop that integrates the action of the TFs and histone modifiers to maintain the undifferentiated ESCs state [106].

The epigenetic control of the undifferentiated-differentiated state transition and the way that the epigenetic barriers are overcome are critical issues in the generation of iPSCs. At the present, molecular mechanisms that underlie epigenetic chromatin remodeling during reprogramming is still unclear, however, several proteins are known to regulate chromatin marks and are associated with the distinct epigenetic states of cells before and after reprogramming [197]. New insights have been gained by treating the cells during reprogramming with agents that promote the chromatin open state. For example, the DNMT inhibitor 5-aza-cytidine, the histone deacetylase (HDAC) inhibitor, valproic acid (VA), and EMHT2 inhibitor lead to increased efficiency of iPSC generation [172, 178] (Figure 10).

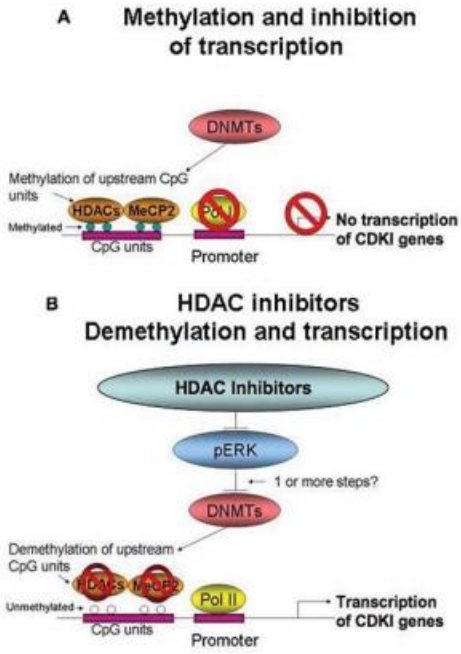

Figure 10: Model of inhibition of transcription directed by methylation of CpG islands in gene promoter regions (A) and HDAC inhibitors [200]

Indeed, Huangfu et al. [172] demonstrated that iPSCs could be generated from primary human fibroblasts by transducing the cells only with Oct4 and Sox2 and adding VA in the culture medium. These results have opened the possibility to reprogram cells without c-Myc and Klf4. More recently, several epigenetic studies focus on enzymes that regulate this process. For example, Onder and collaborators, using shRNA approach, demonstrated that inhibition of the core components of polycomb repressive complex 1 and 2reduced reprogramming efficiency, whereas suppression of the H3K79 histone methyltransferase accelerates reprogramming and increase the yield of iPSC colonies [182]. These studies demonstrated that the knowledge of epigenetic mechanisms in order to act on the chromatin and histones status to be able to trigger the iPSC reprogramming process is crucial to improve reprogramming efficiency and to develop new strategies to avoid oncogenes employment.

Applications of iPSCs

iPSC technology can lead to several clinical applications. iPSCs and iPSC-derived differentiated cells can be useful to study disease biology, to model diseases *in vitro* and to develop new drugs, being a tool for both screening and toxicity tests. iPSCs, because of their versatility, can be differentiated into any of several cell types, which can be used as a model to study molecular mechanisms underlying disease development in a target cell type. Moreover, these cells can be studied in a developmental stage biologically relevant to phenotype analysis of diseases such as in trisomy 21 [201] or Alzheimer's disease [202] studies, or in the observation of multistage oncogenesis, cellular transformation [203] or in hematological malignancies [204]. These models allow a better understanding of disease pathogenesis and the comparison between affected and healthy cells.

iPSCs can also be used as platform for drug discovery, affording advantage to the high cost of generating new drugs. For example, hepatotoxicity and cardiotoxicity are two principal causes of drug failure during preclinical testing. iPSCs could be differentiated into cell targets having a primary tissue-like phenotype and unlimited availability; then it could be possible to assess multi-parameter readouts of general and mechanism-specific hepatotoxicity or cardiotoxicity [205]. Moreover, the variability in individual responses to potential therapeutic agents is another problem in effective drug development. The use of human iPSCs would also allow the study of single nucleotide polymorphisms that influence the ability of an individual to metabolize and clear drugs and toxins. The accurate prediction of human drug toxicity is a vital element of the drug discovery process. iPSC technology allows the screening of a library of human cell lines that may represent

the genetic and potentially epigenetic variation of a broad spectrum of the population [206]. Moreover, because iPSCs grow indefinitely in culture, they can provide an unlimited source of any desired specialized cells. Finally, since iPSCs share similar properties to ESCs, this type of pluripotent stem cell holds great promise for regenerative medicine. Recently, in Japan the first clinical application using iPSC-derived differentiated cells was approved. In July 2013, Japan's regulatory authorities gave permission for a team led by ophthalmologist Masayo Takahashi at the RIKEN Center for Developmental Biology (CDB) in Kobe to proceed to collect cells to be used in a clinical iPSC pilot study. iPSCs were generated from skin fibroblasts of a 70 years old woman affected by age-related macular degeneration. Then iPSCs were differentiated into retinal cells, certified for genetic stability, safety and transplantation in a primate model [207]. In September 2014, the differentiated retinal cells, were transplanted into the patient and no side effects have been reported to date [208].However, the clinical trial was put on hold because the reprogrammed cells obtained for the second patient were mutated. A total of 6 mutations were found and the Takahashi team decided to stop and perform further analysis even though the investigators found that the rate of tumor formation due to these mutations is low [209]. These mutations were not present in the genome of the patient, indicating that they arose during the reprogramming process. At the moment, researchers around the world are watching to see whether the transplanted cells have slowed the retina degeneration progression and whether further side effects will occur. Moreover, another relevant step forward to iPSC clinical application is the HLA-haplotype banking of these cells. Indeed, personalized medicine based on patient-specific iPSC generation, correction and differentiation is expensive and time consuming; on the other hand, cell therapy approaches are limited by immunological rejection. The main immunologic barrier to overcome between two individuals is the human leukocyte antigen (HLA). The HLA system is the most polymorphic locus with almost 10,000 HLA-I and -II alleles. Matching donor cells and recipients for HLA would be an endless work, due to the countless phenotype possibilities. An alternative strategy could be the generation of a bank of HLA homozygous iPSC lines [210]. It was estimated that to find at least one homozygote for each of 50 different haplotypes, it would be necessary to examine a database of 24,000 individuals [211]. Thus, it is feasible and would cover a large majority of the Japanese population. Interestingly, HLA homozygote donors could be easily identified from the HLA databases already used in bone marrow donor or cord blood banks [211]. In this way, HLA-haplotypes banking of iPSCs would allow to overcome cost and immunological limitation of iPSC-based cell therapy, making iPSCs closer to clinical application. By 2020, Yamanaka is planning to generate 75 iPSC lines, starting from 64,000 people, that are sufficient to match with 80% of the population [212].

Finally, several remarkable preclinical works demonstrated that the possibility of generating iPSCs from patients affected by a monogenic disease, to differentiate them into the best cellular target and to correct them introducing the functional form of mutated gene, thus make iPSCs an interesting tool for regenerative medicine.

iPSCs in hematological diseases

iPSCs technology have several applications in hematology, ranging from disease modeling to the development of cell therapy strategies to correct monogenic diseases, as Diamond Blackfan Anemia (DBA), Thalassemias, Fanconi Anemia (FA) and Hemophilia. DBA is a rare congenital bone marrow failure syndrome characterized by red cell aplasia and reduced or absent erythroid precursors. Haploinsufficiency for ribosomal proteins (RPs) is identified to trigger the disease [213] but the mechanism that leads to DBA phenotype is unknown. To study the correlation between RP haploinsufficiency and DBA phenotype and remission mechanism, in vitro models were developed. In this context, iPSC-based model resulted to be useful as demonstrated by the generation of iPSCs from DBA patient's fibroblasts [214]. These cells had the same aberrations of cells from DBA patients: aberrant ribosomal biogenesis and defective red cell production. iPSCs represented an unlimited source of cells recapitulating DBA defects [215]. Recently, iPSCs were generated from DBA patients carrying heterozygous mutations for RLP19 and RLP5 that recapitulate biochemical and phenotypic features of the disease. Transcriptome analysis revealed that genes involved in focal adhesion and extracellular matrix interaction were significantly altered in both affected iPSC lines in comparison with healthy iPSCs [216].These models could also be used for drug testing or discovery. It would be interesting to investigate the effect on DBA-derived iPSCs of drugs previously used for different treatments; for example valproic acid, calcineurin inhibitors, and metoclopramide or new drugs such as leucine [217] and lenalidomine [218].Several works demonstrated that is possible to generate iPSCs from DBA patients and that they can be useful to better understand the pathogenic mechanism of the disease or as a platform to test new drugs for the treatment of the DBA.

Other than to model disease, iPSCs could represent a virtual infinite source of cells to be differentiated, corrected and transplanted in animal disease models, in order to design cell therapy strategy for genetic diseases. For example, Raya et al. [219] generated iPSCs from somatic cells isolated from gene corrected Fanconi anemia (FA) patients. Somatic cells from FA patients were reprogrammed after genetic correction with LVs encoding FANCA and FANC2. Indeed, it appeared that the correction of the FA mutation was a necessary for iPSCs generation from somatic cells of FA patients. Then, FA-iPSCs were differentiated in hematopoietic progenitors of the

erythroid and myeloid lineages that maintained the disease-free phenotype of FA-iPSCs. These data offered proof-of-concept that iPSCs technology can be used for the generation of disease-corrected, patient-specific cells with potential value for cell therapy applications. To genetically correct cells derived from monogenic diseases affected persons several gene editing strategies were investigated. For example, to revert hemoglobin defect in thalassemic patient-derived cells, Ma et al. efficiently used TALENs to genetically correct iPSCs-derived HSCs to transplant [220]. The α–thalassemia major was also corrected in transgene-free iPSCs using zinc-finger nuclease-mediated insertion of a globin transgene in the AAVS1 site of human chromosome. Homozygous insertion of the best of the four constructs tested led to a complete correction of globin chain imbalance in erythroid cells differentiated from the corrected iPSCs[221]. Ma et al., combined the efficient generation of integration-free patient-specific β-thalassemia iPSCs and TALEN-based correction of hemoglobin mutations. These iPSCs differentiated into HSCs and erythroblasts expressing functional β-globin. Interestingly, the correction process did not generate TALEN-induced off-target mutations a critical issue in this technology [220]. Recently, Xu and colleagues compared gene correction of hemoglobin mediated by TALEN and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 in β-thalassemia iPSCs to test specificity and safety of the two methods. In this work, whole genome-wide sequencing analysis demonstrated that both TALENs and CRISPR/Cas9 have minimal off-target effects in iPSCs after gene targeting [222], although high off-target risk was reported for CRISPR/Cas9 in other cell types. Thus, both methods were feasible to correct β-thalassemia iPSCs, but further studies had to be done to check the transplantability and the globin-switch in iPSC-derived HSCs. In summary, the choice of gene-editing strategy to correct disease-specific iPSCs is an important step toward a future therapy of monogenic disease.

iPSCs and hemophilia A

The actual therapy high costs and the side effects including the risk of FVIII neutralizing antibodies development led to the study of new cell or gene based therapeutic strategies. Moreover, further elucidations in the development of anti-FVIII antibodies mechanism are needed. To these aims, iPSCs HA models were developed. Jia and colleagues established human HA models using hepatocytes-like cells differentiated from integration-free-iPSCs. iPSCs were generated from HA patients with integration-free episomal vectors containing human genes OCT4, SOX2, SV40LT and KLF4 and were differentiated into hepatocyte-like cells that displayed the intron 22 inversion in the FVIII gene, and failed to secrete FVIII [223]. These patients-specific iPSCs-derived hepatocytes provide a good model to study FVIII function and allow preliminary vector characterizations for the gene therapy strategies. Despite the generation of patient-specific iPSCs could be useful for the

development of personalized medicine it is not necessary the use of hemophilic samples for the generation of a cellular model. Thus, other strategies were investigated to generate a FVIII-deficient model cell line avoiding the use hemophilic patient samples using gene-editing technology. An HA model cell line was developed using a transcription activator-like effector nuclease (TALEN) pair to invert a 140-kbp chromosomal segment that spans the portion of the FVIII gene in human healthy iPSCs [224]. These cells did not produce FVIII mRNA and protein, showing that TALEN technology can be used to recapitulate genotypes responsible for severe hemophilia A. However, when the inverted segment was reverted, cells were able again to produce the mRNA. This study demonstrated that TALEN could be used not only to model HA, but also to correct genetic defects. Other methods of gene editing such as RNA-guided clusters of the regulatory interspaced palindromic repeats (CRISPR)-Cas9 system [225] and PiggyBac transposon technology [226] were recently used to recapitulate FVIII mutations in iPSCs. The opportunity to combine this gene correction approaches to iPSCs technology lead to the development of iPSC-based cell-therapy to cure HA.

To date the use of cell therapy in the treatment of hemophilia consisted mainly in the transplantation of healthy cells to repair or replace the coagulation factor deficiency [32, 33, 42, 227-229]. These procedures was led mainly with adult stem cells and, more recently, with progenitor cells partially differentiated from iPSCs, but the mechanisms by which transplanted cells engraft, proliferate and function remain unknown [230]. The goal of the combining gene and cell therapy is to correct the defective gene sequence *ex vivo*, resulting in a phenotypic correction of the disease after cell transplantation. In HA cell therapy perspective, LSECs were identified as the optimal cell target because of their ability to produce and secrete FVIII. Moreover, it has been demonstrated that LSEC induced tolerance when they presented an antigen [231]. Xu and colleagues [232], evaluated the therapeutic application of iPSCs using iPSC-derived endothelial cells for treatment of a preclinical mouse model of HA. In particular, they derived murine iPSCs from tail-tip fibroblasts, by a retroviral vectors carrying three reprogramming factors (Oct4, Kl4, Sox2), and differentiated them into endothelial cells and their precursors. These iPSC-derived cells expressed specific markers for these cells such as CD31 and Flk1, as well as FVIII. Moreover, following transplantation of these cells into the HA mice liver, the levels of plasma FVIII in treated mice increased to 8%-12% and, when the tail-clip bleeding assay was performed, HA mice survived. Finally, they examined the liver distribution of GFP-labeled transplanted cells and demonstrated that these cells appeared to reconstitute structurally the sinusoidal endothelial compartment in the liver.

These studies provides the proof of concept for the potential to develop cell therapy strategies based on iPSCs technology correcting the bleeding phenotype of the disease. Indeed, the possibility to differentiate patient-derived iPSCs into endothelial cells and correct them by gene transfer could represent an unlimited source of transplantable FVIII producing cells to treat hemophilia A. In this context the goal of our study is to generate autologous iPSCs from HA patients' peripheral blood. Then we want to correct the established iPSCs for FVIII production and differentiate them to obtain patient-specific disease-free ECs for cell therapy development and application.

Materials and Methods

MNC and CD34+ cell purification and culture

Mononuclear cells (MNCs) were obtained from 5 healthy donors and 20hemophilic patients. CD34+ cells were isolated from 2 healthy donors, 1 heterozygous control and 4 patients. The donors were not treated with any mobilizing agent and peripheral blood was obtained in heparin tubes by venipuncture. MNCs were purified from peripheral blood (PB) by Ficoll(GE Healthcare)gradient. Briefly, 20 ml of PB were diluted (1:3) with phosphate buffered saline (PBS; Sigma Aldrich). Then diluted PB were stratified on Ficoll in a ratio of 2:1 and centrifuged at 650 x g for 20'. MNC ring was harvest, washed with PBS and centrifuged at 350 x g for 10'. Cells pellet was recovered and plated in α-MEM (Euroclone)with hIL-3, hIL-6, hIL-7, hGM-CSF (all Immunotools) 10 ng/mL each. Cells were expanded for 4days.Every 2 days cytokines were added.

CD34+ cells were isolated from MNC using the MACS® CD34 MicroBeadKit (MiltenyiBiotec) according to manufacturer's protocol. Isolated cells were expanded for 4 days in HPGM medium (Lonza) with 1% human serum albumin (Sigma-Aldrich), 50 ng/mL of hSCF, hFlt3-ligand, hTPO and hIL-3 (all Immunotools).

Culture and irradiation of human foreskin fibroblasts

Human foreskin fibroblasts (HFF; ATCC® SCRC-1041™) were used as feeder layer for iPSCs culture. Specifically HFF were cultured in IMDM (Sigma-Aldrich) with 10% of fetal bovine serum (FBS, Euroclone), 2mM glutamine (Sigma-Aldrich), 50 U\ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). Before their use as feeder layer they were mitotically inactivated by gamma ray irradiation (25 Gy) and freezed in aliquots of 10^6 or 2*10^6 cells/ml of freezing medium (90% FBS and 10% DMSO, Sigma-Aldrich). The day before iPSCs expansion, irradiated HFF were plated on a 0.1% gelatin (Sigma-Aldrich) coated plates in IMDM.

iPSCs and embryoid bodies (EBs) culture

iPSCs were cultured and characterized using standard techniques[233]. Specifically, iPSCs were cultured at 37°C with 5% CO_2 on irradiated HFFs in HES medium, consisting in KnockOut DMEM (Life Technologies) supplemented with 20% KnockOut Serum Replacement (Life Technologies), 2 mM Glutammine (Sigma-Aldrich), 50 µM 2-mercaptoethanol (Life Technologies), non-essential amino acids (Sigma), and 10 ng/ml basic fibroblast growth factor (bFGF) (Immunotools). HES medium was changed daily. Once a week, iPSCs were detached mechanically and plated onto fresh HFFs in HES medium.

Vector transduction for reprogramming and FVIII correction

5 days after isolation, both healthy and hemophilic MNC were transduced with third generation self-inactivating Cre-exisable polycystronic lentiviral vectors LV-SFFV-Oct4-Sox2-Klf4 (LV-SFFV-OSK) by two consecutive spinoculation at MOI 5 for each at 300 x g for 1 hour. CD34+ cells were transduced by the LV-SFFV-OSK and another Cre-exisable polycystronic LV carrying miRNA cluster 302\367 followed by OSK cassette (LV-SFFV-miR-302\367-OSK) by a single spinoculation at MOI 5 at 300 x g for 1 hour. 2 days after transduction cells were seeded on the top of HFF feeder layer in α-MEM or HPGM and 2 days later medium was changed with HES medium. From 20 up to 45 days colonies appeared. iPSCs were maintained on HFF feeder layer in HES medium. Medium was changed every day. Individual iPSCs colonies were passed by mechanical dissociation.

HA MNC were genetically corrected by transduction at MOI 10 with a LV carrying the human B domain deleted (hBDD)-FVIII under the control of ubiquitous promoter of phosphoglycerate kinase (PGK). Alternatively, we corrected HA MNC- and HA CD34-derived iPSCs by transduction with a LV carrying the B domain deleted form of FVIII under the control of VEC endothelial specific promoter (LV-VEC-hBDDFVIII), LV-VEC-GFP was used as transduction control.

RNA isolation and RT-PCR

RNA was isolated by Isol-RNA Lysis Reagent (Invitrogen). 1μg 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 min followed by 30 cycles (25 cycles for β-actin) of denaturation at 94°C for 30", annealing at 50-62°C for 30-45", extension at 72°C for 60", and final extension at 72°C for 7 minutes. Primers, annealing temperatures and product sizes are listed in the table 1. PCR products were resolved in 2% agarose gels.

Alkaline phosphatase staining

For Alkaline Phosphatase (AP) staining, iPSCs were fixed and stained using the Alkaline Phosphatase (AP) detection kit (Millipore) according to the manufacturer's protocol.

Vector integration, copy number analysis and Cre/LoxP excision

LV-SFFV-OSK and LV-SFFV-miR-302\367-OSK integration in iPSCswas quantified using genomic DNA purified from cells using ReliaPrepgDNA Tissue Miniprep System (Promega) and diluted to 25ng/mL. Primer used were: Wpre5'-TGGATTCTGCGCGGGACGTC-3' and dNEF 5'-GGCTAAGATCTACAGCTGCCTTG-3', GAPDH 5'-AACGTGTCAGTGGTGGACCTG-3' and 5'-AGTGGGTGTCGCTGTTGAAGT-3'.qPCR for copy number was performed using the GoTaq® qPCR Master Mix (Promega) using primers previously described. qPCR protocol was: denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15" and annealing/extension at 60°C for 60" according to the manufacturer's protocol. To excise the LoxP-flanked reprogramming vector cassette in ECs, cells were transduced with the integrase defective lentiviral vector (ID-LV) carrying Cre recombinase at MOI 30. Excision efficiency was assessed by qPCR as described above.

Telomere length analysis

Genomic DNA was purified from freshly isolated MNC and CD34+, from iPSCs and ECs after 5, 10, 15, 20 passages in cultures using ReliaPrepgDNA Tissue Miniprep System (Promega). Telomere length was assed using qPCR Multiplex on genomic DNA in a collaboration with Dr. Donato Colangelo from our Department as previously described [234].

NANOG promoter methylation analysis

Genomic DNA was purified from MNC, CD34+ cells and iPSCs using ReliaPrepgDNA Tissue Miniprep System (Promega). Then 1 µg genomic DNA was bisulfite-converted using EpiTect Kit (Qiagen).A total of 150 ng of converted gDNA was used for PCR using primer amplifying 8 CpG-islands in the Nanog promoter (Forward: 5'-TGGTTAGGTTGGTTTTAAATTTTTG-3'; reverse: 5'-ACCCACCCTTATAAATTCTCAATTA-3'). Amplified products were subcloned into pCR2.1 vectors using the Topo TA cloning Kit (Invitrogen). Individual colonies were picked, plasmid DNA was purified using the NucleoSpin® Plasmid (Machery-Nagel), and DNA was sequenced using M13 Rev and M13 (-20) For primers.

Adipogenic, osteogenic and chondrogenic differentiation

EBs were formed, plated on 0.1% gelatin (Sigma-Aldrich) coated plates and cultured in Mesenchymal Stem Cell Adipogenic Differentiation Medium (MSC, LONZA) or osteogenic medium consisting in α Minimum Essential Medium (αMEM, Euroclone), FBS 10% (Euroclone), 0.4 mM ascorbic acid, 1 mMβ-glicerophosphate, and 10 nM dexamethasone (all Sigma-Aldrich).

Media were changed every 3 days. After 14–20 days, cells were washed in PBS, fixed with 4% PAF and stained with Oil Red O (ORO, Sigma-Aldrich) for adipogenic and with Alizarin Red (ARS, Sigma-Aldrich) 40mM pH 4.1 for osteogenic differentiation. The presence of lipid vacuoles and the production of calcium deposits was examined in light microscopy (Leica ICC50HD, 200x, 400x magnification).

For chondrogenic differentiation, iPSCs were cultured for 30 days in 15 mL centrifuge tubes in Chondrogenic Medium (LONZA). The medium was changed every 2/3 days. Cells were then washed, fixed in 4% PAF, included in OCT (Fisher), and frozen at-80°C. 4 µm sections were cut, stained using the primary goat antibody against collagen II (Santa Cruz Biotechnology, Inc.;1:200), and secondary AlexaFluor546 donkey-anti-goat-IgG antibody (Invitrogen; 1:500) following standard protocol. Nuclei were stained with DAPI (SIGMA; 1:1000) and observed at fluorescecence microscope (LEICA DM5500B).

Endothelial Cell Differentiation

For endothelial differentiation, EBs were generated and plated on 6-well tissue culture gelatin coated plates (0.1% gelatin) in EB medium with 50 ng/ml of VEGF (Immunotools) until the end of differentiation (20 days).

Flow cytometry analysis

ECs were characterized by flow cytometric analysis. Cells were resuspended in staining buffer (PBS, FBS 0.5% and NaN3 0.1%) and incubated with the antibody of interest for 30' on ice. Antibodies used are listed in Table 2. For each sample, $1x10^5$ events were acquired by FACScalibur. Data were analyzed by Windows Multiple Document Interface for Flow Cytometry (winMDI, v. 2.9; Joseph Trotter, The Scripps Institute).

In vitro tubulogenesis assay

Pure Matrigel (BD Bioscience) was added to each well of a 24-well tissue culture plate and allowed to solidify at 37°C for 1 hour. Then 0.3 ml of a cell suspension containing 10^5 endothelial cells in EB medium was placed on top of the Matrigel. Plates were incubated at 37°C, 5% CO_2, and observed at 16, 18 and 20 hours for observation of cellular formation into capillary-like structures.

LV transduction of endothelial cells

Endothelial cells were transduced with LVs containing GFP under the control of endothelial specific promoters: Tie-2, VEC and Flk1. As positive control LV containing GFP under ubiquitous

promoter (PGK) was used. As negative control LVs containing GFP under hepato-specific and myeloid promoters were used (CD11b and TTR respectively). All LVs were used at MOI 10.

Animals and procedures

Hemophilic NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (HA-γNull), generated in our laboratory [33], of 6-8 weeks of age mice were used for cell transplantation studies. Animals received 200 mg/Kg MCT (Sigma-Aldrich) in saline i.p. 24 hours before intraportal cell transplantation. To blunt immune responses against GFP transgene reporter, mice were given 30 mg/kg cyclophosphamide (CYTOXAN; Bristol-Myers Squibb) in normal saline i.p. twice per week for the duration of the study starting from the day before of surgery. Mice were anesthetized with isoflurane. For cell transplantation, $2x10^6$ of endothelial cells were injected into portal vein as previously described [78]in 0.3 ml serum-free Dulbecco's Modified Eagle Medium (DMEM). Controls received serum-free medium.

For beads transplantation, cells were mixed with Cytodex 3 microcarriers beads (Amersham Pharmacia Biotech) in a ratio of $10 \cdot 10^6$ cells mL^{-1} rehydrated microcarriers and injected intraperitoneally.

Immunostaining

For immunofluorescence staining iPSCs were cultured into slide flasks (NUNC) on irradiated HFF in HES medium, ECs were plated on 12 mm Ø dish glass pre-coated with 0.1% gelatin (Sigma-Aldrich) at concentration of $2x10^4$. Cells were fixed in PAF 4% for 5', for nuclear staining 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). Mouse tissues were fixed in 4% PFA for 2h at 4°C, equilibrated in sucrose, and embedded in cryostat embedding medium (Bio-Optica). Cryostat sections of 4μm thickness were blocked in buffer containing 5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS, incubated with primary antibody for 2 hours RT and then incubated for 45' RT in dark with the secondary antibody. For nuclei detection DAPI or TOPRO were added to the secondary antibodies solution. Primary and secondary antibodies used and dilutions are reported in Table 3.

FVIII activity

Plasma samples of transplanted mice and supernatants of LV-VEC-hBDDFVIII corrected ECs were analyzed for FVIII activity by aPTT. Standard curves were generated by serial dilution of pooled human plasma in hemophilic mouse plasma for aPTT assay. Results were expressed in

percentage of correction. Bleeding assay was performed on anesthetized mice by cutting the distal portion of the tail at a diameter of 2.5-3 mm; the tails were then placed in a conical tube containing 14 ml of saline at 37 °C and blood was collected for 3'. 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.

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 RT-PCR and Real Time

	Synthetic oligonucleotide	Attempted band
hβ-actina	S: 5’-GAGAAAATCTGGCACCACACC-3’	400 bp
	A: 5’-CGACGTAGCACAGCTTCTC-3’	
Oct4	S: 5’-CGTAAGCAGAAGAGGATCACC-3’	179 bp
	A: 5’-GCTTCCTCCACCCACTTCTGC-3’	
Sox2	S: 5’-GCAGCTACAGCATGATGCAGG-3’	134 bp
	A: 5’-AGCTGGTCATGGAGTTGTACTGC-3’	
Klf4	S: 5’-CCAGAGGAGCCCAAGCCAA-3’	130 bp
	A: 5’-CGCAGGTGTGCCTTGAGATG-3’	
NCAM	S: 5’-ATGGAAACTCTATTAAAGTGAACCTG-3’	178 bp
	A: 5’-TAGACCTCATACTCAGCATTCCAGT-3’	
Nestin	S: 5'-CAGCGTTGGAACAGAGGTTGG-3'	388 bp
	A: 5'-TGGCACAGGTGTCTCAAGGGTAG-3'	
αSMA	S: 5’-CTGTTCCAGCCATCCTTCAT-3’	316 bp
	A: 5’-CGGCTTCATCGTATTCCTGT-3’	
Brachiury	S: 5’-CGGAACAATTCTCCAACCTATT-3’	357 bp
	A: 5'-GTACTGGCTGTCCACGATGTCT -3'	
AFP	S: 5'-ACTCCAGTAAACCCTGGTGTTG-3'	255 bp
	A: 5'-GAAATCTGCAATGACAGCCTCA-3’	
FoxA2	S: 5’-ATGCACTCGGCTTCCAGTAT-3’	577 bp
	A: 5’-GGTAGATCTCGCTCAGCGTC-3’	
KDR	S: 5'- TGCAAGGACCAAGGAGACTATGT -3'	457 bp
	A: 5'- TAGGATGATGACAAGAAGTAGCC -3'	
Tie-2	S: 5’-AGACCAGCACGTTGATGTGA-3’	126 bp
	A: 5’-TGGGTTGCTTGACCCTATGT-3’	
VEC	S: 5'-CAGCCCAAAGTGTGTGAGAA-3'	162 bp
	A: 5'-TGTGATGTTGGCCGTGTTAT-3'	
CD105	S: 5’-GCCAGCATTGTCTCACTTCA-3’	134 bp
	A: 5’-GGCACACTTTGTCTGGATCA-3’	
CD31	S: 5'-AGGTCAGCAGCATCGTGGTCAACAT-3'	450 bp
	A: 5'-GTGGGGTTGTCTTTGAATACCGCAG-3'	
VWF	S: 5'- GTTCGTCCTGGAAGGATCGG -3'	697 bp
	A: 5'- CACTGACACCGTAGTGAGAC -3'	
FVIII	S: 5'- GGAGAGTAAAGCAATATCAGATGC -3'	400 bp
	A: 5'- GGTGAATTCGAAGGTAGCGAC -3'	
MRC1	S: 5’- GGGCAGTGAAAGCTTATGGA-3’	162 bp
	A: 5’- CCTGTCAGGTATGTTTGCTCA-3’	
Wpre/ dNEF	S: 5’-TGGATTCTGCGCGGGACGTC-3’	200 bp
	A: 5’- GGCTAAGATCTACAGCTGCCTTG-3’	
GAPDH	S: 5’-AACGTGTCAGTGGTGGACCTG-3’	160 bp
	A: 5’- AGTGGGTGTCGCTGTTGAAGT-3’	
NanogMet	S: 5’- TGGTTAGGTTGGTTTTAAATTTTTG-3’	336 bp
	A: 5'- ACCCACCCTTATAAATTCTCAATTA-3’	

Table 2: Antibodies used for FACS

Antibody	Reactivity	Manufacturer	Format
CD45	human	MiltenyiBiotec	FITC
CD3	human	Immunotools	FITC
CD11b	human	Immunotools	PE
CD19	human	MiltenyiBiotec	PE
CD14	human	MiltenyiBiotec	PE
KDR	human	MiltenyiBiotec	PE
Tie-2	human	MiltenyiBiotec	PE
CD31	human	Immunotools	APC/PE
VE-cadherin	human	MiltenyiBiotec	PE

Table 3: Antibodies used for immunofluorescence

Primary antibodies	Host	Reactivity	Manufacturer	Dilution
OCT4	Rabbit	Human	Abcam	1:100
SOX2	Rabbit	Human	Abcam	1:100
TRA-1-81	Rabbit	Human	Abcam	1:100
SSEA3	Mouse	Human	Millipore	1:100
FVIII	Mouse	Human	Green Mountain	1:100
CD31	Mouse	Human	BD Bioscience	1:100
VEC	Goat	Human	Santa-Cruz	1:100
GFP	Rabbit		Life Technologies	1:300
F4/80	Rat	Mouse	AbDSerotec	1:500
Secondary antibodies	**Fluorophores**		**Manufacturer**	**Dilution**
Goat anti-Rabbit	AlexaFluor 488 or 546		Life Technologies	1:500
Goat anti-Mouse	AlexaFluor 488 or 546		Life Technologies	1:500
Goat anti-Rat	AlexaFluor 546		Life Technologies	1:500
Donkey anti-Goat	AlexaFluor 546		Life Technologies	1:500

Results

Generation of iPSCs from MNCs of healthy and hemophilic donors

We isolated peripheral blood MNCs from healthy and hemophilic donors and we characterized freshly isolated cells and after 5 days of culture. Freshly isolated cells were CD3 (76%), CD11b (42%), CD14 (18%) and CD19 (8%) positive after flow cytometry analysis (Fig. 1a). Meanwhile, after 5 days of culture, cells were mainly CD3+ (86%), CD11b (19%) and CD19+ (10%) (Fig. 1b), thus were mainly lymphocytes and monocytes. Hemophilic MNC were corrected for FVIII expression with an LV carrying the B domain deleted form of FVIII, under the control of phosphoglycerate kinase (PGK) promoter (LV.PGK.hBDD-FVIII; Fig. 2c). Then, we reprogrammed cells with a policystronic LV carrying Oct4, Sox2 and Klf4(LV.SFFV.OSK; Fig. 2a).We obtained iPSCs from 2 of 5 healthy donors and 2 of 20 hemophilic patients (MNC-iPSCs and MNC-HA-iPSCs, respectively).We obtained 1-4good quality clones for each successful reprogramming. These clones reached high passages in culture (more than 50) and were positive at pluripotency assays. Healthy and hemophilic iPSCs showed ESC-like morphology, they were compact and with defined borders (Fig. 3a and c, respectively). AP staining positivity (Fig. 3b and d) displayed the reactivation of the enzyme. iPSCs expressed endogenous reprogramming factors, Oct4, Sox2 and Klf4 (Fig.3e and f), while RT-PCR, using primers specific for LV cassette, confirmed that exogenous factors were turned off (Fig. 3g). iPSCs expressed nuclear and surface pluripotent cells antigens, Oct4, Sox2 and SSEA-3 as shown by immunofluorescence (Fig. 3h and i). Moreover, an increase in telomere length demonstrated the reactivation of telomerase complex, crucial for the stem cells self renewal, both in healthy and hemophilic iPSCs (Fig. 3 k and l). However, the analysis of NANOG promoter methylation profile showed that only the 30% of analyzed CpG islands in NANOG promoter were unmethylated in iPSCs (Fig. 3j), suggesting that cells did not reach a complete reprogramming at the epigenetic level.

Interestingly, we obtained hemophilic iPSCs only from patient FVIII-corrected MNC. However, early-passage iPSCs did not express FVIII (Fig. 3m), although LV.PGK.hBDD-FVIII (Fig. 2c) was integrated in transduced cells (Fig. 3n). We supposed that PGK promoter was silenced during reprogramming process, indeed deep epigenetic changes occurred that probably influenced the PGK promoter activity in the LV expression cassette. To overcome this problem we decided to correct HA cells after cell reprogramming with a LV carrying the hBDD-FVIII transgene under control of an endothelial specific promoter (VE-cadherin promoter, VEC) (LV.VEC.hBDD-FVIII; Fig. 2d).

Next, we evaluated the differentiation potential of the obtained iPSC colonies by *in vitro* embryoid body (EB) formation and differentiation assay. RT-PCR analysis on EBs showed the expression of markers of the three germ layers (Nestin, NCAM and Otx2 for ectoderm; αSMA,

Brachiury and Tbx6 for mesoderm; AFP, FOXA2 and SOX17 for endoderm; Fig. 3o). Moreover, EBs efficiently differentiated in adipogenic, osteogenic and chondrogenic cells as assessed by OilRed O staining, Alizarin Red staining and immunofluorescence for collagen II, respectively (Fig. 3p, q and r).

Differentiation of healthy iPSCs into endothelial cells

Since LSECs were recognized as FVIII producers and sinusoidal endothelial cells could act as non-professional antigen presenting cells and induce immunotolerance[42-44, 78] we decided to differentiate obtained iPSCs in this cell type. During endothelial differentiation MNC-iPSCs changed morphology acquiring the cuboidal shape typical of endothelial cells (Fig. 4a and b, 10 and 20 days after the beginning of differentiation, respectively). Analysis of gene expression showed an increase in endothelial markers such as KDR, CD31, VEC and, interestingly, FVIII (Fig. 4c). Immunofluorescence showed the co-staining between FVIII and vWF (Fig. 4d). As further demonstration of endothelial differentiation, we transduced the obtained cells with LVs expressing GFP under the control of endothelial specific promoters Tie2 and Flk-1 and we used LV.PGK.GFP as positive control. 60% of transduced cells expressed GFP under the control of Flk1 promoter, 50% under Tie2 promoter and 85% under PGK (Fig. 4e). These results confirmed that our cells started to differentiate in EC. To investigate the engraftment capability of differentiated cells, we transplanted Flk-1-GFP+ cells by portal vein injection in MCT (200mg/kg) –treated NOD-SCID HA mice. Immunofluorescence staining on liver sections of transplanted mice showed the presence of GFP+ cells 96 hours after transplantation. By confocal analysis, we detected cells near blood vessels without a significant mononuclear infiltration around transplanted cells as shown by F4/80 staining (Fig. 4f).

Genetic correction of hemophilic iPSCs and differentiation into endothelial cells

Before EBs formation, MNC-HA-iPSCs were transduced with the LV carrying the hBDD-FVIII under the control of the endothelial-specific VEC promoter and, as control of VEC promoter activation during differentiation, with LV carrying GFP instead of FVIII. During the differentiation cells changed morphology and started to express endothelial markers (KDR, Tie2, CD31) as shown by RT-PCR and FACS analysis (Fig. 5a, b). Interestingly, LV.VEC.hBDD-FVIII transduced cells expressed FVIII (Fig. 5a), indicating that correcting cells after reprogramming with an endothelial specific promoter allowed to circumvent the obstacle of promoter silencing. Telomere length analysis showed a progressive shortening in iPSC-derived ECs, demonstrating the telomerase complex switching off typical of differentiated cells (Fig. 5c). Despite these results, VEC was not

expressed, indicating that obtained endothelial cells did not reached a mature stage of differentiation and this was also confirmed by tubulogenesis assay. Indeed, MNC-iPSCs-derived ECs did not give rise to a stable tubules network when plated in matrigel (data not shown).

All together, these results on both healthy and hemophilic iPSCs differentiation suggested that we obtained a mixed population of differentiated cells, which did not reach the mature stage. Indeed, cells expressed early endothelial markers such as KDR, but low levels or no mature ones, such as VEC. Given the fact that iPSCs from MNC were not reprogrammed at the epigenetic level, we suppose that the epigenetic memory of iPSCs made these cells resting and not able to differentiate. Thus, we decided to generate iPSCs starting from other cell types more prone to be reprogrammed.

Generation of iPSCs from different cell sources of healthy and hemophilic donors

The results obtained from MNC reprogramming showed that we generated iPSCs with a low efficiency. The first strategy to improve the protocol was to use a new reprogramming vector. Indeed, we reprogrammed cells using both LV.SFFV.OSK and a LV carrying, other than reprogramming factor, the miR302/367 cluster (LV.SFFV.OSK.miR302/367, Fig. 2b), master regulators in the maintenance of hESCs stemness [235]. Moreover, we considered different cell sources for the generation of iPSCs to evaluate the less invasive procedure for the donors to recover the starting cells to obtain iPSCs with higher efficiency. Thus we started reprogramming hemophilic patients' skin fibroblasts. The generated iPSCs came out after 2 months of culture showing ES-like morphology (Fig. 6a), were positive for alkaline phosphatase (Fig. 6b), expressed the endogenous stem cells transcription factors (Fig. 6c) while the exogenous reprogramming factors were silenced (Fig. 6d). Immunofluorescence showed the expression of stem cells nuclear and surface antigens (Oct4, Sox2, Tra1-81, Ssea-4; Fig. 6e) and Nanog promoter analysis evidenced that 40% of analyzed CpG were in an unmethylated state (Fig. 6f). In parallel, we generated iPSCs from CD34+ cells isolated from cord blood and we obtained good quality clones (Fig. 7a) as shown by AP staining (Fig. 7b), positive RT-PCR for endogenous markers but negative for exogenous factors (Fig. 7c and d) and immunofluorescence (Fig. 7e). Because CD34+ cells from peripheral blood are an easy cell source to obtain compared to fibroblasts, we utilized CD34+ cells from peripheral blood and in parallel we reprogrammed MNCs using LV.SFFV.OSK.miR302/367. From MNC reprogramming we did not establish iPSC clones. In contrasts, starting from CD34+ cells we obtained better results. In 7 independent experiments (2 healthy donors, 1 heterozygous control and 4 hemophilic patients), we isolated a mean of 250.000 non-mobilized CD34+ cells from 20 ml of peripheral blood. After culture and expansion we obtained about 300,000-350,000 cells that we

transduced with both reprogramming LVs. Colonies appeared about 20 days after reprogramming. We obtained about 20 clones from the reprogramming with LV.SFFV.OSKand more than 30 clones from LV.SFFV.OSK.miR302/367. LV.SFFV.OSK-derived iPSCs degenerated rapidly. Indeed, we were able to culture only four of these clones. On the contrary, almost all from LV.SFFV.OSK.miR302/367-derived iPSCs reached advanced passages.We established a mean of 11 clones for each donor. In preliminary experiments, we used different MOI (5 and 10) for transduction reprogramming and the yield of iPSC colonies did not increase as MOI was increased. Thus, we chose to use a MOI of 5 because the efficiency of transduction was high enough so that the yield of iPSCs was adequate but low enough so that most iPSC clones had a copy number between 1 and 2 (Table 1). Indeed, when copy number was determined by Real Time analysis for the high quality iPSC clones, they showed a mean of 1.6 for healthy iPSCs and 1.4 for hemophilic iPSCs inserted copies/cell (Table 1).These good quality clones reached high passages (more than 50) maintaining a stable karyotype. iPSCs colonies showed ESC-like morphology (Fig. 8a and b).We also analyzed iPSCs for pluripotency markers including AP positivity (Fig. 8c and d), expression of endogenous (Fig. 8e and f) and not exogenous reprogramming factors (Fig. 8g and h) and stem cells nuclear and surface antigens (Oct4, Sox2, Tra1-81, Ssea-4; Fig. 8i and j). Moreover, NANOG promoter methylation profile showed that the 63% of analyzed CpG islands were unmethylated both in healthy and hemophilic iPSCs while in CD34+ cells from healthy and hemophilic donors the 92% and 95% respectively of sites were methylated (Fig. 8k and l). Telomere length increased between P5 and P20 (the longest passage analyzed) demonstrating the reactivation of telomerase complex both in healthy and hemophilic iPSCs (Fig. 8 m and n). These results showed that CD34+-derived iPSCs underwent to a complete reprogramming, expressing stem cells markers at deeper level than previously described MNC-iPSCs. Moreover, iPSCs were able to generate EBs which expressed three germ layers markers (Nestin, NCAM and Otx2 for ectoderm; αSMA, brachiury and Tbx6 for mesoderm; AFP, FOXA2 and SOX17 for endoderm; Fig. 8o) and were able to differentiated into adipogenic, osteogenic and chondrogenic cells as assed by OilRed O staining, Alizarin Red staining and immunofluorescence staining for collagen II, respectively (Fig. 8p, q and r).

Differentiation of CD34+-iPSCs into endothelial cells

During differentiation cell acquired an endothelial-like morphology (Fig. 9a). We extracted RNA at day 10 and 20 from the beginning of differentiation. RT-PCR analysis showed an increase of endothelial markers, both early, such as KDR, and mature, like Tie-2, CD31 and VEC (Fig. 9b). We further confirmed a good endothelial gene expression was by FACS analysis (Fig. 9c). Indeed,

37% of ECs were positive at KDR staining, 40% for Tie-2, 65% for CD31 and 64% for VEC (Fig. 9c). Interestingly, ECs expressed at higher levels mature markers CD31 and VEC than the earlier KDR, showing that the obtained cells were not progenitors but reached advanced stage of differentiation. To confirm that we obtained mature differentiated cells we analyzed telomere length and NANOG methylation profile comparing the ECs to the parental iPSCs. ECs showed a decrease telomere length meaning the telomerase complex switching off, typical of differentiated cells(Fig. 9d). NANOG methylation profile analysis indicated a 97 % of methylated CpG islands at NANOG core promoter (Fig. 9e). These data documented that ECs can be efficiently derived from CD34+-iPSCs. Moreover, to assess that obtained ECs were able to form vessels-like structures, *in vitro* tubulogenesis assay was performed. iPSC-derived ECs were able to form good tubules network after 16-18 hours in matrigel, demonstrating the acquired endothelial functionality (Fig. 9f).

To demonstrate the efficiency of the endothelial differentiation protocol used on these cells, we transduced the ECs with LVs expressing GFP under the control of Flk-1, Tie-2 and VEC endothelial specific promoters (LV.Flk-1.GFP, LV.Tie-2.GFP, LV.VEC.GFP), the ubiquitous PGK promoter (LV.PGK.GFP) as positive transduction control. We used TTR hepatocytes specific and CD11b myeloid specific promoters as negative control. FACS analysis showed that 72% of transduced cells were positive for GFP under Flk-1 promoter, 61% under Tie-2, 55% under VEC, while only 6% and 4% were positive under TTR and CD11b promoters respectively (Fig. 9f). These results demonstrated that the cells became endothelial by the functionality of the endothelial promoters in terminally differentiated iPSC-derived ECs. This means that during the differentiation specific endothelial transcription factors were expressed to push cells to acquire the desired phenotype. The differences in the expression reflected the heterogeneous populations we obtained from the endothelial differentiation. Indeed, KDR and Tie-2 are early stage markers of endothelial differentiation, while VEC is a more mature markers.

In order to remove the exogenous reprogramming cassette introduced by the reprogramming transduction, the obtained ECs were transduced by the ID-LV carrying the Cre recombinase. The recombinase efficiently excised the reprogramming LV LoxP-flanked cassette, indeed the copy number analysis revealed a mean of 0.05 integrated copy/cells, while ECs pre-transduction contained 2.49 copies/cell (Table 1b). An efficient excision of exogenous stem cell factors was crucial to avoid their reactivation in terminally differentiated iPSC-derived ECs.

Hemophilic CD34+-iPSCs were genetically corrected for FVIII expression and differentiated into endothelial cells

Before endothelial differentiation induction, we genetically corrected iPSCs by transduction with LV.VEC.FVIII (Fig. 2d). In parallel we transduced cells with an LV carrying GFP under VEC

promoter control (LV.VEC.GFP) in order to check the reactivation of the promoter during differentiation through GFP expression evaluation. Then, EBs were formed from both transduced HA-CD34+-iPSCs and not transduced (NT) iPSCs. At day 20 of differentiation, 31% of ECs derived from VEC-GFP-iPSCs expressed GFP at FACS analysis (Fig 10a), demonstrating that the endothelial specific promoter VEC was turned on during differentiation. Obtained VEC.FVIII, VEC.GFP and NT-ECs acquired endothelial-like morphology. RT-PCR (Fig. 10b) and FACS analysis (Fig. 10c) showed the expression of endothelial markers such as CD105, KDR, Tie-2, CD31 and VEC, both in transduced and not transduced ECs. Moreover, immunofluorescence staining (Fig. 10d and e) revealed the expression of endothelial mature markers as CD31 and VEC. Moreover, VEC-FVIII-ECs expressed FVIII and, interestingly, vWF, another endothelial marker and FVIII carrier in the plasma (Fig. 10b). FVIII expression was confirmed by immunofluorescence staining that revealed FVIII presence near nuclei and, in smaller amount, in the cytoplasm of VEC.FVIII.ECs (Fig. 10e). Telomere length analysis showed a progressive shortening overtime in differentiated cells and NANOG promoter returned in a methylated state after differentiation. These results showed the mature stage of differentiation reached by HA CD34-iPSCs-derived ECs (Fig. 10f and g, respectively). As previously described for healthy ECs, also in hemophilic ECs the exogenous reprogramming cassette was efficiently removed by Cre-recombinase. Post-transduction HA-ECs showed a copy number of 0.07 integrated copies/cells while pre-transduction they had 2,48 copies/cells (Table 1b). *In vitro* tubulogenesis assay showed that ECs were able to form tubules network (Fig. 10h). To assess if FVIII was not only produced but also efficiently secreted, aPTT assay was performed on culture medium of VEC.FVIII., VEC.GFP. and NC-ECs. This analysis revealed a shortening in aPTT of VEC-FVIII-ECs compared with NT- and VEC-GFP-ECs (Fig. 10i). All together, these results demonstrated that HA-CD34-iPSCs acquired the morphology, the gene expression pattern and the functionality of mature endothelial cells. Moreover, they were genetically corrected by LV transduction with high efficiency. Then, during differentiation VEC promoter was switched on and FVIII was produced and secreted by HA-CD34-iPSCs-derived ECs at the end of differentiation process.

***In vivo* FVIII expression and hemophilic phenotype correction after transplantation of genetically corrected iPSCs-derived ECs in a mouse model of HA**

Since iPSCs were successfully differentiated into FVIII-expressing ECs we evaluated FVIII expression and secretion into our mouse model of HA. First, we assessed that ECs injected in peritoneal cavity survived and were able to secrete FVIII at therapeutic level. 10^7GFP+ healthy iPSCs-derived ECs, hemophilic NC-ECs and VEC-FVIII-ECs were injected in association with

microcarrier beads (n=4 each condition). Then FVIII activity was evaluated by aPTT assay 3 and 7 days after injection. At 3 days FVIII activity in mice injected with healthy iPSCs-derived ECs was 2,7±0,5% and with VEC-FVIII-ECs 4,9±1,3% while negative controls (beads only and beads associated with HA NC-ECs) did not show FVIII activity (Fig. 11a). At 1 week after injection FVIII activity was maintained, indeed healthy iPSCs-derived ECs injected mice showed 2,1±0,4% FVIII activity and VEC-FVIII-ECs injected mice 5,0±0,8% (Fig. 11a). These results demonstrated that VEC-FVIII-ECs were able to secrete FVIII *in vivo* at therapeutic levels superior than healthy iPSCs-derived ECs. Then immunofluorescence on recovered beads demonstrated that GFP+ cells were still present near the beads (Fig. 11b). These findings confirmed that iPSCs-derived ECs survived and secreted functional FVIII when injected in the peritoneal cavity.

Following the successful peritoneum injection, we transplanted 2x10^6 healthy GFP+ iPSCs-derived ECs into the livers of monocrotalin (MCT)-conditioned γNull mice to evaluate the engraftment and proliferation of transplanted cells into liver of host mice. We observed cell engraftment and proliferation at 1 week, 4, 8 and 12 weeks after transplantation and we evaluated the presence of GFP+ cells by immunofluorescence on liver section. 1 week after transplantation GFP+ iPSCs-derived ECs engrafted in liver parenchyma without a significant immune response (Fig. 11c). 4 weeks after transplantation cells proliferated in transplanted mice liver (Fig. 11c). The co-staining with human CD31 and the spindle shape morphology confirmed the endothelial phenotype of transplanted cells (Fig. 11c). The proliferation went on up to 3 months after transplantation, the longest time point analyzed (Fig. 11c). Transplanted cells maintained endothelial phenotype, expressing human CD31 (Fig. 11c). Moreover, transplanted cells formed vessels-like structure in the host liver (Fig. 11c). These results demonstrated that healthy iPSCs-derived ECs were able to engraft and proliferate in mouse liver after transplantation. Thus, we transplanted VEC-FVIII-ECs in MCT-condition γNull-HA mice to evaluate FVIII secretion and phenotype correction. The engraftment was also confirmed by immunofluorescence staining, indeed after 1 week GFP+ iPSCs-derived ECs engrafted in liver parenchyma. Nine weeks after transplantation cells proliferated in transplanted mice liver (Fig. 11d). The co-staining with human CD31 and human VEC confirmed the endothelial phenotype of transplanted cells (Fig. 11d). To evaluated FVIII activity aPTT assay was performed at 3, 6, 9 and 12 weeks after transplantation. The relative FVIII activity in mice transplanted with VEC-FVIII-ECs was 2,8±0,5% after 3 weeks and increased at 4,2±0,7% after 6 weeks and remained stable at 9 and 12 weeks (4,6±0,3% and 4,7±0,7 respectively), while in mice transplanted with non-corrected cells no coagulation activity was detected (Fig. 11e). Moreover, to evaluate the improvement of coagulation, bleeding assay was performed on treated mice 12 weeks after transplantation. Mice were challenged by tail clip and

blood loss was measured after 3 minutes of bleeding. Compared to mice transplanted with non-corrected HA-ECs, mice who received VEC-FVIII-ECs stopped to bleed before the end of the assay, because the FVIII levels restored in these mice was sufficient to sustain the primary hemostasis. As result, the amount of blood lost by mice transplanted with corrected cells was reduced, demonstrating an improvement of coagulation capability (Fig. 11f). The transplantation experiment was recently repeated to reproduce data and to evaluate a longer time points (up to 6 months). In this case the relative FVIII activity in mice transplanted with VEC-FVIII-ECs was about the 2% after 3 weeks and then remained stable. Additionally, at one month a fewer number of cells engrafted in the liver parenchyma. This data confirmed that VEC-FVIII-ECs were able to restore FVIII activity, but at a lower level suggesting that the variability of results obtained from differentiation of primary cells influenced the cells capability to engraft and proliferate *in vivo*. However, taken together, these results allowed claiming that hemophilic phenotype could be rescued by transplantation of ECs derived from HA-iPSCs and corrected by LV carrying FVIII under the control of endothelial-specific promoter VEC.

Figure 1.

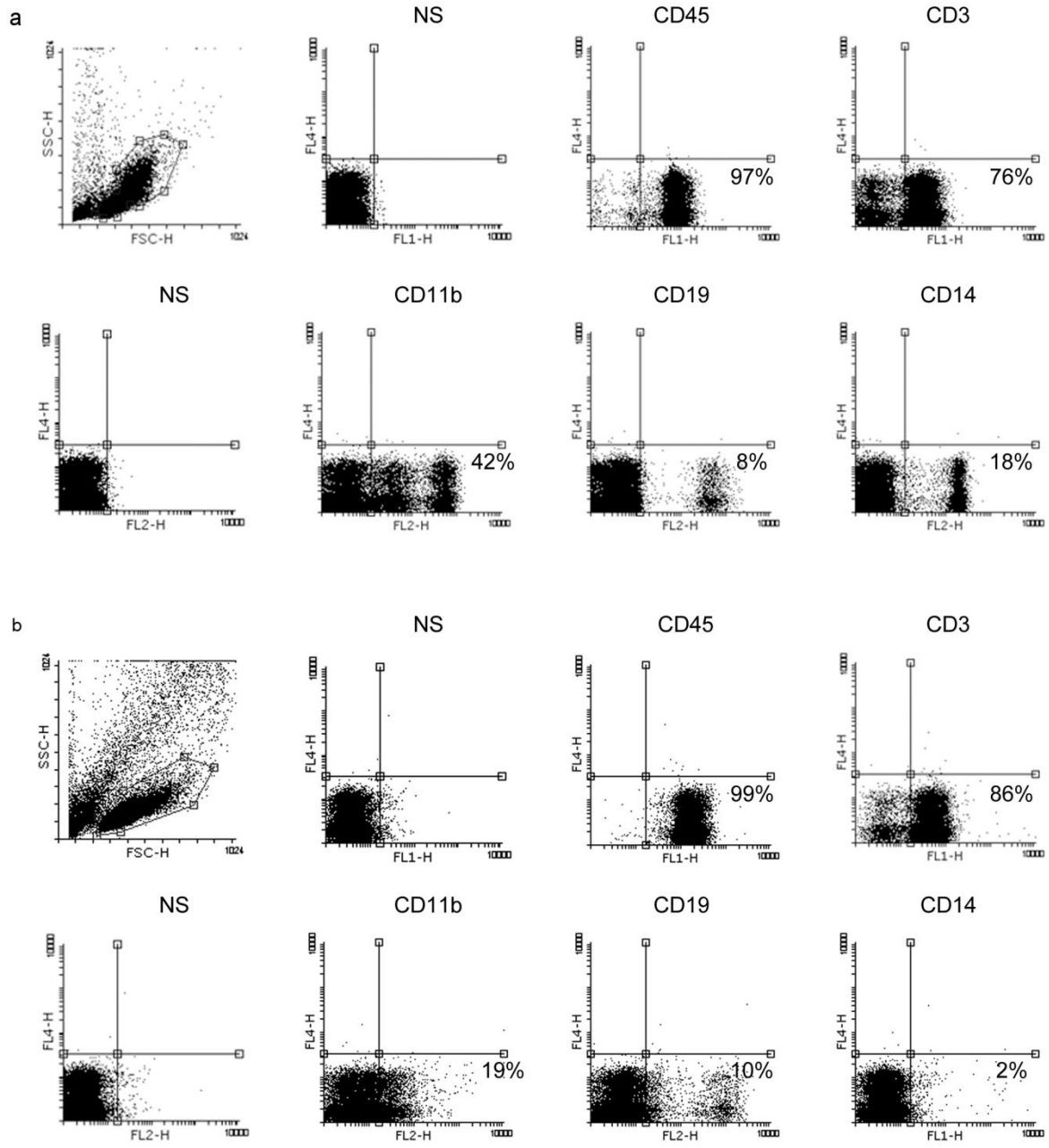
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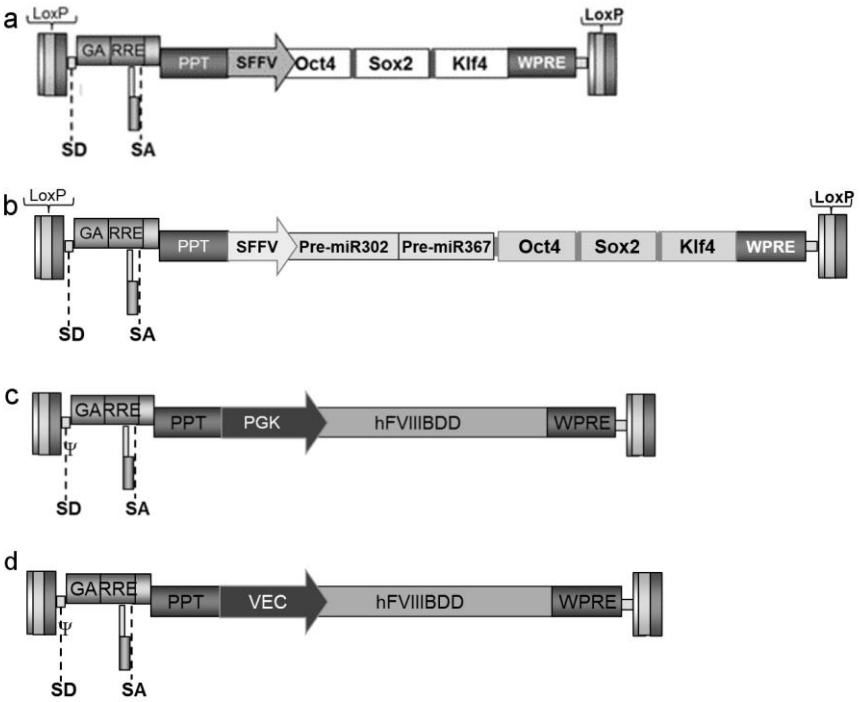
Figure 2.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PPT
SFFV
Pre-miR302
Pre-miR367
Oct4
Sox2
Klf4
WPRE
LoxP
SD
SA
c
GA
RRE
PPT
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VEC
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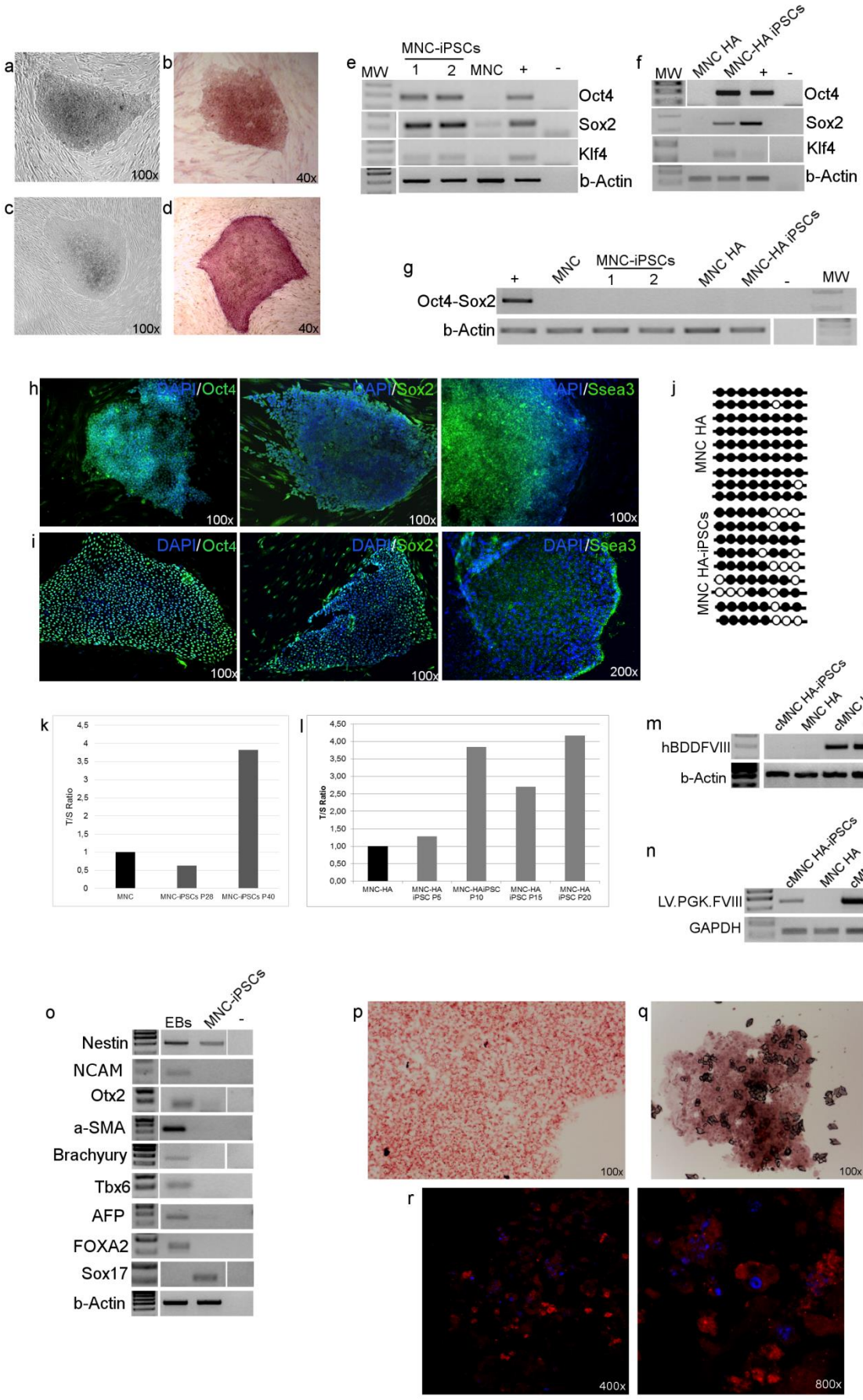
WPRE
SD
SA

Figure 3.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2
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+
-
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b-Actin
f
MW
MNC HA
MNC-HA iPSCs
g
Oct4-Sox2
h
DAPI/Oct4
DAPI/Sox2
DAPI/Ssea3
i
200x
j
MNC HA
MNC HA-iPSCs
k
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MNC-iPSCs P40
l
MNC-HA
MNC-HA iPSC P5
MNC-HA iPSC P10
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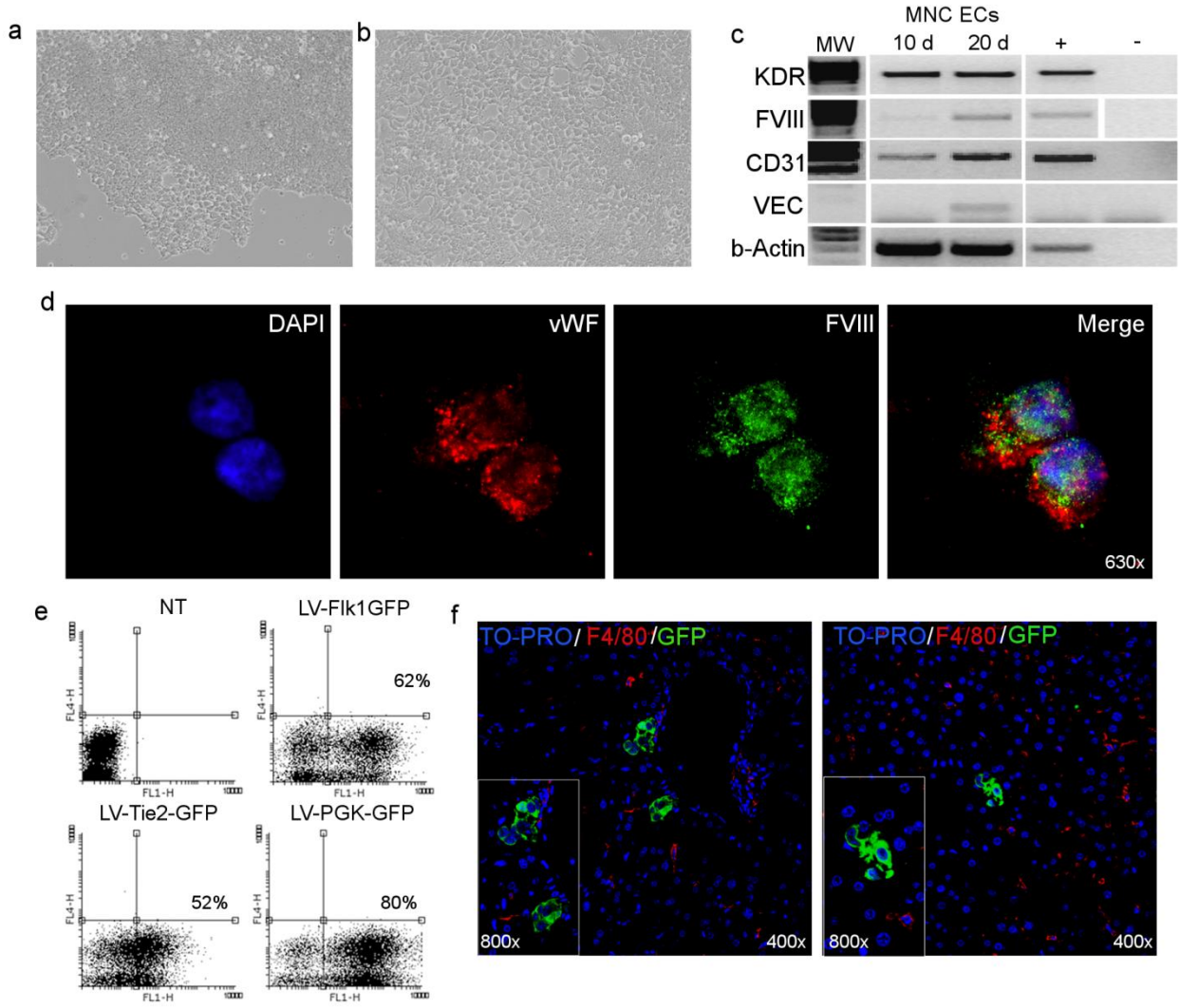
Figure 4.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20 d
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80%
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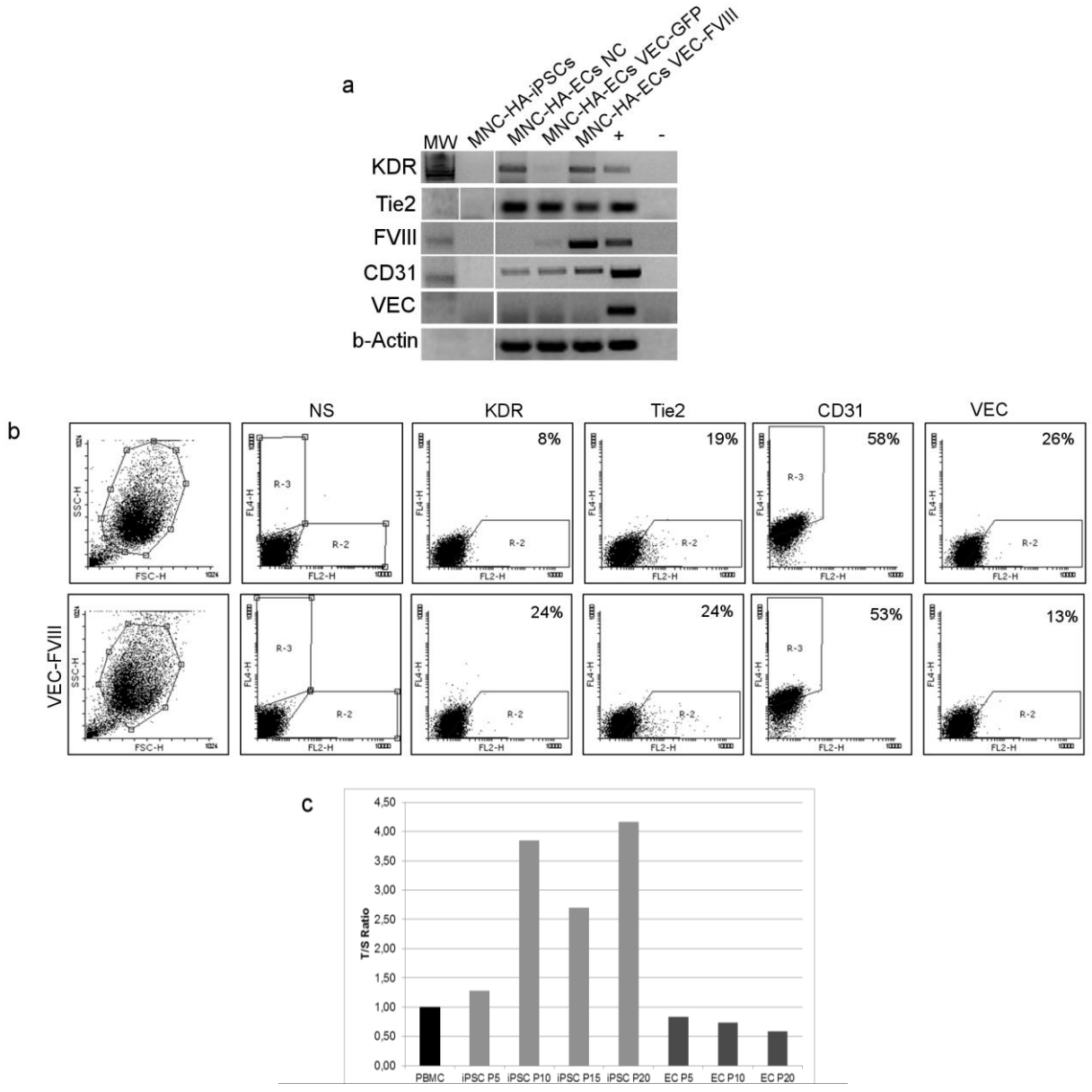
Figure 5.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MNC-HA-ECs NC
MNC-HA-ECs VEC-GFP
MNC-HA-ECs VEC-FVIII
+
-
KDR
Tie2
FVIII
CD31
VEC
b-Actin
b
NS
KDR
Tie2
CD31
VEC
VEC-FVIII
8%
19%
58%
26%
24%
24%
53%
13%
R-2
R-3
SSC-H
FSC-H
FL4-H
FL2-H
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T/S Ratio
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1,00
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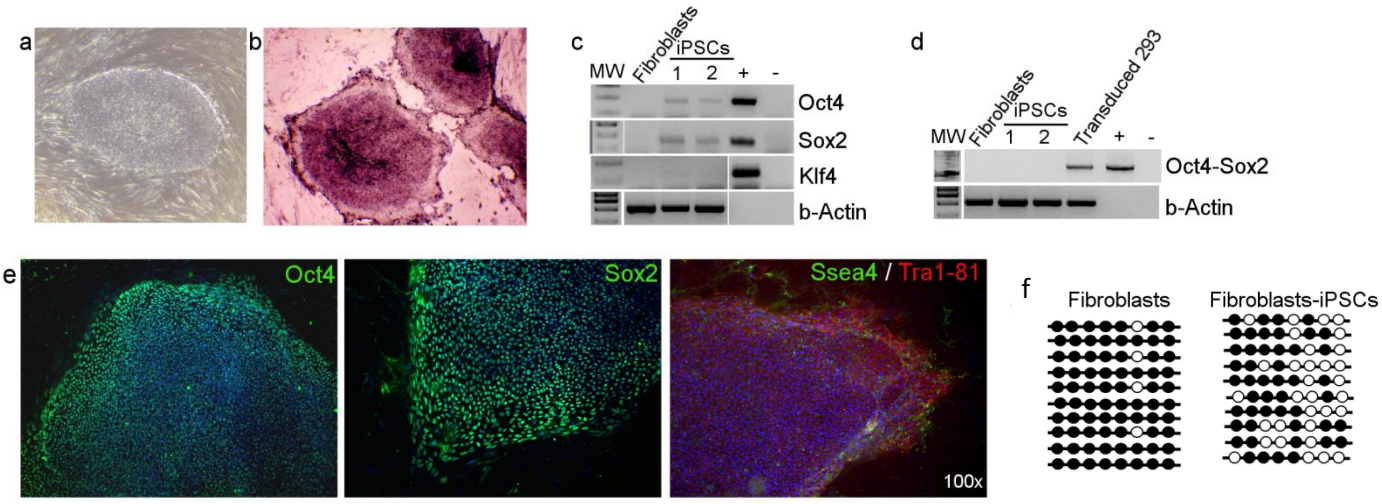
iPSC P5
iPSC P10
iPSC P15
iPSC P20
EC P5
EC P10
EC P20

Figure 6.


a
b
c
MW
Fibroblasts
iPSCs
1
2
+
-
Oct4
Sox2
Klf4
b-Actin
d
MW
Fibroblasts
iPSCs
1
2
Transduced 293
+
-
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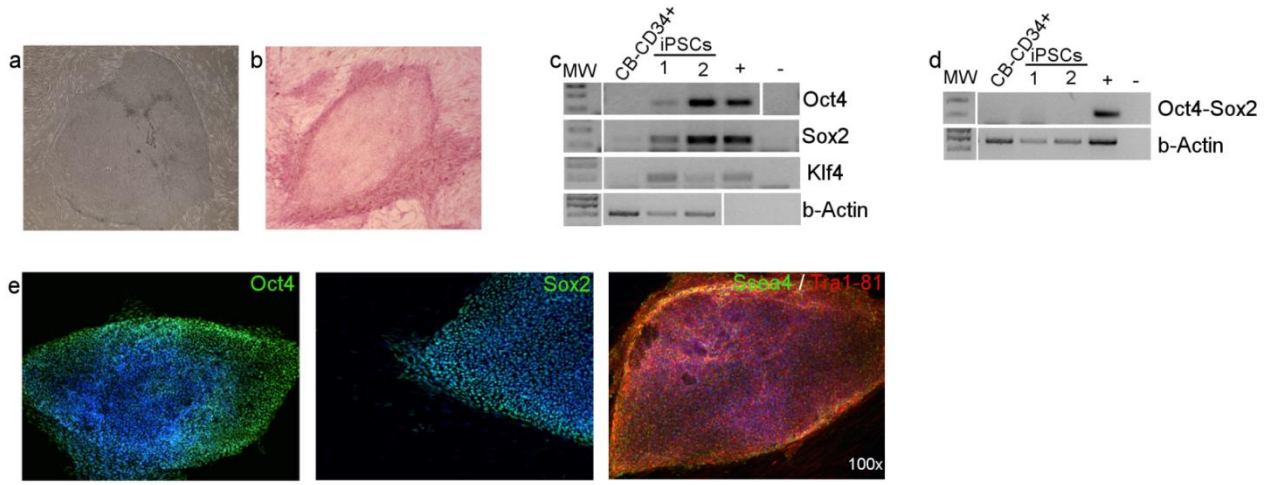
100x
f
Fibroblasts
Fibroblasts-iPSCs

Figure 7.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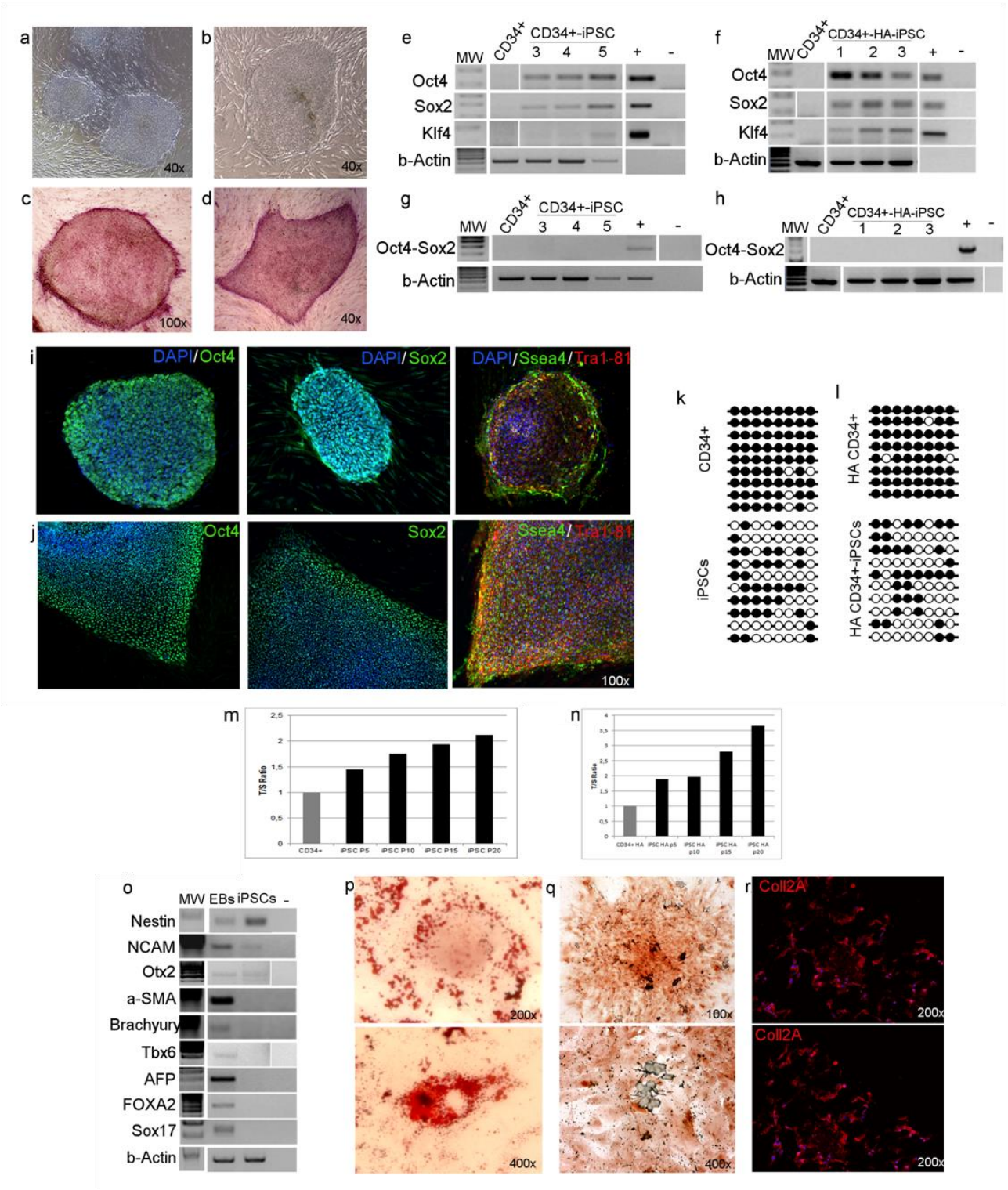
b
c
MW
CB-CD34+
iPSCs
1
2
+
-
Oct4
Sox2
Klf4
b-Actin
d
MW
CB-CD34+
iPSCs
1
2
+
-
Oct4-Sox2
b-Actin
e
Oct4
Sox2
Ssea4 / Tra1-81
100x

Figure 8.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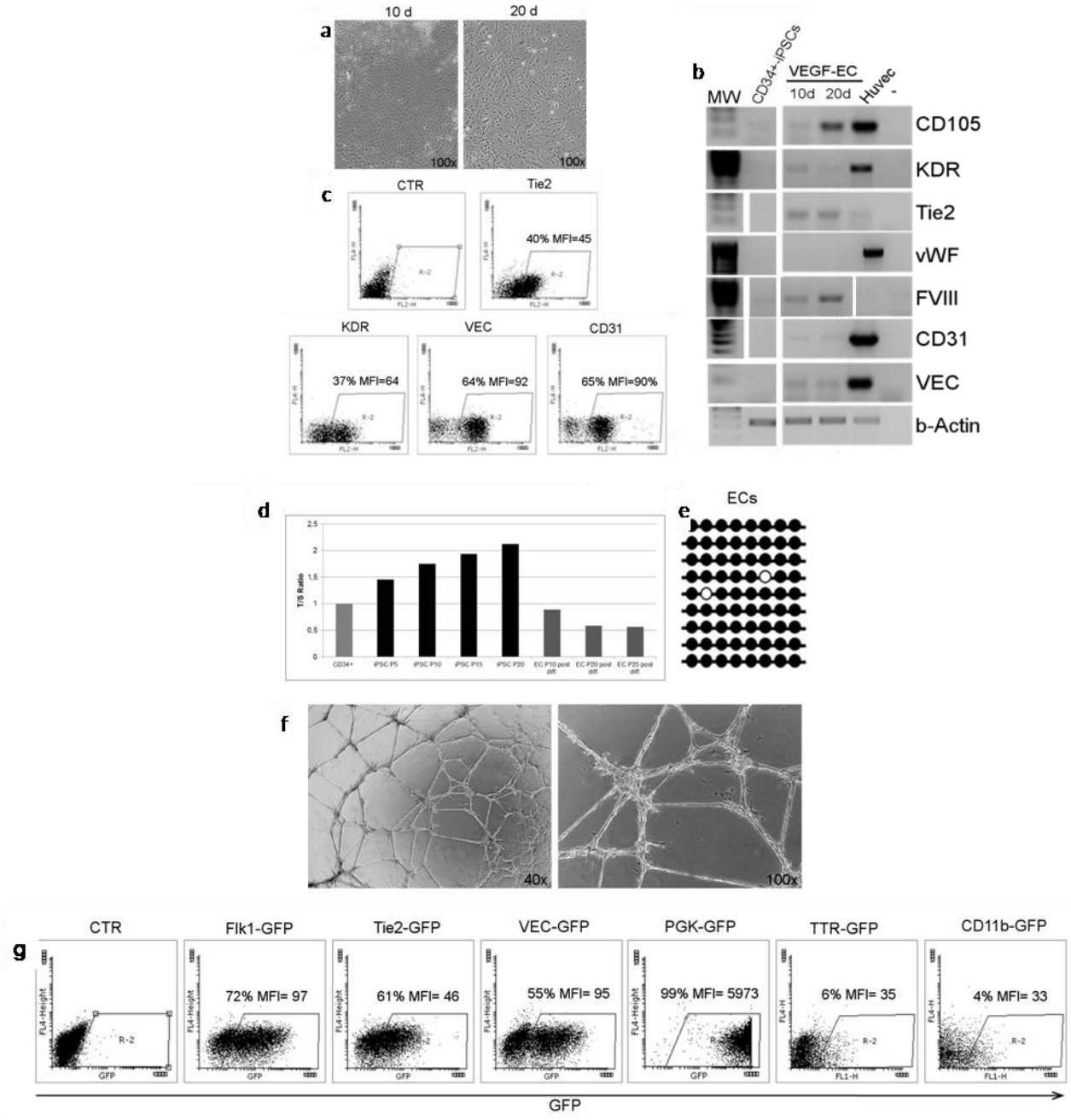
b
c
d
40x
40x
100x
40x
e
MW
CD34+
CD34+-iPSC
3
4
5
+
-
Oct4
Sox2
Klf4
b-Actin
f
MW
CD34+
CD34+-HA-iPSC
1
2
3
+
-
Oct4
Sox2
Klf4
b-Actin
g
MW
CD34+
CD34+-iPSC
3
4
5
+
-
Oct4-Sox2
b-Actin
h
MW
CD34+
CD34+-HA-iPSC
1
2
3
+
-
Oct4-Sox2
b-Actin
i
DAPI/Oct4
DAPI/Sox2
DAPI/Ssea4/Tra1-81
j
Oct4
Sox2
Ssea4/Tra1-81
100x
k
CD34+
iPSCs
l
HA CD34+
HA CD34+-iPSCs
m
T/S Ratio
CD34+
iPSC P5
iPSC P10
iPSC P15
iPSC P20
n
T/S Ratio
CD34+ HA
iPSC HA p5
iPSC HA p10
iPSC HA p15
iPSC HA p20
o
MW
EBs
iPSCs
-
Nestin
NCAM
Otx2
a-SMA
Brachyury
Tbx6
AFP
FOXA2
Sox17
b-Actin
p
200x
400x
q
100x
400x
r
Coll2A
200x
Coll2A
200x

Figure 9.


a
10 d
20 d
100x
100x
b
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CD34+-iPSCs
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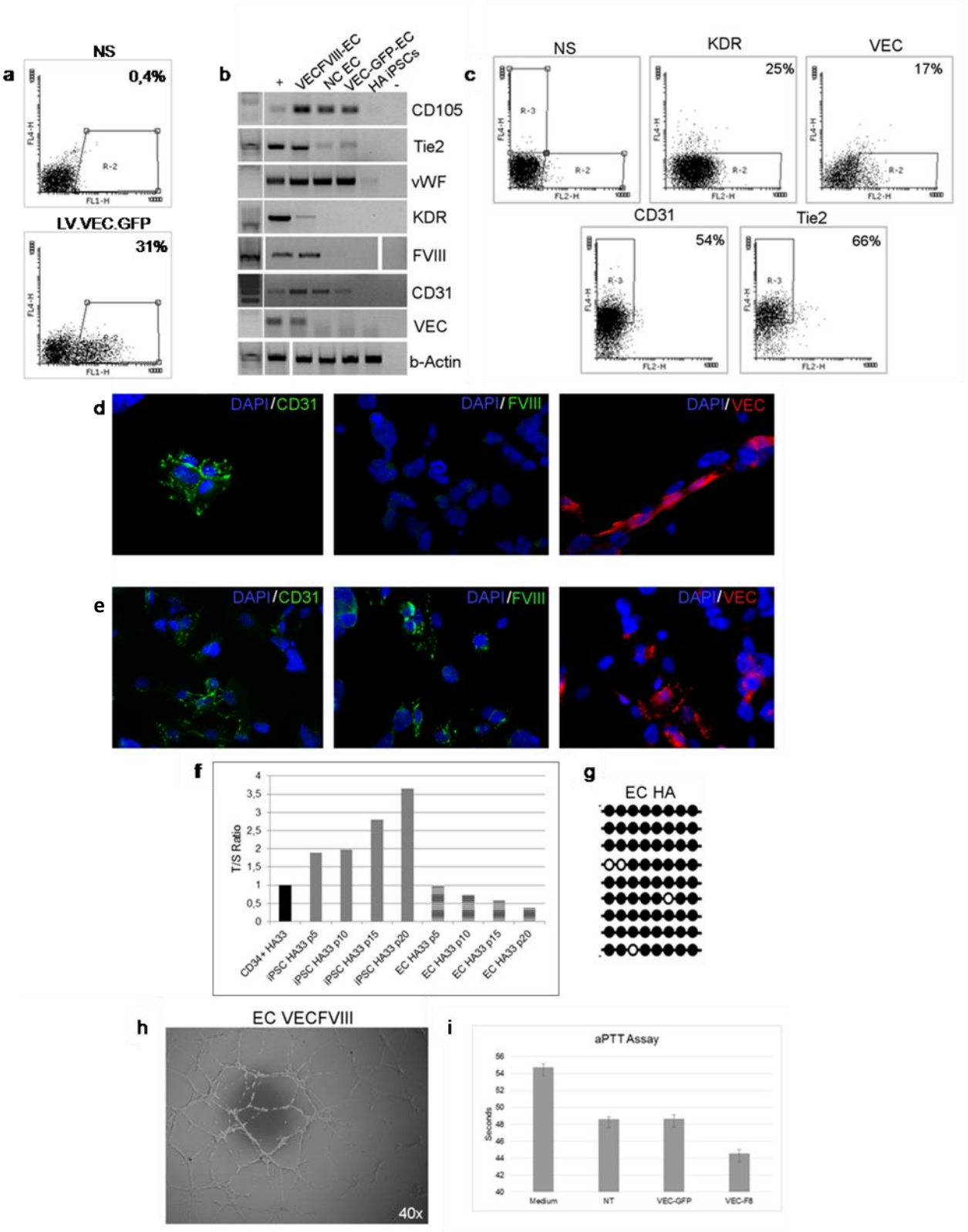
10d
20d
Huvec
-
CD105
KDR
Tie2
vWF
FVIII
CD31
VEC
b-Actin
c
CTR
Tie2
40% MFI=45
KDR
VEC
CD31
37% MFI=64
64% MFI=92
65% MFI=90%
d
T/S Ratio
CD34+
iPSC P5
iPSC P10
iPSC P15
iPSC P20
EC P10 post diff
EC P20 post diff
EC P25 post diff
e
ECs
f
40x
100x
g
CTR
Flk1-GFP
Tie2-GFP
VEC-GFP
PGK-GFP
TTR-GFP
CD11b-GFP
72% MFI= 97
61% MFI= 46
55% MFI= 95
99% MFI= 5973
6% MFI= 35
4% MFI= 33
GFP

Figure 10.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31%
b
+
VECFVIII-EC
NC EC
VEC-GFP-EC
HA iPSCs
-
CD105
Tie2
vWF
KDR
FVIII
CD31
VEC
b-Actin
c
NS
KDR
25%
VEC
17%
CD31
54%
Tie2
66%
d
DAPI/CD31
DAPI/FVIII
DAPI/VEC
e
DAPI/CD31
DAPI/FVIII
DAPI/VEC
f
T/S Ratio
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iPSC HA33 p10
iPSC HA33 p15
iPSC HA33 p20
EC HA33 p5
EC HA33 p10
EC HA33 p15
EC HA33 p20
g
EC HA
h
EC VECFVIII
40x
i
aPTT Assay
Seconds
Medium
NT
VEC-GFP
VEC-F8

Figure 11.

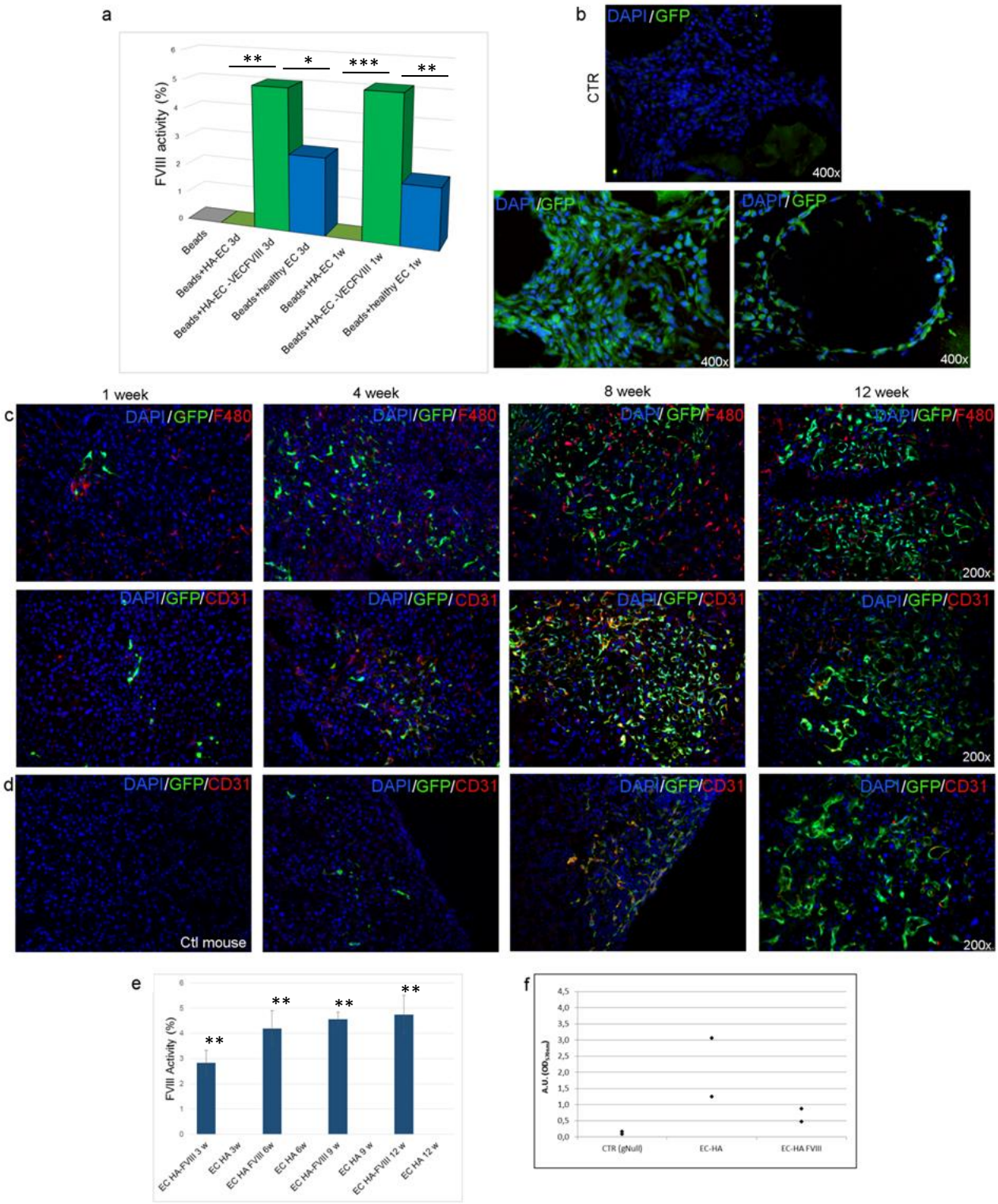
a
FVIII activity (%)
**
*
***
**
Beads
Beads+HA-EC 3d
Beads+HA-EC -VECFVIII 3d
Beads+healthy EC 3d
Beads+HA-EC 1w
Beads+HA-EC -VECFVIII 1w
Beads+healthy EC 1w
b
CTR
DAPI/GFP
400x
c
1 week
4 week
8 week
12 week
DAPI/GFP/F480
DAPI/GFP/CD31
200x
d
Ctl mouse
e
FVIII Activity (%)
EC-HA-FVIII 3 w
EC HA 3w
EC HA FVIII 6w
EC HA 6w
EC HA-FVIII 9 w
EC HA 9 w
EC HA-FVIII 12 w
EC HA 12 w
f
A.U. (OD450nm)
CTR (gNull)
EC-HA
EC-HA FVIII

Table 1.

a

Clone	Copy Number
CD34+ cells	0.00
CD34-iPSC clone 1	4.39
CD34-iPSC clone 2	0.43
CD34-iPSC clone 4	2.29
CD34-iPSC clone 5	1.96
CD34-iPSC clone 6	0.54
CD34-iPSC clone 8	0.29
CD34-iPSC clone 9	2.79
CD34-iPSC clone 10	2.15
CD34-iPSC clone 11	1.03
CD34-iPSC clone 12	0.52
Mean	1.64
HA-CD34-iPSC clone 1	0.69
HA-CD34-iPSC clone 2	1.12
HA-CD34-iPSC clone 3	0.78
HA-CD34-iPSC clone 4	1.74
HA-CD34-iPSC clone 5	2.72
HA-CD34-iPSC clone 6	2.12
Mean	1.45

b

	Copy Number
EC pre-Cre transduction	2.50
EC post-Cre transduction	0.05
EC HA pre-Cre transduction	2.48
EC HA pre-Cre transduction	0.07

Figures Legend

Figure 1-Characterization of isolated MNC.

Isolated MNC were characterized by FACS analysis for blood populations' markers at the day of the isolation and at the day of the reprogramming. Freshly isolated MNC were CD3 (76%), CD11b (42%), CD14 (18%) and CD19 (8%) positive (a). Meanwhile, after 5 days of culture, cells were mainly CD3+ (86%), CD11b (19%) and CD19+ (10%), thus were mainly lymphocytes and monocytes (b).

Figure 2 - Schematic representation of lentiviral vectors used for reprogramming and gene correction.

Healthy and hemophilic MNCs, fibroblasts, cord and peripheral blood CD34+ cells were reprogrammed using third generation LVs carrying a LoxP-flanked polycystronic cassette containing Oct4, Sox2 and Klf4 (a) or the three factors and pri-miR302 and pri-miR367 (b). HA cells were corrected using a third generation LV carrying hBDD-FVIII under the control of PGK ubiquitous promoter (c) or VE-cadherin endothelial specific promoter (d).

Figure 3 - Characterization of healthy and hemophilic MNC-derived IPSCs.

After about 30 days healthy and hemophilic colonies (a, c) were picked and characterized by AP staining (b, d). Cells expressed stem cell markers at RNA (e, f) and protein (h, i) levels. RT-PCR for exogenous factors gave negative results (g) demonstrating that expressed stem cell factors were endogenous. Nanog promoter was in a methylated state in starting MNC while the 30% of CpG islands were unmethylated in iPSCs (j). Telomere length showed an increase during passages in culture both in healthy and HA iPSCs (k, l). HA iPSCs corrected with LV.PGK.hBDD-FVIII did not express FVIII (m) although the LV was integrated in transduced cells (n). Healthy and hemophilic iPSCs, cultured in low adhesion plate, gave rise to embryoid bodies (EBs) expressing markers of the three germ layers (o): Nestin, NCAM and Otx2 (ectoderm), α-SMA, brachyury and Tbx6 (mesoderm), AFP, FOXA2 and Sox17 (endoderm). EBs were able to differentiate in adipose (p), osteogenic (q) and chondrogenic tissues (r).

Figure 4 - Endothelial differentiation of healthy MNC-derived iPSCs.

During differentiation, cells acquired an endothelial-like morphology (a, b; 10 and 20 days of differentiation respectively). RT-PCR (c) showed the expression of several endothelial markers. Immunofluorescence showed the co-staining of FVIII and vWF (d). Differentiated cells expressed GFP when transduced with LVs carrying GFP under the control of endothelial-specific promoters Flk1 and Tie-2 (e). LV.PGK.GFP was used as transduction positive control. ECs were transplanted by portal vein injection in monocrotalin (MCT) conditioned NODSCID HA mice. Immunofluorescence on liver sections at 96 hours post transplantation showed GFP+-cells presence without a significant immune response (F4/80) (f).

Figure 5– Endothelial differentiation of HA-MNC-derived iPSCs.

MNC HA-iPSCs were differentiated by EBs induction formation. RT-PCR (a) and FACS analysis (b) showed the expression of several endothelial markers. Telomere length decreased in differentiated cells (c).

Figure 6 - Characterization of iPSCs obtained by reprogramming of HA fibroblasts.

Fibroblasts-derived iPSCs showed ESC-like morphology (a) and were positive at AP staining (b). RT-PCR showed that iPSCs expressed endogenous stem cells factors (c) but not the exogenous (d). Stem cell markers expression was confirmed by immunofluorescence for Oct4, Sox2, SSea-4 and Tra 1-81 (e). Nanog promoter methylation analysis showed that the 40% of CpG islands in iPSCs were unmethylated while the 96% in the starting fibroblasts were methylated (f).

Figure 7 - Characterization of iPSCs obtained by reprogramming of cord blood CD34+ cells.

Cord blood CD34+ cells-derived iPSCs showed stem cells-like morphology (a). Colonies were positive at AP staining (b). iPSCs expressed endogenous stem cells factors (c) but not the exogenous (d). Immunofluorescence confirmed the expression of stem cells markers like Oct4, Sox2, SSea-4 and Tra 1-81 (e).

Figure 8 - Characterization of healthy and hemophilic CD34+-derived iPSCs.

Both healthy and hemophilic peripheral blood CD34+ cells-derived iPSCs showed ESC-like morphology (a, b; healthy and HA respectively) an positive at AP staining (c, d). iPSCs expressed stem cell markers at RNA (e, f) and protein (i, j) levels. Negative RT-PCR for exogenous factors (g, h) demonstrated that expressed stem cell factors were endogenous. Nanog promoter was in a methylated state in starting CD34+ and, as expected, in an unmethylated state in iPSCs (k, l). Telomere length analysis showed an increase during passages in culture both in healthy and HA iPSCs (m, n). iPSCs, cultured in low adhesion plate, gave rise to embryoid bodies (EBs) that expressed markers of the three germ layers (o): Nestin and NCAM (ectoderm), alpha-fetoprotein and FOXA2 (endoderm), brachyury and AFP (mesoderm) and were able to differentiate in adipose (p), osteogenic (q) and chondrogenic tissues (r).

Figure 9 - Endothelial differentiation of healthy CD34+-derived iPSCs.

During differentiation, cells acquired a typical endothelial morphology (a). RT-PCR (b) and FACS analysis (c) showed the expression of several endothelial markers. Telomere length analysis showed a progressive shortening in differentiated cells (d). Nanog promoter returned to a methylated state in ECs (e). Matrigel assay demonstrated that differentiated cells were able to form tubules in vitro (f). EC efficiently expressed GFP when transduced with LV expressing GFP under the control of endothelial-specific promoters Flk1, Tie-2 and VEC (g).

Figure 10 - Endothelial differentiation of hemophilic CD34+-derived iPSCs.

iPSCs were corrected by LV transduction with a LV carrying FVIII under the control of VEC promoter and in parallel with an LV carrying GFP under the control of VEC promoter. During differentiation the 31% of cells transduced by LV.VEC.GFP start to express GFP (a). RT-PCR (b) and FACS analysis (c) showed the expression of several endothelial markers. Corrected cells showed FVIII expression at RNA level (d). Immunofluorescence confirmed CD31, FVIII and VEC expression (d, e ; NC EC and VEC-FVIII-EC, respectively). Telomere length showed a progressive shortening in differentiated cells (f). Nanog promoter returned to a methylated state in ECs (g). Obtained ECs were able to form tubules when plated on matrigel (h). aPTT on the supernatant of cultured ECs showed a reduction in the clotting formation time in VEC-FVIII-ECs medium (i).

Figure 11– iPSC-derived ECs efficiently secreted FVIII *in vivo*.

GFP expressing VEC-FVIII-ECs, HA-ECs and healthy ECs were intraperitoneally injected in NOD/SCID HA mice in association with cytodexmicrocarrier beads. aPTT, performed after 3 and 7 days after injection, showed that LV-FVIII-corrected ECs restored FVIII activity around 5%, more than healthy ECs (* P value<0.05, ** P value<0.01, *** P value<0.001) (a). Immunofluorescence on beads recovered from injected mice, showed the presence of GFP+-ECs (b). Healthy ECs were transplanted by portal vein injection in MCT conditioned γNull HA mice. Immunofluorescence on liver sections showed the presence of GFP+-cells from 1 week to 12 weeks (c) without a significant immune response (F4/80) and a gradual liver cell repopulation. Moreover, the co-staining between hCD31 and GFP confirmed endothelial phenotype of human transplanted cells. Corrected and not FVIII corrected HA-ECs were transplanted in MCT conditioned γNull HA mice. Immunofluorescence on liver sections confirmed the results obtained with healthy ECs (d). aPTT on plasma of transplanted mice, performed from to 3 weeks to 12 weeks after transplantation, showed that the relative FVIII activity in mice transplanted with VEC-FVIII-ECs was 2,8% after 3 weeks and increased at 4,8% after 12 weeks, while in mice transplanted with non-corrected cells no coagulation activity was detected. ** P value<0.01 (e). Mice transplanted with HA-ECs showed an increase in bleeding volume compared to VEC-FVIII-ECs transplanted mice (f).

Table 1–Copy number analysis of CD34+cells, iPSCs and ECs.

Real Time for the analysis of the LV integrated copies per cell showed a mean of 1,6 for healthy iPSCs and 1,4 for hemophilic iPSCs inserted copies/cell. Moreover, after Cre transduction in ECs the lentiviral cassette was efficiently removed.

Discussion

Currently hemophilia A is treated by the administration of plasma-derived or recombinant FVIII. This procedure is expensive, it is not a cure and 20-30% of patients with the severe form develop inhibitory antibodies against FVIII, worsening patients' condition. Researchers worldwide are looking for new strategies for a definitive HA treatment. In this context, cell and gene therapy could represent an interesting and promising resource for the medical treatment of the disease. In particular, a valid approach could be combining both strategies. In this perspective, the possible use of gene corrected autologous stem cells and in particular iPSCs can be appealing. To develop an iPSCs cell-therapy strategy to correct hemophilia A, several issues need to be solved: i) to identify the best cells source to be reprogrammed, ii) to define a reprogramming method both efficient and safe, iii) to identify the best cellular target to get after iPSCs differentiation to be employed in HA cell therapy, iv) to establish the more suitable method and time point to correct cells for FVIII expression. In the perspective of therapeutic application, it is necessary to define a method to generate iPSCs with high efficiency starting from a modest patient sample. Since iPSCs discovery, a large number of different somatic cells derived from the three germ layers and from different species were successfully reprogrammed. However, although iPSCs generated from a wide range of cell types have been shown to express pluripotency genes and to differentiate into all cell types of the three germ layers, several studies revealed substantial molecular and functional differences among iPSCs derived from distinctive cell types [236, 237]. Persistent donor cell–specific gene expression patterns in human iPSCs were identified, suggesting an influence of the original somatic cells on the molecular properties of resultant iPSCs [238]. Different cells undergo reprogramming with different kinetics and efficiencies. It became evident that if cells to be reprogrammed are terminally differentiated, the reprogramming process is less efficient [239].It was also demonstrates that the reprogramming efficiency can be increased when the starting cells to be reprogrammed are precursors. For example, Eminli et al., [137] showed that hematopoietic stem and progenitor cells give rise to iPSCs up to 300 times more efficiently than terminally differentiated B and T cells. We generated iPSCs starting from several cells: fibroblasts, MNC and CD34+ cells of peripheral and cord blood and accordingly to cited literature we appreciated different reprogramming efficiency and speed. Fibroblasts gave rise to iPSCs after 2 months from the reprogramming, MNC after about 4-5 weeks but only from 4 of 24 reprogramming performed and more important CD34+ cells after 3 weeks generated iPSC colonies from all reprogrammed samples. This observation highlighted that the differentiation stage of the starting cells has a critical influence on the return to pluripotent state. Moreover, while CD34+ cells and fibroblasts gave rise to iPSCs that underwent to a complete reprogramming at morphological, molecular and epigenetic levels, MNC gave rise to non-completely reprogrammed colonies. They were reprogrammed at transcriptional but not epigenetic

level, as shown by the analysis of Nanog promoter methylation profile. This result demonstrated that the induction of pluripotency in MNCs was not sufficient to remodel the epigenetic signature of DNA methylation pattern characterizing the differentiation status of these cells. Probably, MNCs have strong epigenetic memory that did not allow cells to return to a stem cell state. It is clearly reported that epigenetic memory of original cells can be retained in iPSCs. A genome-wide methylome analysis of human iPSC lines compared to ESCs showed a significant reprogramming variability including aberrant reprogramming of DNA methylation in large genomic regions [240]. From this first analysis fibroblasts and CD34+ cells resulted good cell sources for reprogramming. However, fibroblasts and cord blood CD34+ cells are not optimal in the perspective to develop a cell therapy strategy for HA. Indeed, to harvest a biopsy is risky for an hemophilic patient and CD34+ cells from cord blood do not allow the establishment of autologous iPSCs colonies. Thus, we identified CD34+ cells from peripheral blood as the best cells source to be reprogrammed for our purpose. The second issue to deal with is the optimization of the reprogramming strategy with the aim to obtain an ideal balance between safety and efficiency. iPSCs can be derived by several reprogramming strategies and introducing a variety of factors to create a magic "cocktail". For safety concern, we generate iPSCs omitting c-Myc employment. Actually, human iPSCs can be generated without c-Myc, but the reprogramming efficiency is markedly reduced [121]. Actually, using a lentiviral vector carrying only Oct4, Sox2 and Klf4 we obtained about20 clones from healthy CD34+ reprogramming but only 4 clones remained stable overtime. However, the association of Oct4, Sox2 and Klf4 with the miRNAs 302/367 allowed us to improve the efficiency and generate iPSCs from CD34+ cells of peripheral blood in a range of time and efficiency comparable to a reported study that used vector carrying the four factors [146]. This is due to the tight interplay between miRNAs 302/367 clusters and Oct4 and Sox2 transcription factors in the induction and maintenance of pluripotency [188]. Indeed, depletion of the miR-302/367 family reduces reprogramming efficiency in response to transduction with Oct4, Sox2, Klf4 with or without c-Myc suggesting that the miR-302/367 family plays essential roles in the process [191]. Then, once iPSC clones were established the endogenous transcription machinery is sufficient to sustain pluripotency gene expression and exogenous reprogramming factors have to be removed. We demonstrated that the expression of exogenous LV expression cassette was spontaneously silenced in established iPSC clones. However, the integrative nature of LVs requires excision of transgenes after reprogramming to avoid an aberrant reactivation of exogenous factors that could increase iPSCs oncogenic potential and interfere with differentiation and the maintenance of differentiated state [241]. The LVs carrying a LoxP-flanked exogenous cassette allowed us to efficiently remove it by the transient transduction of recombinase Cre carried by an integration

defective LV. Using this strategy we removed transgene sequences in differentiated cells allowing iPSCs-derived ECs to maintained their differentiated state until about 25 passages in culture. All together, our results showed that we identified a successful strategy to generate autologous iPSCs from hemophilic patients.

In the future, this proof-of-concept study could be improved in terms of efficacy and safety using newer methods for reprogramming. Indeed, iPSCs technology is still evolving because of the need for efficient and reproducible protocols that enable the generation of clinically safe iPSCs. Efficiency, reproducibility and safety are determined mostly by the methods used for the reprogramming. Currently, in addition to the stable integration approach, non-integrating methods, such SeV and adenoviral vectors, episomal vectors or minicircle DNA,were employed[158, 159, 165, 168]. Moreover, viral-and DNA-free approaches were developed. They consists in the introduction of reprogramming-inducing molecules into cells in form of: i) recombinant proteins [151, 152], ii) mRNA [169], and iii) mature microRNA [242]. The efficiency of non-integrating reprogramming methods is greatly enhanced by the use of low oxygen level conditions [243] and small molecules such as histone deacetylase inhibitors [172] and/or DNA methyltransferase inhibitors [244]. The choice of the reprogramming method relies on the application for which the iPSCs will primarily be used and the integration-free methods have high potential for therapeutic application. Moreover, another key point for iPSCs clinical application for genetic disease is the gene correction. We used lentiviral vector carrying hBDD-FVIII to correct hemophilic iPSCs. However, random integration of the transgene into the genome can be complicated due to silencing or altered regulation of expression due to genomic effects. Alternatively, site-specific methods targeting transgenes in genomic loci (safe harbors) that supports long-term expression, stem cell proliferation and differentiation. For example, theAAVS1 locus is considered to be one of the safe harbors that can accommodate a foreign gene without disrupting normal cell homeostasis. There are several reports utilizing this approach, such as the insertion of the α-globin gene for α-thalassemia [221], the FANCA gene for Fanconi anemia [245], and the RPS19 (ribosomal protein S19) for Diamond Blackfan anemia patients [214]. A highly precise and accurate editing of the genome driven by homology can be achieved using traditional methods as well as the newer technologies such as zinc finger nuclease [246], TALEN [247] and CRISPR/Cas system [248].

The next step in the design of iPSC-based cell therapy strategy for HA is to identify the optimal cellular target to get after iPSCs differentiation. For HA treatment it is necessary to identify cells able to secrete FVIII and, at the same time, is able to meet the necessary conditions to ensure the transplant success. The cellular origins of FVIII is still controversial [249]. Liver is the main organ producing FVIII. In particular, among liver cell types, although several authors described

both mRNA and protein presence in hepatocytes [28, 36, 37], LSECs were recently proposed as the main source of FVIII [44-46]. Importantly in the perspective to develop a cell therapy approach, several studies demonstrated that LSECs transplantation in HA mice corrected the bleeding phenotype restoring FVIII activity at therapeutic level [42, 78]. Other than liver, FVIII expression was detected in ECs from other organs such as kidneys, spleen and lungs [45, 250, 251] but not in heart and brain isolated ECs [45]. A very recent work analyzing the transcriptomic profiling of subsets of stromal cells, showed that F8 mRNA is expressed by LSECs, lymphatic ECs, and some high endothelial venules but not by capillary ECs or fibroblastic reticular cells[252] as previously reported from our group as well[33].In this context LSECs, that are the natural site of FVIII production, represent one of the best cellular models for our studies. In addition, LSECs allowed overcoming one of the major obstacles for hemophilia A treatment: anti-FVIII antibodies development. Indeed, liver was from a long time target of HA gene therapy because of its immunotolerance role. For hemophilia B successful results were reached expressing FIX in the hepatocytes [47, 72]. However, because of the great immunogenicity of FVIII, this strategy for hemophilia A was not sufficient to definitively avoid immune response[62]. Thus, LSECs can represent a valid alternative. Indeed, it is reported that in the liver the endothelial sinusoidal cells can act a as antigen presenting cells, and possibly induce immunotolerance[253].In this context, the optimal cell type toward which differentiate iPSCs for HA cell therapy is represented by LSECs. In 2009 endothelial progenitors were efficiently generated from murine iPSCs. These cells were able to correct hemophilic phenotype when injected in liver parenchyma [232]. This proof-of-concept work paved the way for the development of strategies to correct the hemophilic phenotype involving iPSCs. In 2014, Park and colleagues created a model cell line using a TALEN pair to invert a 140 kb chromosomal segment that spans the portion of FVIII gene in iPSCs. More importantly, they reverted the inverted segment back to its normal orientation in the HA model iPSCs using the same TALEN pair demonstrating that these enzymes could be used to correct hemophilic mutation in patient-derived cells [224]. More recently, the same group efficiently corrected HA iPSCs using CRISPR-Cas9 technology. ECs obtained from the inversion-corrected iPSCs expressed FVIII and were able to rescue hemophilic phenotype in short term experiments when injected in the hind limb of HA mice [225]. In this study, we efficiently obtained ECs from healthy and hemophilic peripheral CD34+ cells-derived iPSCs, while from MNC-iPSCs we did not obtain a pure population of mature cells. The difficulty to obtained differentiated cells from MNC-iPSCs can be related to the persistence of the epigenetic imprinting of the original cells. Moreover, it is clear that DNA methylation is a key and stable mechanism in specifying cell identity or plasticity [254]. MNC-iPSCs showing a methylated state of the Nanog promoter, did not display the

epigenetic plasticity necessary to remodel the methylome defining a new endothelial cell identity. CD34+ cells being progenitor cells are probably more prone to be completely reprogrammed to iPSCs and then differentiated into endothelial cells. The obtained differentiated cells acquired endothelial gene expression pattern and functionality. Indeed, expressed endothelial cell markers and were able to form tubules both *in vitro* and *in vivo*. Importantly, genetically corrected HA-ECs secreted FVIII and correct the bleeding phenotype when injected in our mouse model of hemophilia A. We efficiently corrected hemophilic ECs introducing the functional form of FVIII under the control of VEC endothelial specific promoter by LV transduction. For iPSCs or iPSC-derived differentiated cells gene correction it is crucial to identify the optimal step to genetically modified cells. For example, it was reported that the correction of the FA mutation was necessary for iPSCs generation from somatic cells of FA patients [219]. On the contrary, in our case we obtained best results correcting iPSCs instead of the original cells. Indeed, correcting cells before reprogramming we obtained iPSCs that integrated the expression cassette delivered by LV but did not express FVIII. This was probably due to the epigenetic remodeling that occurred during reprogramming process that probably silenced the transgene promoter. In addition, the choice of the promoter that drive FVIII expression was crucial. The use of the VEC (cadherin 5) endothelial specific promoter allowed us to restrict FVIII expression in the desired cell type and to assess that iPSC-derived differentiated cells were effectively ECs. Indeed, if cells after differentiation were able to express FVIII after gene correction they demonstrated the transcriptional activation of the endothelial specific promoter driving FVIII expression in the LV expression cassette. On the contrary, if cells did not properly differentiated into endothelial phenotype they could not turn on the promoter. In this study, FVIII was efficiently produced and secreted by corrected ECs. *In vivo* experiments were favored by the use of the mouse model generated in our laboratory [33], in which HA phenotype was back-crossed with the NOD/SCIDγNull immunocompromised (NSG) mice that is the best suitable model for xenotransplantation. iPSC-derived ECs transplanted into HA-γNull mice liver repopulated the parenchyma, forming new vessels and improving hCD31 expression, confirming the endothelial phenotype. More importantly, ECs transplantation rescued hemophilic phenotype both at short and long term up to 4,7% in the best experiment among the 2 performed. However, we observed some differences in engraftment and consequently in correction in a second transplantation experiment. This showed the variability characterizing the differentiation of primary cells, such as iPSCs derived from different donors. Indeed, we observed that each differentiation lead to ECs with an heterogeneous level of markers expression or that reached the mature stage after a different number of passages in culture. This variability reflected on their capability to engraft and proliferate *in vivo*. Thus, it is very important to perform a complete *in vitro*

characterization of the ECs obtained from different differentiation experiments. In any case, this proof-of-concept study showed that endothelial iPSC-derived ECs expressed the FVIII protein effectively, engrafted within the hepatic parenchyma, and integrated to provide the therapeutic benefit necessary for phenotypic correction of hemophilia A. Importantly, ECs restored FVIII activity not only when transplanted directly into the liver but also when injected intraperitoneally in association with micro-carrier beads. This result is very interesting from a translational point of view. Indeed, it could be possible to transplant autologous gene corrected ECs into the patient using immune-protecting and retrievable encapsulation medical device. These devices, some currently in clinical trials, are implantable and form a natural environment for the housing and long-term survival and function of encapsulated cells. Thus patient-specific gene-corrected iPSC-derived ECs could be encapsulated into the device, transplanted into the patient and release FVIII into the host. At the moment, a phase I clinical trial for type 1 diabetes is ongoing in which pancreatic progenitor cells were encapsulated into a device, which prevents immunoreactions (http://www.eurostemcell.org/commentanalysis/making-insulin-producing-beta-cells-stem-cells-how-close-are-we). Moreover, it was recently published that macro-encapsulation devices loaded with neonatal pancreatic tissue and transplanted into RIP-LCMV.GP mice prevented disease onset in a model of virus-induced diabetes mellitus [255]. Thus, the idea to put our FVIII-secreting ECs into a device seems to be feasible and it is clinically attractive.

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