Università degli Studi del Piemonte Orientale "Amedeo Avogadro"

PhD Program
in
Molecular Medicine
Cycle XXV

Phd Thesis

Cell therapy approaches to cure of hemophilia A

Tutor:
Prof. Umberto Dianzani

PhD Student:
Gabriella Ranaldo

Co-tutor:
Dr. Antonia Follenzi

Coordinator: Prof. Umberto Dianzani

Abbreviations:

HA: Hemophilia A; FVIII: Factor VIII; LSEC: Liver Sinusoidal Endothelial Cell; vWF: von Willebrand Factor; FXa: activated Factor X; FIXa: activated Factor IX; aPTT : activated partial thromboplastin time; HIV: immunodeficiency virus; HCV: hepatitis C virus; rFVIII: recombinant FVIII; APCC: Activated Prothrombin Complex Concentrates ; ITI: Immune Tolerance Induction; PEG: Polyethylene Glycol; pFVIII: porcine FVIII; ADA: adenosina deaminase; APCs: Antigen Presenting Cells; RV: Retroviral Vector; MLV: Murine Leukemia Virus; BDD-FVIII: B Domain Deleted FVIII; MSC: Mesenchymal Stem Cell; HSC: Hematopoietic Stem Cell; BM: Bone Marrow; LV: Lentiviral Vector; EC: Endothelial Cell;BOEC: Blood Outgrowth Endothelial Cells; cFVIII: canine FVIII; SIV: Simian Immunodeficiency Virus; HP-FVIII: human porcine FVIII; SFFV: Spleen Focus Forming Virus; miRNA: micro RNA;Ad: Adenoviral; AAV: Adeno-Associated Virus; ITR: Inverted Terminal Repeat; HD-Ad: Helper Dependent Ad; SB: Sleeping Beauty; iPS: induced Pluripotent Stem; GP: glycoprotein; MK: megakaryocytes; MNC: Mononuclear cells; TTFs: Tail Tip Fibroblasts.

Summary

Hemophilia A (HA) is an X-linked bleeding disorder caused by mutations in the coagulation factor VIII (FVIII) gene. Currently, there is no definitive cure. Identification of cells capable of synthesizing and releasing factor VIII (FVIII) is critical for insights into pathophysiological mechanisms and for developing therapeutic approaches in hemophilia A. Endothelial cells, particularly liver sinusoidal endothelial cells (LSEC), express FVIII most in the body. However, recent studies of bone marrow (BM) transplantation suggested that additional cell types could synthesize and release FVIII, and also correct bleeding in hemophilia A mice. Therefore, to establish the ability of circulating blood cells in expressing FVIII, we analyzed several murine and human hematopoietic cell types. We found by that FVIII was present in both in mouse and in human hematopoietic cells isolated from peripheral blood or BM, as well as in cells from human cord blood (CB). These peripheral blood, BM and CB cell types, included mainly myeloid cells, e.g., monocytes, macrophages and megakaryocytes. Another solutions for HA therapy could be the reprogramming of genetically corrected somatic cells. Towards this goal, we first generated induced pluripotent stem (iPS) cells from human fibroblasts derived from healthy donors by retroviral transduction with four factors (Oct4, Klf4, Sox2 and c-Myc). These cells were phenotypically similar to human embryonic stem cells (hESC) and differentiated into endothelial cells (EC), a cell type that, when transplanted in HA mice, allows correcting the hemorrhagic phenotype of this model. However, in hemophilic patients, to harvest fibroblasts from skin biopsies is risky; for this reason, we utilized peripheral blood cells as an easy-to-access source of cells and reprogrammed mononuclear cells (MNC) from donors and hemophilic patients with HA. After genetic correction and reprogramming with lentiviral vector carrying Oct4, Sox2 and Klf4 without c-Myc, the iPS cells differentiated into EC and engrafted into NOD-SCID HA mice. Contemporary, we reprogrammed and differentiated in EC mouse fibroblasts from hemophilia A mouse after correction by gene transfer of FVIII to have an autologous model to study. Overall, these data confirmed the presence of extra-hepatic sources of FVIII. These additional cell types offer further opportunities for understanding mechanisms in FVIII synthesis and replenishment. Contemporary, the generation and differentiation of iPS cells from hemophilic mice and patients give a new concept in hemophilia A research and future therapy.

Index of contents

Introduction

Hemophilia A and coagulation factor VIII

Hemophilia A (HA) is an X-linked bleeding disorder in which the functionally active coagulation factor VIII (FVIII) is partially or totally deficient. It has an incidence of 1-2 per 10000 males. It is characterized by frequent spontaneous bleeding episode, mostly into joints. Hemophilia A is typically divided into three classes, which are defined by factor plasma activity: severe, when FVIII activity is less than 1%; moderate when the activity is between 1% and 5% and mild, when the activity is greater than 5% but less than normal.[1] People with severe hemophilia A have spontaneous bleedings that cause complications as hemorrhages in joints, in muscles, brain and other organs.[2] However, there is a type of acquired hemophilia, a rare autoimmune bleeding disorder, which arises as a result of the spontaneous production of auto-antibodies against endogenous FVIII. The breakdown in immune tolerance is thought to be a result of a combination of genetic and environmental factors. It is associated with various autoimmune diseases, pregnancy, cancer or drug ingestion. Acquired hemophilia occurs in about one person per million, and affect mainly females.[3]

Hemophilia has a very long story. References to excessive bleeding episodes, occurred frequently in males of a family, were describe already in the Talmud and by an Arabic physician in the twelfth century.[4] However, only in the nineteenth century appeared the first modern description of haemophilia and the word emerged in 1828 by Hopff.[4,5] Initially it was believed that hemophilia were caused by vessels fragility and later by defect in platelets, but in 1937 Patek and Taylor corrected the coagulation defect by adding a substance extracted from plasma. In 1944 Pavlosky demonstrated that blood from an hemophilic could ameliorate coagulation of another hemophiliac with a deficiency in a different protein and vice-versa.[6] Only in 1964 Judith Pool discovered that cryoprecipitated plasma contained a considerable amount of FVIII.[7,8] The protein was completely purified and cloned in 1984.[9]

FVIII is a complex plasma glycoprotein that initially was demonstrated to be synthesized by hepatocytes,[10,11] although extrahepatic FVIII production has been confirmed by mRNA detection in spleen, kidney, liver sinusoidal endothelial cells (LSEC), pulmonary endothelial cells, lymphatic tissues. [12-14]

The FVIII gene is localized on the long arm of chromosome X and it comprises 26 exons, which encode a polypeptide chain for a signal peptide of 19 amino acids and

a mature protein of 2332 amino acids.[15] FVIII is synthesized as an inactive single chain with the domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2 (Fig.1).[16] The aminoacid sequence is conserved among species except for B domain, a large domain encoded by exon 14. Its function is not required for clotting activity, but it has a role in the trafficking and intracellular processes of the molecule.[17] This domain has a 30% homology with factor V (FV), prothrombin cofactor, and ceruloplasmin.[18]a1,a2 and a3 contain cutter sites for thrombin necessary for FVIII activation[19] and C domains are related to FV, milk globular protein and bond lipid of Discoidin I domains.[20]

Biosynthesis and secretion studies of FVIII were limited by low expression of protein in cell lines due to instability of mRNA and insufficient secretion.[21,22] FVIII synthesis consists in post-translational modification within cell. In particular proteolytic digestion at aminoacid1648 in the Golgi apparatus results in the formation of a 80 kDa light chain, containing C-terminal, and an heavy chain of 200 kDa.[22] Other processes essential for FVIII maturation are introduction of oligosaccharide chains in the B domain, modification of saccharide groups added in the endoplasmic reticulum and sulfation of tyrosine residues critical for proteolytic activity of thrombin.[23] N-glycosylation is important for the correct protein folding, prevents aggregation between intermediate forms and allows interactions with enzyme and chaperon essential for intracellular maturation, vesicular trafficking, exocytosis and secretion.[18]

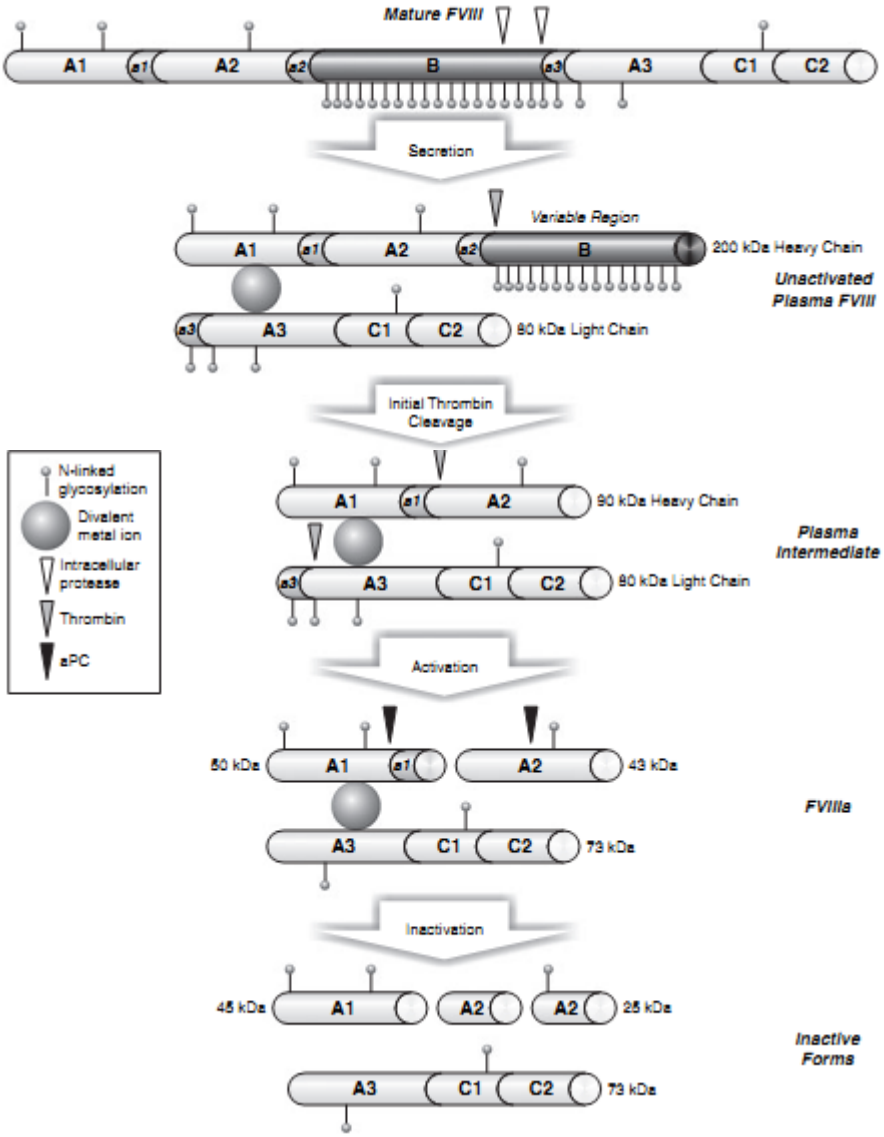

Figure1. Domain structure and processing of FVIII (Pipe,Haemophilia 2009)**.**

Since FVIII is not stable, it circulates in plasma in a non-covalent complex with von Willebrand factor (vWF), which protects factor VIII from premature proteolytic degradation and concentrates it at sites of vascular injury. In this complex ever monomer of vWF binds FVIII with an high affinity (kd 0,5 nmol/L),[24]interaction sites are in a3, c-terminal of B domain and c2.[25] In healthy people FVIII concentration in plasma is 200-300 ng/mlwhile vWF is 8 ug/mL therefore they complex in a molar ratio of 1:50.[26,27]FVIII is activated by thrombin (FIIa) with a proteolitic cut allowing dissociation from vWF. In this way FVIII is able to stabilize itself on platelets surface through hydrophobic interaction between c2 domain and phospholipids.[28]Activated FVIII (FVIIIa) functions as a non-enzymatic cofactor for activated factor IX (FIXa) in the activation of factor X (FXa) forming a complex called tenase.[29] Thrombin cleaves

fibrinogen in fibrin monomers that polymerize and form clot. Hemostasis ends with the dissolution of fibrin plug due to activation of protein C by thrombin in a feed-back loop. Protein C is a protease that inactivates several coagulation factors including FVIII (Fig.2).Secreted FVIII in the plasma has a short half-life of about 12 h in adults (shorter in children).[30]

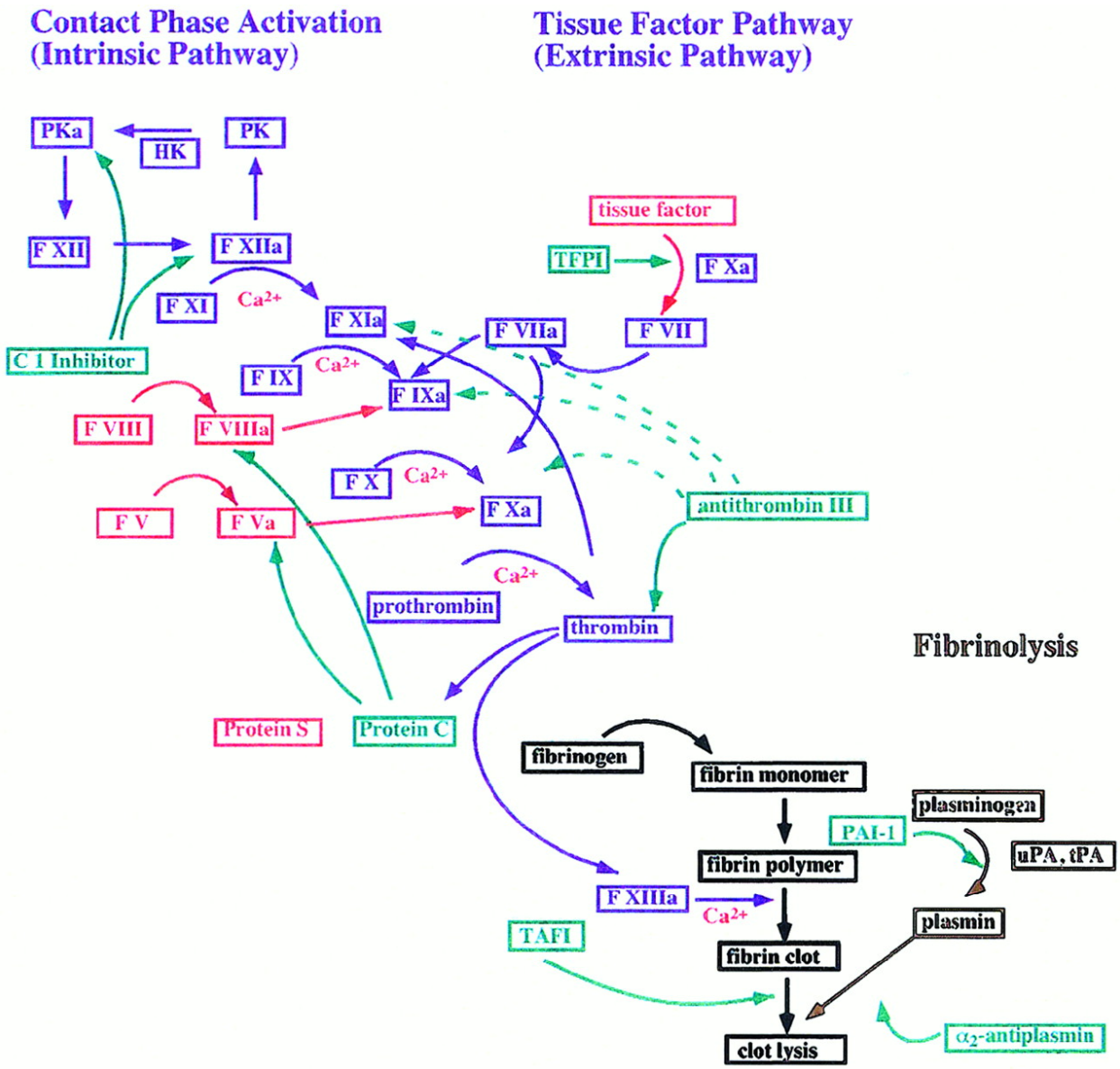

Figure 2. **The blood coagulation cascade**. (Tapper, Blood 2010)

The most common mutation in patients with hemophilia A is a large inversion and translocation of exons 1–22 (together with introns) away from exons 23–26, due to homologous recombination between the *F8A* gene (with F8B additional gene whose

functions is unknown) in intron 22 and one of the *F8A* copies lying away from the FVIII gene. Other mutations are point mutations and small insertion or deletion.[1] All mutations known to date are listed in the site http://www.cdc.gov/ncbddd/hemophilia/champs.html.Diagnosis is made by family history or at the age of appearance of the first hemoartrosis episode: 0-3 years for the severe form, 2-7 for the moderate and 5-14 for the mild.[1] Main assays are the measure of activated partial thromboplastin time (aPTT), the two-stage clotting in the form of a cromogenic assay and genetic analysis.[31]

At the moment, hemophilia A is a disease without a definitive cure. Initially, hemophilia was treated with whole blood or fresh plasma transfusions, but the amount of coagulation factors was not enough concentrated to stop severe bleeding.[32] Only in 1964 with the discovery that cryoprecipitate obtained from healthy plasma were enriched of large amount of FVIII and infusing them, severe bleeding could be stopped.[8] These findings allowed to develop programmes of comprehensive care management with involvement of several medical specialities in particular correcting musculoskeletal abnormalities.

In the ’80s, a major side effect of using cryoprecipitate was the infection of blood derived clotting factors by immunodeficiency virus (HIV) and hepatitis C virus (HCV) infecting 60-70% of people with severe hemophilia. This event had led an improvement in viral inactivation techniques adopted and increased the controls of blood donors’ health conditions. However, the most important advance was the production of high amounts of recombinant FVIII (rFVIII) after cloning the gene in 1984.[9] This advance was important for the implementation of prophylactic regimens in order to prevent bleedings and reduce joint damage in young patients.[33]

Unfortunately, this regimen was not optimal: 30-50% of patients with the severe form of disease developed inhibitory antibodies that made ineffective replacement therapies. Some studies demonstrated that patients treated with recombinant protein have the higher incidence of neutralising antibodies,[34] probably due to insufficient amount of vWF in the plasma, to protect the recombinant protein from antigen presentation. Indeed, vWF present in plasma-derived FVIII concentrates decreased FVIII immunogenicity masking epitopes and preventing endocytosis by antigen-presenting cells.[35-37] A solution to circumvent this effect was the use of bypassing agents as activated prothrombin complex concentrates (APCC) and recombinant FVII. Another attempt was the immune tolerance induction (ITI) protocol adopted,

that was the eradication of inhibitors trough the long-term daily treatment with large doses of coagulation factors. This approach is the only proven therapy against inhibitors but it has difficulties for patients in terms of venous access and for community in terms of a treatments of high costs.[38] In the future further advances need to be realized for the hemophilia treatment. The most likely progress is the availability of molecules with a longer half-lives. The main strategies adopted are the modification of FVIII by the addition of polyethylene glycol (PEG) polymers, polysialic acids or PEG-modified liposome.[39] Additional research has been made to prolong the life of recombinant FVII and the patients treated with the latter new formulation improved haemostatic efficacy without increasing the risk of thrombosis.[40,41] To improve FVIII pharmacokinetics, FVIII was fused with albumin and IgG Fc moiety.[38,42] Finally recombinant porcine FVIII (pFVIII) was produced and tested in phase II trial in hemophilia A patients with inhibitors resulting in bleeding control and tolerance.[38]

Gene therapy for hemophilia A

Cell and/or gene therapy are alternative to supplemental therapy.in gene therapy genes are used to treat diseases by restoring the function of a mutated allele adding a functional copy of it. To reach this aim there are rules to be followed: stable and high expression level for long term to reach correction and to have a biological effect, tissue-specific expression to restrict transgene activity in a target cells and inducible expression. Several attempts of gene therapy were performed over the years to cure diseases as adenosine deaminase (ADA) deficiency, hemophilia B, X-linked SCID and thalassemia, Leber congenital amaurosis.[43-51]Unfortunately, some of these had side effects as the development of T-cell lympho proliferative syndrome within 2 to 5 years after treatment in 5 out of 20 patients treated for X-linked SCID.[52,53] However, in 2009 an Italian group reported the results of a clinical trial were patients affected by ADA-SCID were treated with a retroviral vector containing the ADA transgene resulting in the cure of 8 out of 10 patients with an excellent and persistent immune reconstitution.[54]

Hemophilia A is a good candidate for gene therapy because it is a single-gene disorder; therapeutic coagulation factor levels may well be in a wide range (5-100%) and many well characterized animal models of disease exist to explore new

therapeutic approaches.[55] Over the years two approaches were followed: ex-vivo gene therapy,in which cells isolated from an individual are genetically modified in vitro and then returned to the same individual, and in vivo therapy, the direct infusion of vectors carrying the transgene in the patient. Although ex-vivo gene therapy is more invasive than in vivo gene therapy, it might potentially be safer because gene delivery should be restricted to desired cell types and inadvertent gene transfer into additional undesirable cells, e.g., germline cells or antigen presenting cells (APCs), might be excluded.[56]

Ex-vivo gene therapy

Retroviralvectors. First delivery systems used in ex-vivo gene therapy were retroviral vectors (RV), which have an RNA genome based on the murine leukemia virus (MLV). One in cytoplasm, ssRNA is reverse-transcribed in dsDNA and then integrated in the genome of the host during cell division. AB domain-deleted FVIII (BDD-FVIII) was cloned in a retroviral vector and different transduced cells were transplanted in NOD-SCID mice. For the first time expression of FVIII persisted up 1 week,[57] but this period is very shorter for an effective and long term therapy for hemophilia. Therefore, for ex-vivo gene therapy is important to find the exact cell population to be transduced. Bone marrow-derived cells are a promising target for gene therapy. In particular hematopoietic stem cells (HSCs) are easily transplanted, undergo self-renewal and repopulate the bone marrow (BM) following transplantation. It has been shown that transplantation of modified-HSCs facilitate the introduction and the tolerance of new antigens in many diseases, resetting host immune response providing long term expression.[58] Initially, Moayeri *et al.* demonstrated that total bone marrow FVIII-engineered cell transplantation, corrected up to 16 weeks haemophilia A first in immunecompromised mice and then in immunecompetent mice sub lethal irradiated.[59,60]

Moreover, mesenchymal stem cells (MSCs) are interesting cells to be used in cell therapy since they are easy to be isolated, cultured, and expanded in vitro and could be transduce with RV. However, MSC encoding BDD-hFVIII were able to produce FVIII in vitro but, when injected in hemophilia A mice, FVIII expression was detectable only for 6 days probably due to a transcription repression, since transplanted MSC were found 3 months after transplantation.[61] Also platelets are a

good target for FVIII expression. Platelets are the main players involved in primary hemostasis. Together with endothelial cells they are considered to be the main FVIII storage compartment.[62] Moreover, megakaryocytes (MK,platelets' progenitors) and endothelial cells synthesize and store vWF. Therefore, targeting expression of FVIII in these cells could establishing a pool of FVIII secreted along with vWF, which will be released in the sites of injury. Thus, the inhibitory activity of antibodies might be circumvented and it could limit the exposure of *the novo* FVIII to immune system.[63]However, since megakaryocytes have a finite lifespan, hematopoietic stem cells are better targets to obtain long term expression of FVIII in gene transfer.CD34+ cells isolated from mouse BM and human peripheral blood were transduced with a RV encoding the BDD-hFVIII and then differentiated with cytokines to form multiple lineage including megakaryocytes. In vivo and in vitro analysis showed co-staining of FVIII with vWF in a-granules in human platelets isolated from peripheral blood of transplanted NOD-SCID mice.[62]

Besides using the right target cells, in gene therapy it is important to reach therapeutic levels of FVIII and one strategy was to bioengineer FVIII itself. HSCs and MSCs were transduced with RV to express porcine FVIII. The porcine FVIII, which is made from pig plasma, is similar enough to its human counterpart to work effectively in the human clotting system.Its production is 10- to 100- fold higher than the human and it is less immunogenic then hFVIII. Transplantation of modified-HSCs expressing pFVIII in immunecompromised hemophilia mice promoted high level of FVIII activity for several months.[58,64]

Lentiviral vectors. Another delivery system used to genetically-modify cells was lentivirus-based vector (LV), generated from lentiviruses, ssRNA virus of retroviridae family. Their capability to transduce non-dividing cells, the lack of viral protein and accommodation of expression cassette up to 10 kb render LV a good tool for gene therapy in particular for HA in which the gene to be inserted is large. Since LV could theoretically transduce all cells, they were used to deliver therapeutic transgenes in several cell populations. In particular endothelial cells (EC), platelets and hematopoietic stem cells were the main targets. Endothelial cells differentiated from CD34+ isolated from peripheral blood were transduced with a LV expressing BDD-hFVIII and high levels of active FVIII were produced.[65] An easy source of endothelial cells is blood outgrowth endothelial cells (BOEC), characterised by long half-life and

high degree of proliferation index.Restricted expression of canine FVIII (cFVIII) in BOEC achieved a long term expression both in immunocompromised then in immunocompetent mice.[66]

Good results were obtained by targeting expression of hFVIII in platelets with a simian immunodeficiency virus (SIV) based-lentiviral vector using a platelets specific promoter, the human platelet glycoprotein (GP) Ibα.Lethal irradiated HA mice were transplanted with this vector and 1-2 % of correction in plasma of treated mice was detected.[67] Targeting expression of a modified FVII in platelets with SIV vector corrected hemophilia A phenotype also in presence of inhibitory antibodies.[68] No inhibitor formation was observed in HA mice transplanted with a LVαIIb-BDD-hFVIII transduced-bone marrow cells and expression of FVIII was sustained for at least 5 months demonstrating a successful transduction of hematopoietic stem cells.[69] Despite these important results, studies in mice suggest that both spatial and temporal aspects of clot formation may differ, based on whether FVIII is supplied from plasma versus a gene-modified platelet affecting the outcome of hemophilia gene therapy.[70]

An alternative approach to overcome antibodies formation is the expression of FVIII in B cells. Ramezani et al. demonstrated that hemophilic mice, transplanted with Sca1+ cells expressing a modified FVIII only in B-cells using a LV containing an immunoglobulin heavy chain enhancer-promoter, reached therapeutic level of FVIII, did not mount an immune response and survived in a tail clip assay. Interestingly, correction was observe up to 24 months.[71] Stem cells transduced by LV were also used to express hybrid form of human-porcine FVIII (HP-FVIII) and after transplantation therapeutic levels of FVIII and clotting times were restores in HA mice.[72]

In vivo gene therapy

Retroviral and lentiviral vectors. In parallel to ex-vivo gene therapy RV and LV were applied in attempts of *in vivo* therapy.As mentioned before an important aspect to be analyzed in treatment of hemophilia A is the development of FVIII antibodies, that it was observed occurred mainly after gene therapy in adult immunocompetent animal models. An alternative approach could be the treatment in neonatal age. Indeed, intravenous injection of a RV expressing cBDD-FVIII under a liver specific promoter

corrected the phenotype both in neonatal mice and dogs without antibody formation.[73] Treatment after birth gave good results also in disease models injected with a LV codon-optimised FVIII in comparison with the non modified molecules, although the ubiquitous promoter Spleen Focus Forming virus (SFFV).[74]Together with modification in the structure of the protein, it was possible to restricted expression of FVIII in specific compartments introducing in the vector specific microRNA (miRNA) target sequence and promoters. Matsui et al. constructed a LV containing a liver restricted promoter enhanced-transthyretin (ET) to drive the expression of the cFVIII with a mir-142-3p target sequence, which prevents transgene expression in antigen presenting cells, in combination of GP64-pseudotyped selecting hepatocytes transduction only. This vector was injected in HA mice and expression was detected mainly in the liver and was maintained for 60 weeks. However, better results were obtained when mice were injected with this LV and treated with clodronate, which depleted liver macrophages.[66] Improvement in hemostasis was finally obtained in hemophilic mice after the injection of the LV containing hybrid human-porcine (HP) FVIII resembling clinically administrated products.[72]

The most used viral vectors for in vivo gene therapy areadenoviral vectors (Ad) or adeno-associated vectors (AAV).

Adenoviral vector. Ad derived from adenovirus, a double strand linear DNA genome virus. They are efficient vectors for gene therapy due to their capability to transduced dividing and non-dividing cells can accommodate a large expression cassette and persist in the episomal form as long as cells do not actively proliferate. In 1996 Connelly et al. improved the expression cassette of FVIII and this allowed a lower dose administration in mice of the vector resulted in a sustained gene expression due to the presence of the hepatocyte-specific promoter from mouse albumin.[75] Same effects were observed in dogs. To obtain good results in large animal it is important for future application in human because it is possible to correlate the amount of vector necessary and the size of the host to be cured.[76] However, the animals developed a strong antibody response again the human FVIII (hFVIII). The generation of hemophilia A mouse model confirmed the efficacy of Ad for phenotypic correction of the disease with therapeutic levels of human FVIII up to 9 months.[77,78] Later Ads were manipulate to obtain safer vectors. A MiniAdFVIII was built with a

minimal viral *cis* elements and it carried a 20-kb expression cassette that contains the full length hFVIII cDNA coding sequence under the transcriptional control of albumin promoter. This vector was able to sustained expression of hFVIII at human physiological levels in HA mice in the absence of antibodies production.[79] Later helper-dependent adenovirus (HD-Ad) were built which were missing all the viral genes and maintained only the two inverted terminal repeats (ITRs) and the packaging signal. All proteins needed for replication and assembly were supplied *in trans* and for production an helper virus was present in culture as well.[80] Sustained expression of human or canine FVIII and phenotype correction were observed in immunocompromised hemophilia A mice injected with the HD-Ad. In immunecompetent mice stable expression was maintained only after an in vivo transient depletion of Kupffer cells and lymphoid macrophages. However, serum transaminase levels and cytokine profiles confirmed safety of these vectors.[81] Then an HD-Ad expressing cFVIII under hepatocyte nuclear factor 1 (HFN-1) promoter were injected in HA dogs. Liver restricted expression resulted in long term expression without formation of inhibitors but treating the dogs with high vector dose resulted in transitional elevation of transaminase levels and thrombocytopenia.[82] A better correction up to 2 years was obtained when dogs were injected with an high dose of HD-Ad containing cFVIII under the control of the phosphoenolpyruvate carboxykinase (PEPCK) promoter, a stronger hepatocytes promoter.[83] This promoter in HD-Ad vector was also used in further studies to analyze which variants of human FVIII hadhigher activity in combination with apolipoprotein E1 (ApoE1) enhancer for prolonged expression up to 16 months in HA mice.[84,85]

Adeno-associated vector. Finally, adeno-associated viral (AAV) vectors, derived from non-enveloped small single strand DNA genome virus, were studied for gene transfer application. Vector production requires the presence of a helper virus and thirteen different serotypes were discovered to date. The different AAV serotypes have tropism for a specific tissue with a distinct efficacy of transduction rendering these vectors a tool with an enormous potential in human gene therapy. The use of an AAV vector to direct expression of FVIII in mice was first reported by Burton in 1999. To overcome limited genome packaging capacity of AAV they used two vectors, one for the heavy chain and the other for the light chain injected simultaneously in normal mice. A discrete amount of hFVIII was made functional, but only 5-10% of the total

protein produced was biologically active because an unbalanced ratio of the two chains was produced in wild type mice.[86] Hereafter, the same group extended their work to the hemophilia A model. Two AAV2encoding for light and heavy chain of human FVIII under the control of liver-specific regulatory sequence were injected in HA mice. They confirmed production of supraphysiologic levels of biologically active FVIII, but the imbalance production of the two chains and the increased immunogenicity of FVIII rendered this approach not suitable for HA gene therapy.[87] Therefore to circumvent these difficulties, a single AAV expressing B domain-deleted FVIII was used to obtain long-term expression with therapeutic levels of hFVIII in vivo in wild type mice.[88] When injected in HA dogs, it was demonstrated that multiple AAV serotypes had a long-term efficacy and safety up to 4 years suggesting the possible cure in humans. Although levels of FVIII achieved in mice did not reach the same therapeutic levels in dogs showing some difficulties in scaling up positive results obtaining in mice to a large model.[89] The results obtained were suboptimal, probably due to the use of a wrong AAV serotype of. Evaluation of several AAV serotypes demonstrated that serotype 8 was superior to deliver cFVIII to the liver and to correct hemophilia status in mice both with a single-chain then with two-chain vectors.[90] Also in dogs liver-restricted expression of cFVIII improved disease phenotype and there was no evidence of immune response to FVIII even after antigen challenge of the AAV-injected dogs.[91] Recently, AAV were applied in neonatal gene therapy. As shown for RV and LV vectors, treatment after birth resulted in tolerance using a moderate dose of vector, phenotypic improvement of hemophilia A and lack of immune response.[92]

Non-viral vectors. Viral-vectors described up to now have limits of such as the immune response against components of viral particles, random integration, transactivation of gene, difficulties with the production of high vector titers and difficulty in targeting a specific tissue. Therefore it was necessary to develop of non-viral delivery system for therapy. There are two categories of non-viral gene therapies: physical (needle injection, electroporation, gene gun, ultrasound and hydrodynamic delivery) and chemical (lipid or polymer carriers in complex with nucleic acids). There are at least three essential requirements for establishing an effective method of non-viral release of a DNA: protection against cell nucleases, nuclear location, and very low toxicity.[93]

Hydrodynamic therapy is based on the principle that the physical barriers protecting parenchymal cells from unwanted invasion may be overcome by briefly increasing luminal pressures to promote extravasation. This principle allows naked genetic material to be delivered via high-volume/pressure injection without the need for additional chemicals or formulations.[94] Bluescript-modified plasmid carrying FVIII was injected by tail vein in hemophilic mice. High levels of hBDD-FVIII were produced, but expression decreased after 2-4 weeks in concomitance with the development of neutralizing antibodies. A deeper analysis demonstrated that inhibitory antibodies were most likely mediated by T helper-2-dependent response.[95] Indeed in a following study was shown that treatment with a block agent in B/T cell interaction induced persistent and therapeutic hFVIII levels and no antibody formation.[96] Due the immune responses and the aggressive approach of vector injection, it is difficult to predict the feasibility of these approaches in clinic.

Anon-viral method, that allows a prolonged transgene expression, was obtained by the incorporation of components of a eukaryotic DNA transposon into the vector.

Transposons are naturally occurring genetic elements capable of moving from one chromosomal location to another. Sleeping Beauty (sb) is a synthetic transposable element made from ancestral Tc1/mariner superfamily transposon. It consist of two elements: the transposon, a DNA surrounded by the inverted repeated (IR) direct repeated (DR) element, and the transposase, a protein which recognize IR/DR and facilitate transposition. These elements integrate into a TA target dinucleotide, which is duplicated upon insertion. In gene therapy this two elements are divided in two components: the transposon, which is flanked by IR/DR elements and contains an internal promoter to drive expression of a gene of interest, and the transposase which is either expressed in a *cis* or *trans* configuration.[94] Transposon-mediated transgene delivery was used to correct hemophilia A phenotype and long term FVIII expression was obtained for several months.[97] A very elegant demonstration of transposon delivery in tissue was performed using nanoparticles targeted with a endogenous ligand specific for the hyaluronan receptor (HA) expressed by LSEC. Nanoparticles, carrying a plasmid with cFVIII under hybrid CMV enhancer:chicken β-actin (CAGGS) promoter and SB transposase version 10, were injected in hemophilia A mice. FVIII activity reached levels similar to wild-type mice for 11 months without inhibitors.[98] Moreover, transposons were used to reduce immune response against FVIII. Hemophilia A mice were injected with two transposons, one with hFVIII and

one with indoleamine 2,3-dioxygenase (hIDO) which induced indirect T-cell apoptosis, and transposase. Low titers of antibodies were present over time in mice treated with only hFVIII transposon, while IDO was able to limit but not to eliminate completely the immune response.[99] More delivery systems were studied to be used for therapeutic gene transfer. Bowman and colleagues administered orally chitosan nanoparticles with an expression plasmid expressing FVIII to hemophilia A mice. Plasmid DNA was detected in several organs and despite the modest FVIII levels achieved in mice, detectable FVIII protein persisted for one month and phenotypic bleeding correction was observed in most of the mice given high or medium doses of chitosan-DNA nanoparticles.[100]

In all these studiesgene therapy for hemophilia A consists in the addition of normal FVIII gene.Despite that it has been demonstrated a phenotypic correction of hemophilia A by RNA repair with spliceosome-mediated RNA *trans*-splicing with the direct correction of the mRNA.[101] For hemophilia this demonstration is a proof of concept considering the high number of mutations that need to be corrected rendering this approach quite cumbersome.

Based on excellent results obtained in animal models, between 1998 and 2001 five different Phase I clinical trials were initiated for the treatment of hemophilia A in human with different gene delivery systems included retroviral vector, an adenoviral vector, two adeno-associated viral vectors and non-viral gene-delivery methods, but these approaches showed worse results compared with those obtained in animal models.[102] Moreover there are some problems to be considered: insertional mutagenesis of some integrating viral vectors that randomly insert genes through the genome and immune response against vector's component. And finally an important question remains to be answered: will gene therapy by the production of ectopic FVIII be a risk for inhibitor development?[1]

Cell therapy for hemophilia A

New approaches to cure hemophilia A require insights into cell compartments capable of producing FVIII.Early reports showed that in dogs, only orthotropic liver transplantation (OLT) corrected the phenotype of hemophilia A.[103] It should be

noteworthy that transplantation of fetal porcine spleen, which was obtained from a developmental stage prior to the appearance of mature T cells, and also included endothelial cells, of course, offered a novel treatment modality for hemophilia A without immune responses against the reintroduced FVIII.[104] Similarly, data from a Swedish and Chinese group of investigators, established the safety and efficacy of spleen transplantation in hemophilic patients with remarkable long term therapeutic results.[105,106] However the supply of donor human organs is limited, one solution will be the use of cell therapy, that is the transplantation of new cells into a tissue in order to treat diseases with or without the addition of gene transfer. There are many potential forms of cell therapy, however frequently it is considered cell therapy the use of progenitors cells or multipotent/pluripotent stem cells in order to replace an organ function. Nevertheless, mature cells were used in the treatment of some diseases as hepatocytes for metabolic dysfunction or in place of liver transplantation.[107-116] Interestingly, hemophilia A liver donor were transplanted into a non-hemophilic recipient with alcoholic cirrhosis without develop of disease.[117]

Other cells can be used for the treatment of different diseases, indeed recent studies have demonstrated that transplanted LSEC and BM from healthy donor can correct the phenotype of hemophilia A mice.[104,118-120]

Since the reduced availability of progenitor and mature cells from human, it is necessary to find new cell sources. One could be stem cells because of their major properties: capacity of self-renewal, multipotency, functional, long-term tissue reconstitution and serial transplant ability.[121] These cells could be isolated, expanded, differentiated and finally transplanted. The possibility of using genetically modified autologous stem cells after appropriate expansion in vitro could help to avoid allograft-related issues.

Induced pluripotent stem cell (iPSCs)

Several diseases characterized by damaged cells, tissues or organ could be cure by transplanting healthy cells or tissues generated in laboratory from stem cells of a compatible donor or patient's own cells. Source of stem cells are adult stem cells (ASCs), umbilical cord blood stem cells or embryonic stem cells (ESCs).[122]

ASCs are stem cells that differentiate only in one tissue or lineage in which they reside and they were studied for the application in therapy as in myocardial infarction or leukemia.[123,124]

Umbilical cord blood cells or dental pulp stem cells are a potential source for autologous stem cell therapy, indeed they could be cryopreserved. Due to their origin these cells have the potential to cure a variety of diseases without rejection. For example they were differentiated in b-cells secreting insulin for the therapy of insulin dependent diabetes mellitus (IDDM) in human and mouse.[125]

ESCs are pluripotent stem cells that can differentiate in all three germ layers. Their derivation from the inner cell mass of 4-7 days old embryo is object of a difficult ethic and religious debate. However, two clinical trials with human embryonic stem cells were approved by FDA for spinal cord injury and Macular degeneration with no results so far.[126-128] Anyway these difficulties prompt the scientists to explore alternative ways to generate ESC-like from somatic cells. The first two explored reprogramming methods were nuclear transfer to oocytes and fusion with ES cells. A successful nuclear transfer experiment was reported in 1952, when Brigg and King demonstrated that transferring nuclei from blastula stage embryos into enucleated *Ranapipiens* eggs resulted in normal tadpoles.[129] More recently, Egli *et al*. reported that is possible to generate pluripotent cells using the nucleus of adult somatic cells as donor and zygote as recipient.[130] This protocol implies arresting the recipient zygote in mitosis by drug treatment, removing its chromosomes and replacing them with donor-derived mitotic chromosomes. Recently, same results were obtained with human oocytes and fibroblasts or lymphocytes.[131] Another approach implied the fusion of a somatic cell with a stem cell (Fig.3).[132,133] However these methods had ethical complications, high costs and technical difficulties, but demonstrated that dormant gene expression programs can be dominantly awakened in differentiated cells and that oocytes not fertilized and ESCs containedfactors necessary to confer pluripotency to somatic cells. After several studies these gene were identified, some were important for the maintenance of pluripotency as Oct 3/4,[134,135] Sox2,[136] e Nanog[137,138] and others for ESCs proliferation as Stat3,[139,140] E-Ras,[141] c-myc,[142] Klf4,[143] e β-catenin.[144,145]

The latest development is the demonstration that somatic cells can be reprogrammed to a pluripotent state by the expression of a transcription factors cocktail, generating induced pluripotent stem (iPS) cells. The Nobel prize for medicine in 2012 Yamanaka

showed that retroviral transduction of mouse and human fibroblasts with four transcription factor Oct3/4,Klf4,c-Myc and Sox2, which were narrowed down from a pool of 24 genes, induced pluripotency in somatic cells.[146,147] Other cocktail of factors were able to reprogramming cells as Oct4, Sox2, Nanog and Lin 28.[148]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.[149] Oct4 deficient embryos did not grow beyond the blastocyst stage and lacked pluripotent cells in their inner cell mass (ICM).[134] Oct4 is also activated by nuclear receptor factors as LRH-1, SF-1 and Essrb.[150-152] Despite its vital role in maintaining pluripotency and suppressing differentiation, the myriad processes involved in sustaining this cells state cannot be regulated by Oct4 alone. It acts in concert with other regulatory factors as Sox2 and Nanog. Sox2 is a part of family DNA binding protein known as sex-determining region Y (SRY) related high mobility group (HMG-box) proteins. It is also expressed in ICM and is essential for development. However, Sox2 expression is observed in the development of other cells as central nervous system.[153] Indeed, iPS cells were generated from adult neural stem cells by the transduction of Oct4 alone.[154] Together with Sox2, Klf4 is not necessary in reprogramming when the somatic cells has endogenously high expression levels or when it was substituted by a chemical compound.[155] Klf4 or Kruppel like factor 4 is a zinc finger transcription factor that regulates cell proliferation and differentiation. In particular, it inactivates p53 which repress Nanog and interacts as transactivator with Oct4-Sox2 in the synergistic activation of Nanog.[156,157] Nanog and c-Myc have been shown to be dispensable for reprogramming although they do improve the efficiency of the reprogramming process.[158] 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).[137,159] c-Myc is a proto-oncogene required for cell growth and proliferation and in stem cell maintenance, although the exact mechanism of action for this is not known.[122]

The established iPS cells are similar to ES cells in many aspects and certain standards for identification and characterization are followed. First step in the identification of iPS cells is the morphology. They have high nucleus to cytoplasm ratio, large nucleoli, formation of compact and uniform colonies with well-defined

borders. They 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 and Nanog at comparable levels to those of ESCs. They show same proliferation rate and feeder dependence of embryonic stem cells, stain positive for TRA-2-49/6E (alkaline phosphatase, AP), and have high telomerase activities. A further analysis is the promoters' methylation status, ability to differentiate in vitro and to form teratomas when injected in immunodeficient mice.[160] Although the latter is one of the assay to be done for a complete characterization, iPS cells that form teratomas might not be the best choice for in vivo use.[161,162] Started from fibroblast iPS cells were obtained from several cell types: keratinocytes,[163] neural stem cells,[164] B lymphocytes,[165] adipose stem cells,[166,167] peripheral and cord blood[168-173] and melanocytes.[148]

With this incredible discovery, Yamanaka opened a new research field. In 2008, the removal of c-Myc from the Yamanaka's transcription factors cocktail Idue to its oncogenic activity has been a pivotal action in the generation of safer iPS cells. Despite a reduced efficiency, murine and human iPS cells could be successfully generated using only the other three factors: Oct4, Sox2 and Klf4.[174]

Generation of iPS cells

Since then additional studies reported the derivation of human iPS cells with a variety of methods, viral and non-viral vectors were used, to improve efficacy and safety (Fig.3).

Viral methods. First iPS cells were obtained by retroviral transduction. An advantage of using retroviral vectors is their capability to be spontaneously silenced after the reprogramming induction and activation of endogenous factors. However, these vectors transduce only dividing cells and the use of high doses of multiple viral particles increased the insertional mutagenesis risk with reactivation of transgenes and subsequent tumor formations.[175]

Another approach is the reduction of integration sites by putting the reprogramming factors into a single vector with IRES or 2A self-cleavage peptide.[176] This reprogramming cassette was used with a lentivirus system that had high transduction

efficiency to a wide variety of dividing or non-dividing cells and the stable and reproducible transgene expression. Additionally, factors could be flanking by a loxP sequence in the LTR to produce iPS cells with reduced genome insertions. The expression of Cre recombinase successfully cuts out the cassette. Although it left an incomplete LTR in the iPS genome, this method minimizes the genomic alterations.[177]These vectors have a mutated integrase coding sequence, so do not integrate in the host genome, remaining present in an episomal form in the nucleus, but having comparable transduction efficiency of the integrating counterpart.[178,179]Moreover, it was possible to control reprogramming factors expression by using doxycyline-inducible lentiviral vector. Not integrating viral vectors as adenovirus-based and Sendai virus vector were used to reprogram mouse and human cells. In 2008 Stadtfeld *et al*. reprogrammed mouse fetal liver cells, tail tip fibroblast (TTFs) and finally adult hepatocytes with adenoviral vectors containing the four reprogramming factors. Although the efficiency was lower than integrating vectors, they obtained *bona fide* iPS cells.[180,181] One year later, adenoviral vectors were used for the first time to recover iPS cells from human embryonic fibroblasts. These cells were pluripotent, able to differentiate and most important free from viral DNA integration into the host chromosomes.[182] Sendai Virus (SeV) is a RNA virus that replicate in cytoplasm that do not integrate in the host genome. Using SeV-based vectors, iPS cells were originated with a higher efficiency compared to the other methods. An advantage of SeV is that RNA viruses are diluted during passages even if some residual viruses can still be present after several passages. However, it was possible to eliminate these cells, still containing the virus, by a negative selection using a specific antibody that recognized HN, the major protein expressed in SeV-infected cells, to maintain only virus-free cells.[183] These studies demonstrated that is not necessary vector integration in the genome and it is sufficient a transient expression of the factors to induce reprogramming. Their efficiencies of iPS cell induction are lower than that with retrovirus vectors, possibly due to low transduction efficiency, and unstable expression. However they could potentially become standard methods in the future.[184]

Non-viral methods. Within non-viral methods there is the transfection of cells by lipofectamine or nucleofection with plasmids carrying the factors encapsulated into lipid or cationic polymer.[185,186] One of the most efficient non-viral gene delivery

systems for iPS cells generation make use the *piggyback* (PB) transposon which it is excised from its integration sites by the PB transposase without changing the original DNA sequence. This prevented inadvertent re-expression of the reprogramming factors obviating some of the concerns associated with the use of integrating vectors.[187,188] The transfected PB transposon carries a single construct containing c-Myc, Klf4, Oct4 and Sox2 successfully reprogram the somatic cells to iPS cells.[189,190] All described methods involved the use of genetic materials, which could cause unexpected genetic alterations. Thus, alternative strategies were investigated, like the delivery of reprogramming proteins or RNA into the cells and the manipulation of cell culture conditions parameters. In 2009, the first successful generation of protein-induced iPS (piPS) cells was described. In this system, the purified Oct4, Sox2, Klf4 and c-Myc proteins were fused to polyarginine peptide tags, which allowed plasma membrane passage. The first colonies appeared after four rounds of protein delivery and subsequent 30-35 days of culture.[191] However, this reprogramming method is not as efficient as gene-delivery systems, indeed, the multiple protein transductions required may hamper the process.[192] An alternative strategy is the generation of iPS cells by direct RNA transfection. Synthetic mRNA of classic reprogramming factors and LIN28 were manufactured and modified to overcome antiviral response. Daily transfection gave rise to colonies after only 18 days, showing a higher efficiency and kinetics. This method eliminates risk of genomic integration and insertional mutagenesis and allows the regulation of proteins stoichiometry in culture.[193] Interestingly, Page and collaborators reported successful reprogramming of adult human fibroblasts by manipulating cell culture alone. It was demonstrated that Oct4, Sox2 and Nanog genes are not completely dormant, so they could be activated altering culture condition, such as by exposing cells to a lower amount of atmospheric oxygen or adding fibroblast growth factor 2 (FGF2). However, this short-term induction is not always self-sufficient to induce and maintain a genuine pluripotency.[194] Many chemical compounds as BIX, VPA, 5-aza-cytidine have been shown to either replace certain factor or significantly improve iPS cells generation.[160,195-197] Recently, emerged the crucial role of micro-RNA (miRNA) in the pluripotency mechanism was explored. Expression of ESC specific miRNAs has been found to promote induction of iPS cells.[198] A very important miRNAlet-7 has been implicated in pluripotency maintenance and it is modulated by the gene

Lin28.[199] Another micro-RNA, miR-145 was found to modulate Oct4, Sox2 and Klf4 expression in hESCs by inhibiting self-renewal and inducing maturation.[200]

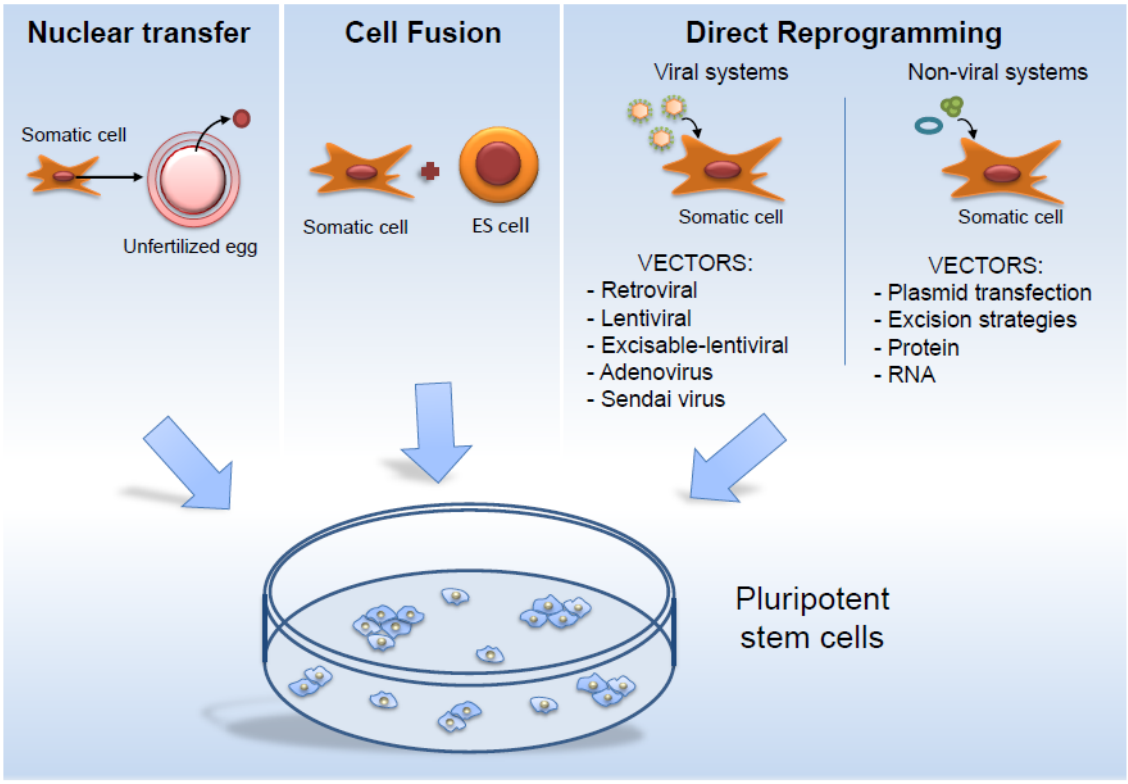

Figure 3 – Methods of reprogramming from somatic to pluripotent stem cells. Different methods are used to reprogram somatic cells to pluripotent stem cells. Nuclear transfer consists in the insertion of a somatic cell nucleus into an unfertilized egg. Another approach is to fuse somatic and stem cells. However, actually gene-delivery is the preferred technique to induce reprogramming. Indeed, either viral or non-viral methods were developed. Lately, a new way of reprogramming without the use of genetic materials has been shown to be applicable for the production of iPS cells.

Epigenetic control of reprogramming

An important topic of discussion on the generation of iPS cells is the epigenetic mechanisms involved in cell reprogramming. Indeed, chromatin status and histone modifications are crucial in the regulation of transcription mechanism. At now, for the improvement of reprogramming to iPS and differentiation in several cell types, it is very important to understand the epigenetic marks and mechanisms which are at the basis of the induction and maintenance of the pluripotent state and if epigenetic memory could influence these processes.

It is well known that cells of the early mammalian embryo, including pluripotent ES cells and primordial germ cells (PGCs), are epigenetically dynamic and

heterogeneous.[201] The histological analysis of stem cell nucleus, progenitors and differentiated progeny showed that several cellular types, like neoblast cells in planaria and hematopoietic stem cells in mammals, are characterized by a chromatin open state.[202,203] This particular state implements the transcription program and allows a rapid switch upon induction of differentiation.[204] Development and differentiation regulatory genes transcription is ruled, at an epigenetic level, by the balance between activating and repressing modification, like residues methylation on histones as H3K4me3 and H3K27me3 respectively.[205] In the case of H3K27me3, methylation is ruled by polycomb group (PcG) proteins.[206] It is interesting to underlying that some targets of PcG proteins tend to be co-occupied by transcription factors like NANOG, OCT4, SOX2.[207] Another histone mark commonly associated with gene repression is H3K9 methylation, which increases with cells differentiation. One enzyme associated with this mark is the methyltransferase EHMT2, which is notably required to silence OCT4 during differentiation.[208] EHMT2 binds directly to OCT4 promoter and leads to H3K9 methylation, which is followed by DNA methyltransferase (DNMT) recruitment leading to a more definitive OCT4 promoter repressive state, by the methylation of CpG islands core promoter.[209] The low level of H3K9 methylation in undifferentiated ES cells 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 H3K9me2 and H3K9me3, respectively, and they maintain the pluripotent state by directly demethylating H3K9 at the promoter regions of ES cell factors, allowing their expression. Interestingly, the genes encoding jMjD1A and jMjD2C are regulated by OCT4, representing a positive feedback-loop that integrates the action of transcription factors and histone modifiers to maintain the undifferentiated ES cell state.[210]

The epigenetic control of undifferentiated-differentiated state transition and the way through which the epigenetic barriers are overcome are critical issues in the generation of iPS cells. The reprogramming process is slow and gradual, with intermediate states that are rare stochastic epigenetic events.[211,212] Reactivation of endogenous pluripotency factors genes such as OCT4 can occur at different time points in different iPS cell lines derived from the same clone and this moment is hard to establish.[213] At now, the molecular mechanism that underlies the 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.[206] New insights are gained by the treatment of cells in 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 iPS cells generation and sometimes can substitute a particular transcription factor [214,215]. More recently, several epigenetic studies focus on enzymes that regulate this process. For example, Onder *et al*., using shRNA approach, demonstrate that inhibition of the core components of polycomb repressive complex 1 and 2, including the H3K27methyltransferase EZH2, reduced reprogramming efficiency, whereas suppression of SUV39H1, YY1 and DOT1L enhanced reprogramming.[216] Specifically, inhibition of the H3K79 histone methyltransferase DOT1L by shRNA or a small molecule accelerates reprogramming increasing the yield of iPS cells colonies. Moreover, the inhibition of DOT1L early in the reprogramming process allows to reprogram cells using only two of the Yamanaka's factor: Sox2 and Oct4.[217] Moreover, Mansour et al. demonstrated that H3K27 demethylase Utx6–9 (also known as Kdm6a) regulates the efficient induction of pluripotency. In particular, Utx safeguards the timely execution of H3K27me3 demethylation observed in mouse embryonic day 10.5–11 PGCs, and Utx-deficient PGCs show cell-autonomous aberrant epigenetic reprogramming dynamics during their embryonic maturation in vivo. In the same way, the timely and authentic execution of H3K27 demethylation is ruled by Utx during the transition to a pluripotent state.[216]

In conclusion, the knowledge of epigenetic mechanisms that underlie the reprogramming process and the possibility to act on the chromatin and histones status are crucial for the improvement of reprogramming efficiency and the development of new strategies that avoid the employment of oncogenes.

Applications of iPS cells

Several human diseases are difficult to study because appropriate *in vitro* cell models, which recapitulate disease features, are lacking and the direct recovery of primary cells from patients is often difficult. Additionally, animal model of disease not always recapitulates the human disease features.

More importantly, iPS cells can bypass the ethical concerns related to ES cell derivation and potentially issues of allogenic immune rejection. They may represent a more ideal source to produce patient-specific and disease-specific adult cells for future clinical applications. As a result, these cells have been regarded as a leading candidate for donor cell source in regenerative medicine (Fig.4).[218]

Recent studies demonstrated that iPS cells indeed can be generated directly from human patients and then differentiated in specific cell type compromised in diseases. iPS-derived cells from amyotrophic lateral sclerosis (ALS), Huntington and Alzheimer diseases, Down syndrome, Long QT syndrome type 2 (LQTS) and Pompe-disease were used to study abnormalities present in patients, to evaluate the efficacy of new drugs and to identify new genes that whose expression monitors the effect of therapies.[219-224] Disease-specific iPS cell lines were also extremely useful to analyse behavioural, morphological differences and effect of compounds in development of disease with normal iPS cells, above all this approach has been used for neurodegenerative and neuropsychiatry diseases.[177,225-230] Application of iPS cells was also investigated in cell therapy for disorders that required a long term cure to ameliorate quality of life's patients and reduce dependence from treatments, as diabetes.[231] Moreover, iPS cells were differentiated in several cell types to correct some diseases: functionally neurons for Parkinson's disease or ischemia,[218,232-235] hematopoietic progenitor for the correction of Fanconi and Sickle Cell Anemia,[236,237] cardiomyocites for myocardial stroke.[238] Besides the known degenerative disease, many people are affected by the loss of vision because of retina damage for disease or injury. There is not a regenerative pathway in this tissue, so stem cells transplantation could be a good opportunity. Preliminary studies demonstrated that iPS cells differentiated in retinal pigmented epithelium and rodopsin cells and engrafted in damage retina even if only injection of latter cells restore neuronal function.[239,240] Interestingly, cells obtained from iPS cells could be used to deliver therapeutic genes, as antibodies against tumor markers or specific proteins.[241,242]

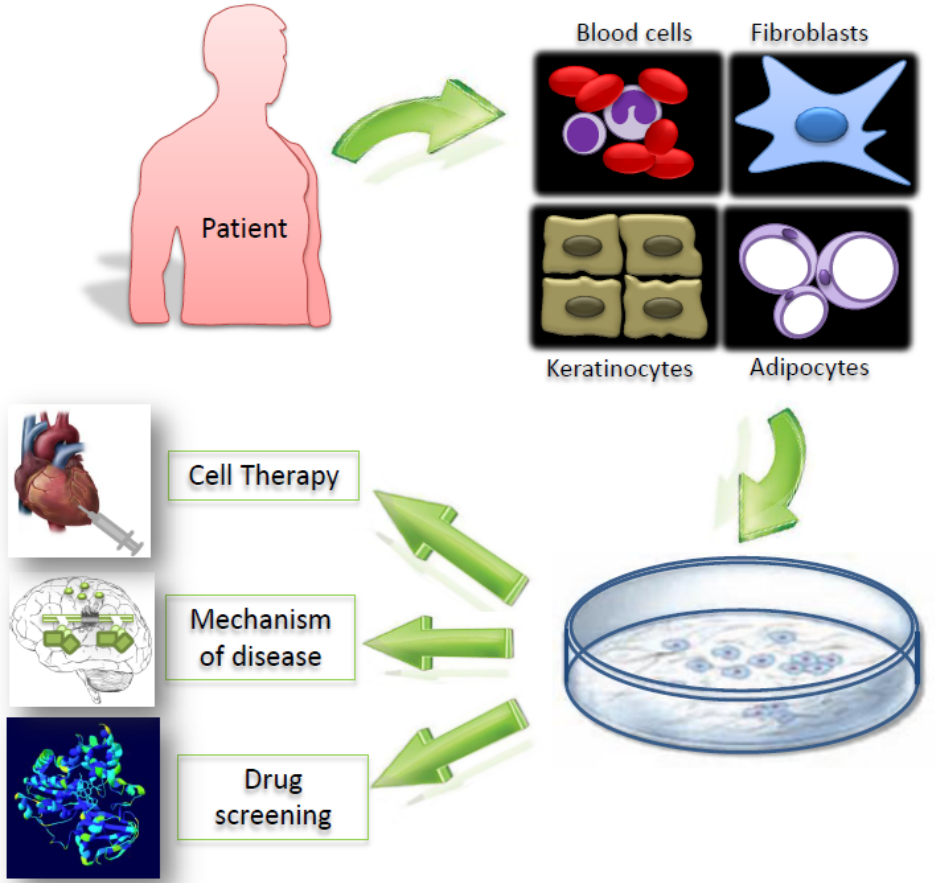

Figure 4 –iPS cells origin and use. Human iPS cells are derived from a variety of cellular types (e.g. fibroblasts, melanocytes, keratinocytes, adipocytes, cells from peripheral, cordonal and menstrual blood). iPS cells originated from patients could be used as model for the study of molecular mechanism of diseases, like neuronal degenerative syndromes. Moreover, another application for iPS cells is the regenerative medicine. Particularly, iPS cells have been used for therapy of long-term cure diseases and for a more reliable drug test *in vitro*.

In conclusion, iPS cell-based therapies are still in their infancy, and many hurdles remain to be overcome before their clinical applications become a reality. With further improvements in derivation technologies, characterization methods, cultivation, differentiation protocols, and a better understanding of the reprogramming mechanisms, therapies using patient-specific iPS cells have the potential to revolutionize regenerative medicine and benefit patients for decades to come.

In this thesis we investigated new approaches in the cure of hemophilia A by cell and gene therapy. In particular we analyzed additional extra hepatic sources able to express and secrete FVIII and in particular we concentrated our efforts on the myeloid lineage. Then we used an iPS-based strategy to obtain iPS cells from hemophilic mouse and human cells. First we optimized the protocol with normal donor cells and derived-iPS cells were differentiated in endothelial cells. Afterward using cells from hemophilic donors we corrected hemophilia A by gene therapy and then we reprogrammed them to iPS cells.

Material and Methods

Cell culture. 293T cells, Fibroblasts, Phoenix (packaging cell line for retroviral production), Mesenchymal-like, ECV and HepG2 cells were cultured in DMEM supplemented with 10% FBS (Hyclone), 2mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C, 5% CO2. Feeder cells used: HFF, human foreskin fibroblast (ATCC) were cultured inin IMDM supplemented with 10% FBS (Hyclone), 2mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin at 37 °C, 5% CO2.HFF were mitotically inactivated by gamma irradiation (55 Gy).
ES (embryonic stem) cells and iPS cells were cultured on top of irradiated human fibroblasts in human ES cell medium (hES) containing Knockout DMEM supplemented with 20% knockout serum replacement, nonessential amino acids, 2-mercaptoethanol, penicillin/streptomycin, GlutaMAX (all reagents from GIBCO), bFGF (Immunotools) and were picked mechanically.

Preparation of mouse megakaryocytes and bone marrow-derived macrophages. Femuri and tibiae of 8-9 weeks-old wt or hemophilia A mice were flushed with DMEM 5%. Cells clumps were broken by three or four passages through a 10 ml-syringe and 20-G needle. Red blood cells were lysed with RBLB buffer (150mM NH_4Cl,10mM $NaHCo_3$, 1 mM disodium EDTA) for 8 minutes on ice, reaction were neutralized with DMEM 10% FBS and cells were centrifuged for 10 minutes at 1300 rpm. For MK c-Kit+ cells were isolated with magnetic beads (Miltenyi Biotec) and cultured in STEM-SPAM medium (STEMCELL Technologies Inc.) with SCF 20 ng/ml for 2 days. Then cells were counted and plated at 1x10^6 cells/ml density for 3-4 days in STEM-SPAM medium with 100ng/ml TPO, 10 ng/ml IL-6 and 10 ng/ml IL-11. For BM-DM total cells from BM were plated at 5x10^6 cells density in 6-wellplate for 7 days with 5 ng/ml mMCSF (Immunotools). Images of cells were acquired with ZEISS Axiovert 40 CFL.

Isolation of tail tip fibroblasts (TTFs). Tail tips of 4 weeks hemophilia A mice were washed twice with ethanol 70% and then with PBS. Tips were cut in small pieces and plated in a 6-wellplate in DMEM 10% FBS. After about 5 days fibroblasts migrated out of the tip. For the correction and reprogramming passage 2 TTFs were used.

Western blot analysis. Cell pellets of MK were lysed on ice with NP-40 buffer (NP-40 1%, Tris/HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1mM, leupetin,pepstatin,

aprotinin and PMSF). Lysates were clarified by centrifugation at 13000 rpm for 20 minutes at 4°C. 100 ug of protein extract were resolved by 7,5 % SDS-polyacrylamide gel electrophoresis; transferred to PVDF and incubated with a 1:250 dilution of anti-hFVIII (produced in our laboratory) for 2 hours at room temperature. After three washes with PBS-Tween 20 0,1%, membrane was incubated with a 1:5000 dilution of secondary antibodies. Blot were developed with the use of Western Lightning ECL (PerkinElmer).10 and 40 ng of purified recombinant protein (KOGENATE, Bayer) was used as positive control.

RT-PCR analyses. Total RNA from MK, bone marrow cells, peripheral blood cells, TTFs, MNC, fibroblasts, iPS cells, EBs and endothelial cells was isolated using Isol-RNA Lysis Reagent (5 PRIME) and 1-2 ug were used to synthesize cDNA with the RevertAid Premiun First Strand cDNA Synthesis Kit Fermentas. 2 ul of the reaction were used to analyze gene expression by PCR (F:forward; R:reverse).

Primers for mouse β-Actin were β-Actin F 5'-gatgacccagatcatgtttga ga-3'; R 5'-gtctcc ggagtccatcacaat-3', 25 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 56°C for 30 seconds 72°C for 30 seconds and 72°C for 7 minutes.

Primers for αIIb, vWF and mFVIII were αIIb F 5'-cagggccaagtgctgatatt-3'; R 5'-ttgaagcagctgactggtgt-3', vWF F 5'-tgttcatcaaatggtgggcagc-3'; R 5'-acagacgccatctccagattca-3' and mFVIII F 5'-ggtatcaaagtgacaatgtacc-3'; R 5'-ccaattaatcccgagtgcatatc-3', with 30 cycles at 94° C for 3 minutes, 94° C for 30'', 54°C for 30'' for αIIb, 62° for 45'' for vWF and 54°C for 30" for mFVIII,72°C for 30 seconds and 72°C for 7 minutes.

Primers for pluripotency were: mMtap-2 F 5'-ctggtgcttttaaacaggcg-3'; R 5'-ttgcagttgatccaggggta-3', mGATA6 F 5'-accttatggcgtagaaatgctgagggtg-3'; R 5'-ctgaatacttgaggtcactgttctggg-3', mBrachyury F 5'-atgccaaagaaagaaacgac-3'; R 5'-agaggctgtagaacatgatt-3' with 30 cycles at 94° C for 3 minutes, 94° C for 30'', 52°C for 30'' for mMtap-2, 61° for 45'' for mGATA6 and 50°C for 30" for mBrachyury ,72°C for 30 seconds and 72°C for 7 minutes.

Primers for endothelial differentiation were: mVEC F 5'-ggatgcagaggctcacagag-3'; R 5'-ctggcggttcacgttggact-3', VEGFR2/Flk-1 F 5'-cgagtctgtctaccttggaggc-3', R 5'-cagcctgagcctttaccgc-3' with 30 cycles at 94° C for 3 minutes, 94° C for 30'', 62°C for

30'' for VEGFR2/Flk-1 and 56°C for 30" for mVEC,72°C for 30 seconds and 72°C for 7 minutes.

PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: β-Actin, 550bp; αIIb, 300 bp; vWF, 270 bp; ,mFVIII, 400 bp; mMtap-2,n300 bp; mGATA6, 334 bp; mBrachyury 870 bp; VEC, 250 bp; VEGFR2/Flk-1*,* 350 bp.

Primers for human β-Actin wereβ-Actin F 5'-gagaaaatctggcaccacacc-3'; R 5'-cgacgtagcacagcttctc-3', with 25 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes. Primers for hFVIII were: F 5'-ggagagtaaagcaatatcaga tgc-3', R 5'-ggtgaattcgaaggtagcgac-3'; with 30 cycles at 94° C for 5 minutes, 94°C for 30 seconds, 55 °C for 30 seconds, 72°C for 30 seconds and 72°C for 10 minutes.

Primers for Oct4, Sox2, Klf4 and c-Myc were: Oct4 F 5'-cgtaagcagaagaggatc acc-3', R 5'-gcttcctccacccacttctgc-3'; Sox2 F 5'-gcagctacagcatgatgcagg-3', R 5'-agctggtcatggagttgtactgc-3'; Klf4 F 5'-ccagaggagcccaagccaa-3', R 5'-5'-cgcaggtgtgccttgagatg-3', c-Myc F 5'-catccaggactgtatgtggag-3'; R 5'-gcgagctgctgtcgttgag-3' with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes. Primers for hBDD-hFVIII were: A2 F 5'-tgccacacctcagactttcg-3', A3 R 5'-gacggcgtttcaagactg gt-3', with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes.

Primers for endothelial differentiation were: KDR F 5'-tgcaaggaccaaggagactatgt-3', R 5'-taggatgatgacaagaagtagcc-3'; CD31 F 5'-aggtcagcagcatcgtggtcaacat-3', R 5'-gtggggttgtctttgaataccgcag-3' with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 55°C for KDR, 65 °C for CD31 for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes.

Primers for pluripotency[169] were: Nestin F 5'-cagcgttggaacagaggttgg-3', R 5'-tggcacaggtgtctcaagggtag-3'; Brachyury F 5'-cggaacaattctccaacctatt-3', R 5'-gtactggctgtccacgatgtct-3', AFP F 5'-actccagtaaacctggtgttg-3', R 5'-gaaatctgcaatgacagcctca-3' 3' with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 62°C for Nestin; 52°C for Brachyury; 54 °C for AFP for 30-45 seconds, 72°C for 30 seconds and 72°C for 7 minutes.

PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: β-Actin, 400bp; Oct4, 179bp; Sox2, 134bp; Klf4, 130bp; hBDD-FVIII, 180 bp;

FVIII, 400 bp; KDR, 457 bp; CD31, 450 bp; Nestin, 388 bp; Brachyury, 357 bp; AFP, 255 bp.

Immunofluorescence and AP staining. For iPS cells, cells were grown on plastic cover slide chamber, fixed with 4% parafolmaldehyde. The following antibodies were used: anti-Oct3/4 (Abcam), anti-SSEA-3 (Abcam), anti-NANOG (Abcam), anti-Sox2 (Abcam), all 1:100. MK were cytospined at 1000 rpm for 5 minutes, fixed with 4% parafolmaldehyde. The following antibodies were used: anti-FVIII (Abcam) 1:100, anti-vWF (SIGMA) 1:100 and anti-CD61 (Santa Cruz Biotecnology)1:250.
BM-DM, monocytes and macrophages were fixed with 4% parafolmaldehyde. The following antibodies were used: anti-hFVIII (produced in our laboratory) 1:150, anti-F4/80 (AB direct) 1:300, anti-CD14 (EBioscience) 1:150. For endothelial differentiation were used anti-hFVIII (produced in our laboratory) 1:150, anti-vWF (SIGMA) 1:100 and anti-vWF (Santa Cruz Biotecnology) 1:50 for mouse differentiation.
Secondary antibodies used were all from Invitrogen (all 1:500). Images were acquired with LEICA DM5500B fluorescence microscope.
Direct AP activity was analyzed using an alkaline phosphatase blue membrane substrate solution kit (Sigma, AB0300) according to the manufacturer's guidelines.

qPCR analysis from human bone marrow. Red blood cells of patient's total bone marrow were lysed with RBLB (red blood lysis buffer, 1,5 M NH_4Cl, 100 mM $NaHCO_3$, 10 mM disodium EDTA,10X). Total RNA was isolated with Isol-RNA Lysis Reagent (5 PRIME) and 2ug was used to synthetized cDNA with OmniScript Qiagen kit.
The quantitative real time PCR was carried out in a 20-ul total volume containing 1X SYBR green PCR master mix (PROMEGA), 1 uM forward and reverse primers (FVIII) and 0,25 uM forward and reverse primers (hβ-Actin), 20 ng of cDNA for hβ-Actin and 30 ng of cDNA for FVIII. Primers sequence are the following: hFVIIILC1 F 5'-caatggctacataatggatacactacct-3', R 5'-tgtccactgaaatgaatagaatggat-3'; hβ-Actin F 5'-gagaaaatctggcaccacacc-3'; R 5'-cgacgtagcacagcttctc-3'. Quantitative PCR were performed by incubation at 95°C for 3 minutes and 40 amplification cycles of 95°C for 3 minutes and then 60°C for 30 seconds.

Preparation of human megakaryocytes and macrophages from cord blood. Hematopoietic stem cells were isolated from cord blood mononuclear cells (CBMC) by immunomagnetic selection using the MACS ® CD34 MicroBead Kit (Miltenyi). To obtained macrophages differentiation from hematopoietic stem cells 10^5 CD34+ cells/ml were plated in STEM-SPAM medium (STEMCELL Technologies Inc.) added with 20% FBS, 2mMglutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 30 ng/ml IL-3, 30 ng/mlhMCSF, 30 ng/mlFlt-3 ligand, 25 ng/mlSCF. Medium were changed every 2 days. After 14 days cells acquired macrophages morphology. To obtain megakaryocytes from hematopoietic stem cells 1,5x10^6 CD34+ cells/ml were plated in STEM-SPAM medium with glutamine 2mM, penicillin 50 U/ml streptomycin 50 µg/ml, 10 ng/ml IL-6, 10 ng/mlIL-11 and 20 ng/mlTPO. The medium was changed at day 3, 7and 10. After 13 days megakaryocytes were harvest and used for experiments. Images of cells were acquired with ZEISS Axiovert 40 CFL.

Preparation of mouse and human peripheral blood cells (PBMC).
Red blood cells of peripheral blood from wt and HA mice were lysed as above described. Total PBMC were plated 5-6x10^6 cells in 6-well plate. The day after monocytes were attached. For macrophages differentiation monocytes were cultured for 1 week with mMCSF 10 ng/ml.
To isolate human peripheral blood mononuclear cells, blood was diluted three times in PBS, added to a density gradient solution (Ficoll-PaqueTM PREMIUM, GE Healthcare) and centrifuged 20 minutes at 650g without brake and acceleration. The cells ring obtained was harvested, washed two time and finally the cells were seeded in serum free RPMI medium to raise the monocytes adhesion. After 1 hour the medium was changed with RPMI supplemented with 5% FBS and cells were cultured for 12 hour. To obtain macrophages from peripheral blood monocytes, cells were cultured for 1 week in DMEM supplemented with 10% fetal bovine serum (FBS), glutamine 2mM, penicillin 50 U/ml streptomycin 50 µg/ml, sodium pyruvate 1mM (Lonza) , non-essential amino acids 1mM (Lonza), HEPES 0,25mM (Lonza), M-CSF 10 ng/ml. Fresh medium was added every 3 days. Images of cells were acquired with ZEISS Axiovert 40 CFL.

Immuno-histochemistry (IHC). IHC were done in collaboration with Prof. Valente using anti-FVIII produced in our laboratory.

Retroviral production and cell's transduction. cDNA's of Oct3/4, c-Myc, Sox2 and Klf4 were in modified pMSCVpuro vector that allows the expression of N-terminal FLAGtagged proteins, green fluorescent protein (GFP) in pSIN vector. Retroviruses for the four factors were independently produced after transfecting the cell line Phoenix Amphotropic using Fugene 6 reagent (Roche) according to the manufacturer's directions. After 24 h, the medium was replaced, cells were incubated at 32 °C, and the viral supernatant was harvested after 24 and 48 h. A 1:1:1:1 mix of retroviruses with FLAG-tagged *Oct4*, *Sox2*, *Klf4* and c-*Myc*, a 1:1:1 mix of *Oct4*, *Sox2*, *Klf4* and a 1:1:1:1 mix of *Oct4*, *Sox2*, *Klf4 and GFP* was added to fibroblasts in the presence of 1 µg/ml polybrene and spin infected for 45 min at 700*g* at 32 °C. This procedure was repeated the following day. After replacing with fresh serum-free low-calcium medium and incubating for 2 days, cells were trypsinized and seeded into 10-cm dishes containing 4 million irradiated mouse fibroblasts and ES cell medium.

Endothelial differentiation of fibroblast-derived iPS cells, hES cells, mesenchymal-like cells. Embryoid bodies (EBs) formation was induced by mechanically picking of colonies and were cultured in low attachments plates in EB medium (ES medium without FGF and with 10% FBS) . After 3-4 days the EBs were transferred to 0.1% gelatin-coated 6-well plate and mesenchymal-like cells were seeded at density 50000 cells/well. Cells were cultured in M199 medium (Sigma) supplemented with 10% heat-inactivated FBS, penicillin-streptomycin (PAA), GlutaMAX (GIBCO), IGF-1 20 ng/ml (Peprotech), ECGS 10 ng/ml (Sigma), VEGF 50 ng/ml (Peprotech), bFGF 20 ng/ml (Peprotech). The medium was replaced every 2-3 days.

For MNC-derived iPS cells or mouse iPS cells, EBs were plated respectively in EB medium with hrVEFG 50 ng/ml or HPGM medium (LONZA) with mrVEGF 50 ng/ml (Immunotools) for 20 days, adding fresh VEGF twice a week. Morphology and specific endothelial markers were analyzed over time.

Isolation of peripheral blood mononuclear cells (MNC) and transduction. 30 ml of peripheral blood was isolated from healthy donors or hemophilic patients and MNC cells were separated by Ficoll-Hystopaque density gradient (GE Healthcare). MNC cells were cultured in α-MEM media containing 10% FBS and 10 ng/ml IL-7, GM-CSF, IL-3 and IL-6 for five days. Blood cells were spin-infected two times for 1h at

1600rpm. After two days, cells were transferred onto HFF feeders in α-MEM media containing 10 ng/ml IL-7, GM-CSF, IL-3 and IL-6 for four more days. After, cells were cultured in standard human ES medium for 25-40 days before iPS colonies were picked.

Flow cytometry analyses (FACS). For immunophenotypic analysis of MNC freshly isolated or after 5 days of culture and of macrophages obtained from CD34+, cellswere incubated with the following antibodies using according to the manufacture's instruction: PE-CD11b, FITC-CD3, FITC-CD45, FITC-CD14 (all from Immunotools), PE-CD19 (Biolegend). Flow cytometry was performed using FACSCalibur (Becton Dickinson). Data were analyzed with WinMDI2.9.

Lentiviral vector production. 293T cells were co-transfected with four vectors by calcium phosphate precipitation; these vectors were the pMDLg/RRE packaging plasmid (12,5 ug); the pMD2.VSV-G envelope-coding plasmid (9 ug); pRSV-Rev (6,25 ug) and transfer vector plasmid included 3 factors (OCT4, SOX2, KLF4, 36 ug). All four plasmids were added to cells in a 15-cm dish and vector particles were concentrated by ultracentrifugation. Titer was calculated by qPCR.

Adipogenic and osteogenic differentiation. EBs were plated on gelatin with adipogenic medium (hMSC Mesenchymal Stem Cell Adipogenic Differentation Medium, LONZA) or osteogenic medium (α-MEM with 10%FBS, 10nM dexametasone, 0,4 mM ascorbic acid, 1 mMβ-glycerophosphate) for 15 days. To detect calcium deposits cells were fixed with PAF 4% for 10 minutes. After wash with PBS, cells were incubated for 15-20 minute with Alizarin Red S. For adipogenic differentiation, cells were fixed with PAF 4% for 10 minutes, incubated with isopropanol 60% for 30 seconds. Oil Red O (ORO) was added for 15 minutes. After ORO elimination, cells were incubated with isopropanol 60% for 30 seconds, then were stained with haematoxylin and wash with ddH20. Images were acquired byLeica ICC50HD.

Transplantation of differentiated cells. NOD-SCID HA mice were pre-treated with monocrotaline (200 mg/kg, MCT). The day after $2x10^6$ of iPS-derived EC were

transplanted by portal vein injection in mice. Mice were killed 96 hours after transplantation and cell engraftment was analyzed using anti-GFP (Invitrogen) and F4/80 (AbDSerotec) primary antibodies and secondary antibodies FITC- and TRIC-conjugatedfor immuno-staining of liver sections. Images were acquired by LEICA DMIRE2 confocal microscope.

Genomic DNA isolation. Genomic DNA was isolated from iPS cells with ReliaPrep gDNA Tissue MiniPrep System (Promega). Primers used for integration analysis were: PGK sense 5'-gttccgcattctgcaagcc-3' and hFVIII5' 5'-atagtttagcggccgcgagtcgactctagaggatcc-3'. PCR products were resolved in 2% agarose gels. Expected product size was 500 bp.

Results

FVIII expression in mouse and human bone marrow-derived cells

Since it has been demonstrated that bone marrow transplantation in mice corrected hemophilia A phenotype,[119,120] we further investigated which population of hematopoietic cells expressed FVIII. Bone marrow from wild type mice of 8-9 weeks was cultured for 7 days with mMCSF to induce macrophages differentiation. cDNAs were prepared from total bone marrow and bone marrow-derived macrophages mRNA(BM-DM, Fig.1A) and RT-PCR was performed to analyze FVIII expression. FVIII transcripts were detected in total BM and interestingly FVIII expression increased in BM-DM after 1 week in culture (Fig. 1B). Since the presence of mRNA does not mean presence of protein, we analyzed FVIII protein in BM-DM by immunostaining. We used anti-F4/80 antibody, a specific marker of mouse macrophages, and anti-FVIII antibody, produced in our laboratory. As showed in Figure 1C, cell positive for F4/80 were also positive for FVIII.

To analyze the presence of FVIII in megakaryocytes, we decided to isolate c-Kit+ progenitor cells from total BM with magnetic separation as described[243] and cultured for 4 days with TPO, IL-3 and IL-6. MK were identified by their large size (Fig.2A) and expression of vWF and αIIb integrin (Fig.2B), MK-specific markers of differentiation. As shown in Figure 1B MK and c-Kit+ expressed FVIII, instead c-Kit negative cells had low level of FVIII mRNA.

Then to investigate the expression of FVIII at protein level, MK were cytospinned onto cover glass and they were stained with vWF and FVIII antibodies for immunofluorescence analysis. FVIII was expressed in MK co-localizing with vWF (Fig.2C) and it was also detected by western blot analysis on MK lysates (Fig.2D).On the contrary hemophilic mice did not express FVIII (Fig.2E,F).

On the same time, we investigated if also human myeloid cells express FVIII. First we investigated if total human bone marrow express FVIII mRNA as well. In collaboration with the haematology division of Prof. Gaidano, we collected 21 bone marrows from “healthy” donors. We considered “healthy” a donor that underwent BM biopsy with non-malignant diagnosis. mRNA was isolated and analyzed for FVIII expression by RT-PCR. All donors expressed human FVIII with exception for donor 2 and 4 because the mRNA quality was not good (not shown). To verify mRNA integrity β-Actin RT-PCR was performed (Fig. 3A). To normalize the expression of FVIII for

some patients, we performed a qPCR. The endothelial cell line ECV was used as a positive control and FVIII expression in total BM was lower than in ECV, but it was present at different level in all donors (Fig. 3B). FVIII was also detected as protein product as shown in Figure 3C.

To analyzed expression in human myeloid cells, an accessible source of progenitor, cells as CD34+ cord blood-derived cells, were utilized. CD34+ cells were cultured with IL-3,M-CSF, Flt-3 ligand, SCF for macrophages differentiation and IL-11, IL-6 and TPO to recover megakaryocytes. The latter after differentiation acquired typical morphology as big,round cells and grown in suspension but human megakaryocytes were smaller than mice ones (Fig.4A). Macrophages appeared as adherent cells with fried egg morphology and abundant cytoplasm (Fig.4A). Cytofluorimetric analysis for CD11b and CD14 markers, two well-known macrophages markers, confirmed the successful differentiation (Fig. 4B). Finally we confirmed that human MK and macrophages expressed FVIII at mRNA (Fig.4C) and protein level as well (Fig.4D).

FVIII expression in peripheral blood cells

Since there are no evidences of FVIII expression in peripheral blood cells, we isolated monocytes and lymphocytes from mouse blood. RT-PCR analysis showed that FVIII mRNA was expressed both in monocytes and lymphocytes (Fig.5A). In particular monocytes expressed FVIII protein as shown in Fig.5B, CD14 was used as specific monocytes markers. As we observed expression of FVIII in macrophages from bone marrow, we analyzed FVIII expression also in monocytes-derived macrophages differentiated from PBMC. Macrophages were staining for F4/80 and all cells showed typical perinuclear FVIII-expression (Fig.5B).

Then we analyzed FVIII expression in hemophilic mice. As previously reported FVIII mRNA (Fig.5A) was not expressed.

Afterward analysis of peripheral blood of healthy donors and one hemophilic patient (in collaboration with Dott. Schinco, Torino) confirmed expression of FVIII in human samples (Fig.6A). This is not surprising because, unlike mouse model in which introduction of neomycin cassette between exon 16 and exon 17 leads to a stop codon, in humans mutations caused partial or total deficiency of protein activity.

FVIII expression was confirmed both in human monocytes and macrophages differentiated from PBMC (Fig.6B,C). A validation of our *in vitro* results were confirmed by immunohistochemistry on several human tissues showing that spleen, lymph node and bone marrow macrophages stained positive for FVIII (Fig.7). In figure 7C is shown a bone marrow tissue slide showing a FVIII-positive megakaryocyte. In conclusion our results clearly indicated that myeloid cells were the main FVIII expressing cells in the hematopoietic compartment both in human and mouse.

Generation and characterization of fibroblasts-derived iPS cells

Since the reduced availability of progenitor and mature cells from human, it is necessary to find new cell sources to cure diseases characterized by damaged cells, tissues or organ. The latest development is the demonstration that somatic cells can be reprogrammed to a pluripotent state by the expression of a transcription factor cocktail, generating induced pluripotent stem (iPS) cells that can be differentiated virtually to all type of cells presents in the body.

The second part of this project was focused on the optimisation of cell reprogramming of mature cells to obtain iPS cells to be used for hemophilia therapy. Initially, human skin fibroblasts were reprogrammed (Fig. 8A) by 4 different retroviral vectors containing the reprogramming factors in a ratio of 1:1:1:1 (Oct3/4, Sox2, Klf4 and c-Myc). Control of transduction was done with the infection of Sox2, Klf4 and Oct4 and GFP (Fig.8B). After two dayd from infection, cells were seeded onto a layer of irradiated HFFs in hES cell medium.

Within 9 days post transduction we detected several cell colonies (Fig. 9A). After about one month from transduction we picked four-factor-induced cells (Fig. 9A) displayed typical human ES cell-like morphology and seeded them onto HFF in order to expand and characterize them. In Figure 9B was showed a typical ES cell-like colony of cells transduced with GFP.

To verify that cells were pluripotent a staining for alkaline phosphatase (AP) activity was performed and cells were positive (Fig. 10A). AP staining is considered one of the most reliable pluripotency marker in hES cells.[244] RT-PCR showed that iPS cells expressed mRNA of reprogramming factor unlike fibroblasts before reprogramming

(Fig.10B). Human iPS cells expressed Oct4, Sox2 and NANOG proteins and typical surface antigens of ES cells including SSEA-3 (Fig. 10C). All these results confirmed that our iPS cells were *bona fide.*

Endothelial differentiationof fibroblast-derived iPS cells

The goal of our study is to differentiated iPS cells in endothelial cells, a cell type that it has been demonstrated could be a good target for cell and gene therapy in hemophilia A.[118] It has been demonstrated that mouse mesenchymal cell can differentiate in endothelial cells using a particular differentiation medium.[245] To induce endothelial differentiation of iPS and ES cells, the cells were plated and cultured in ES medium without bFGF to trigger EBs formation. After 3 days EBs were seeded on 0,1% gelatine coating plate in differentiation medium as described in material and methods. Cells were collected and analyzed for gene expression 3 weeks after differentiation. Mesenchymal-like cells, obtained from differentiation of iPS cells, were used as a control.

During differentiation cells changed morphology and assumed cuboidal shape typical of endothelial cells (Fig. 11A,B,C). Analysis of gene expression showed an increase in endothelial markers such as CD31 and KDR in the three lines tested after 3 week of differentiation. FVIII expression increased in iPS and ES cells, instead mesenchymal-like cells have high levels of FVIII and there was no difference in expression after endothelial differentiation (Fig.11D).

Generation and characterization of MNC-derived iPS cells

We generated iPS cells from fibroblasts by retroviral transduction using the 4 Yamanaka's factors.However, in hemophilic patients to harvest fibroblasts from skin biopsies is a risky procedure for bleeding. Because it has been demonstrated that iPS cells could be generated from blood cells[168-173]we utilized peripheral blood cells as an easy-to-access source of cells and reprogrammed mononuclear cells from donors and hemophilic patients. Moreover, blood cells were transduced with a lentiviral vector containing the reprogramming factors all in one construct (Oct4, Sox2

and Klf4) in a specific order to obtain reprogramming in human cells. In this case we did not include c-Myc among the reprogramming factors.First mononuclear cells were isolated from peripheral blood of healthy donors with Ficoll-hystopaque gradient. Cells were plated in α–MEM with IL-3, IL-6, IL-7 and GM-CSF for 5 days. Cells were characterized both after isolation and after 5 days in culture to know the cell population undergoing reprogramming. As shown in Fig.12A all cells were CD45 positive, a marker of hematopoietic lineage, and the principal cell populations were CD3, CD14 and CD11b positive. It is interesting to note that after 5 days, cells were mainly CD3+ and CD19+ (Fig.12B). For reprogramming, cells were transduced with the reprogramming LV at different multiplicity of infection (MOI). This vector contained LoxP sites to allow the removal of the expression cassette after reprogramming with Cre-recombinase and reduce the possibility to perturb the host genome. After two days cells were seeded onto a layer of HFFs in α–MEM. After 4 days medium was changed with hES medium.

One month after transduction colonies were picked displaying a typical human ES cell-like morphology (Fig.13A) and seeded them onto HFF in order to be expanded and characterized.

These colonies stained positive for AP activity (Fig. 13B). RT-PCR of iPS cells mRNA showed expression of reprogramming factors unlike MNC before reprogramming (Fig.13C). Human iPS cells expressed Oct4, Sox2 proteins and the typical surface antigens of ES cells including SSEA-3 (Fig. 13D) by immunofluorescent staining.

iPS cells were differentiated into EBs and after 1 week mRNA was isolated and RT-PCR of mesodermal (Brachyury), endodermal (Alpha-fetoprotein, AFP) and ectodermal (Nestin) markers was performed to verify the pluripotency of the isolated clones. As shown in Fig. 14A the three lineage markers were up-regulated in the differentiated EBs as compared to the undifferentiated iPS cells. Moreover, EBs were differentiated in adipogenic and osteogenic tissues using specific media. Cells stained positive for Oil Red O (ORO) staining for adipose differentiation and Alizarin Red for the osteogenic differentiation (Fig. 14 B,C).

Endothelial differentiation of MNC-derived iPS cells

To differentiate iPS in endothelial phenotype, we first induced EBs formation. After 1 week EBs were seeded on 0,1% gelatine coating plate in EB medium with 50 ng/ml of hrVEGF for two weeks. Cells were collected and analyzed for endothelial markers expression at different time point during differentiation process. Cells changed morphology and assumed cuboidal shape typical of endothelial cells (Fig. 15A). Analysis of gene expression showed an increase in endothelial markers in two clones analyzed such as CD31 and FVIII, instead KDR did not change (Fig. 15B). The expression of FVIII was also analyzed by immunofluorescence staining. iPS-derived EC expressed FVIII and vWF (Fig. 15C), the FVIII-carrier protein in the plasma, which is a protein typically expressed by endothelial cells and megakaryocytes.[63]
As further demonstration of endothelial differentiation we transduced cells with several LV expressing GFP under the control of endothelial specific promoters, Tie2 and Flk-1. More than 50% of transduced cells expressed GFP using these LV (Fig. 16A). Moreover, we analyzed transduction efficiency of differentiated cells by using a LV containing GFP under the control of the ubiquitous promoter phosphoglycerate kinase (PGK) and in this case over 85% of cells were GFP+. These results confirmed that our cells started to differentiate in EC but at the time of analysis the EC-differentiation was not complete and further experiments are necessary to address the best protocol to obtain endothelial cells. To investigate the engraftment capacity of differentiated cells, we transplanted Flk-1 cells by portal vein injection in MCT – treated NOD-SCID HA mice. After 96 hours, mice were killed and GFP+ cells were detected by immunofluorescent staining of liver sections. By confocal analysis we detected cells near blood vessels without a significant inflammatory response around transplanted cells (Fig. 16B).

Generation and characterization of MNC HA-derived iPS cells

Once optimized the protocol with healthy cells, we isolated MNC from several hemophilic patients. At day 3 from isolation, we corrected MNC with a LV expressing the hBDD-FVIII under the control of PGK promoter at MOI 10. RT-PCR with specific primers showed that corrected cells express hFVIII compared to not transduced cells

(Fig. 17A). After 2 days we reprogrammed corrected and non-corrected cells and after about 5 weeks colonies appeared in corrected cells. Also iPS cells from hemophilic patients were correctly reprogrammed, indeed stained positive for AP activity (Fig. 17B) and expressed reprogramming factors (Fig. 17C,D). Unfortunately, expression of FVIII decreased in iPS cells overtime (Fig. 17E). We are now trying to understand the reason of FVIII silencing in corrected cells because by genomic PCR analysis we found that LV-genome is still present in transduced iPS cells but expression of FVIII driven by PGK was absent (Fig. 17F).

These results confirmed that it is possible to obtain iPS cells from normal and hemophilic hematopoietic cells by LV using only 3 reprogramming factors (Oct4,Sox2 and Klf4) without Myc increasing the biosafety of this process and differentiate them in endothelial cells. Some more work need to be performed because we need to address the reason of FVIII silencing under the transcriptional control of PGK promoter. We are now correcting new iPS cells directly with LV expressing FVIII under the control of an endothelial specific promoter (Vascular-endothelial cadherin, VEC) and we will verify if we are going to obtain therapeutic expression of the clotting factor.

Generation and characterization of mouse fibroblast-derived iPS cells

In parallel with human experiments, we generated mouse iPS cells in order to have an autologous model of disease ready to study.

TTFs were isolated from hemophilic mice and were expanded. The day before transfection cells were seeded and transduced with the LV expressing the human FVIII at MOI 10, as control of transduction efficiency fibroblasts were transduced with LV-PGK.GFP at the same MOI. TTFs were transduced but not all cells were GFP positive (Fig. 18A). After 3 days mRNA was extracted and immunofluorescence staining were performed. As shown in Figure 18 B,D cells were corrected and expressed human FVIII, while TTFs before transduction were negative for FVIII (Fig.18B,C). The remaining cells were plated again and transduced with the reprogramming LV at MOI of 3,5 and 10, similar to experiments with human cells but the order of factors along the expression cassette after the promoter was different:

Oct4, Klf4 and Sox2. After two days cells were plated on irradiated feeder and after 21 days first colonies appeared in cells transduced with MOI 5. As for human cells, colonies were compact and with defined borders but were smaller in size (Fig. 19A). Cells were characterized for pluripotency markers and were positive for AP staining (Fig.19B). Expression of reprogramming factors were analyzed by RT-PCR and immunofluorescence (Fig.19C,D). Expression was due to reactivation of endogenous genes. Indeed RT-PCR performed with primers that amplified a part of the polycistronic mRNA originated from the LV did not give any results (Fig.19E). Interestingly all clones obtained were corrected and maintained FVIII expression over time without silencing of the promoter as seen in the human counterpart (Fig. 19D).

In vitro differentiaton of mouse iPS cells

To test the pluripotency ability, miPS cells were detached and maintained in low-attached plates to form EBs (Fig. 20A). EBs were differentiated in adipogenic and osteogenic tissues with the same media used for human cells. As shown in Figure 20B (left) after ORO staining, cells displayed triglycerides deposits in the form of typical lipid drops. Osteogenic differentiation was demonstrated by the coloration of cells with Alizarin Red and crystal formation (Fig.20B, right). Moreover, EBs expressed markers of three germ layers: GATA6 for endoderm, MTap-2 for ectoderm and Brachyury for mesoderm (Fig. 20C).

Endothelial differentiation of mouse iPS cells

Finally, we evaluated if mouse iPS cells can differentiate in endothelial cells. EBs were plated on gelatine for 1 week in EB medium and once attached (Fig.21A) were cultured for two week with HPGM and mVEGF. Cells acquired typical endothelial morphology (Fig.21A) and at day 12 of differentiation by RT-PCR an increase of endothelial markers as vWF, Flk-1 and VEC was appreciated. As validation of our results, endothelial-differentiated iPS cells co-expressed vWF and FVIII as demonstrated by immunofluorescent staining (Fig.21C).

In conclusion our protocols to obtain iPS cells from mouse and human cells was successful. We were able to obtain cell reprogramming in the absence of myc and by lentiviral vector transduction. We will now implement the endothelial differentiation protocol to obtain large numbers of cells to be transplanted in hemophilic mice to verify the phenotypic correction of the bleeding disorder.

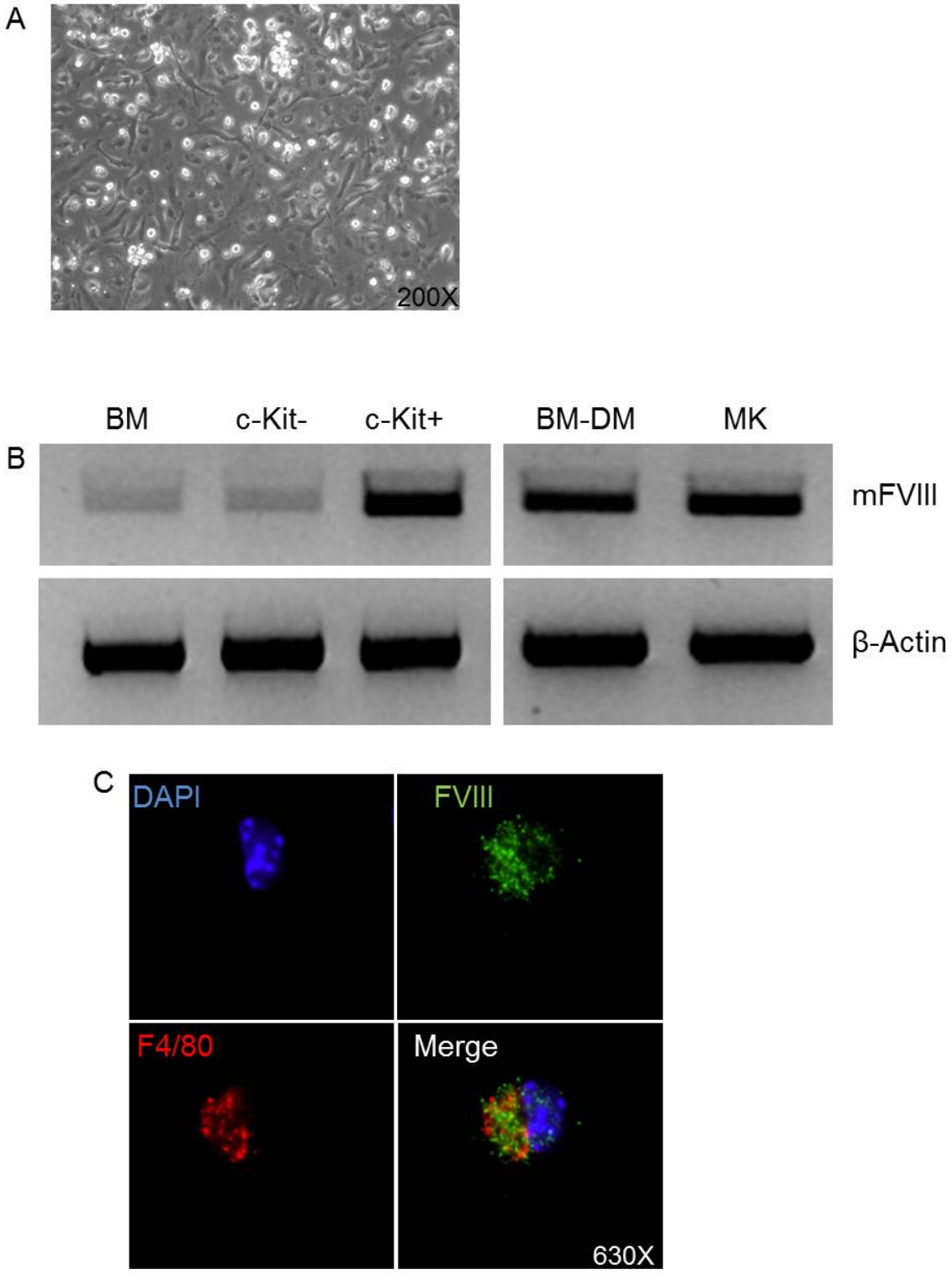

Figure 1. Expression of FVIII in mouse BM-DM cells. BM-DM in culture from C57BL/6 (A) were analyzed for FVIII expression by RT-PCR (B). Also FVIII immunofluorescent staining of BM-DM demonstrated FVIII expression. Anti-FVIII (green), F4/80 antibody (red) DAPi for nuclei (blue) (C).

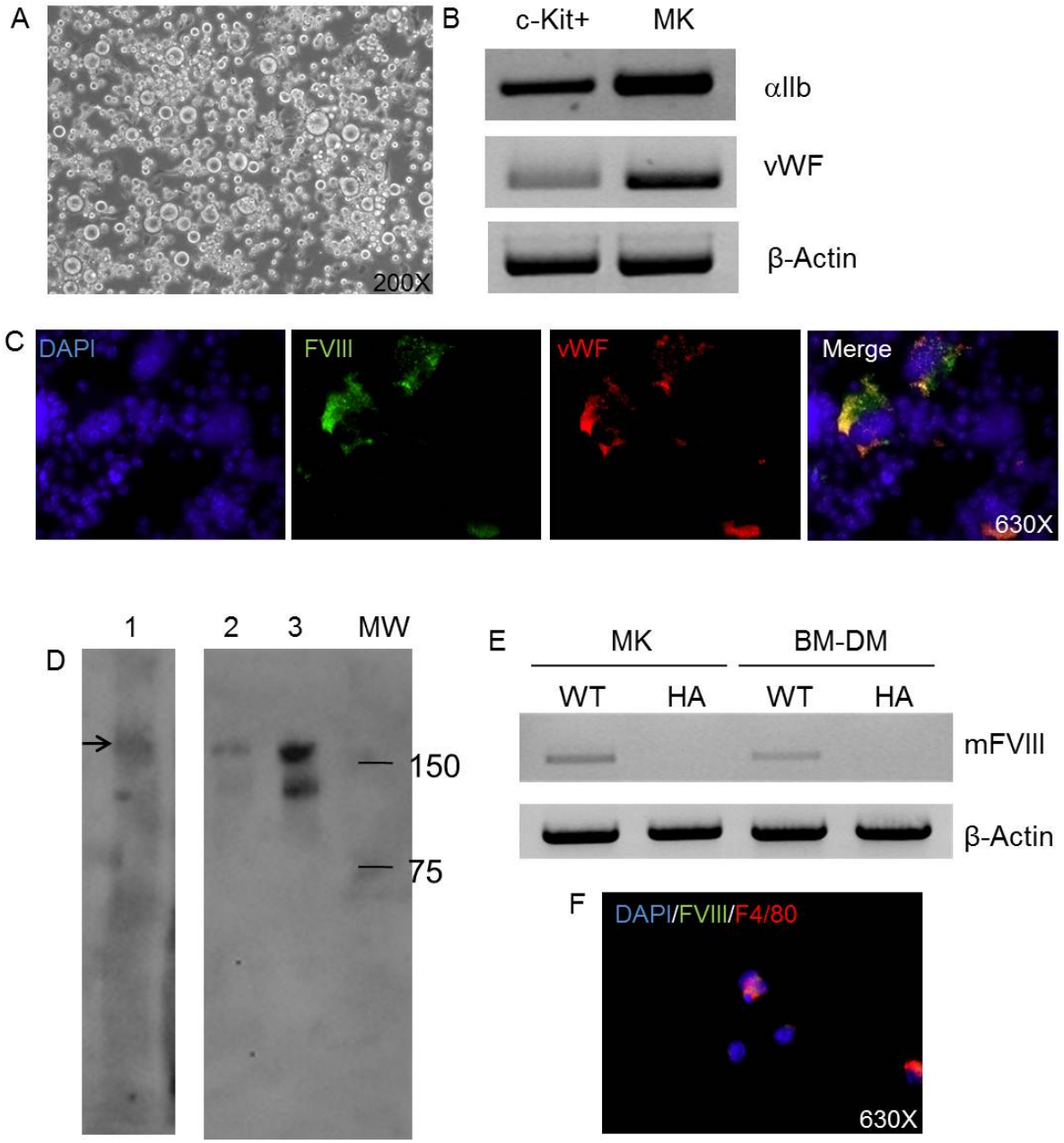

Figure 2. Expression of FVIII in mouse MK. Culture of MK differentiated from bone marrow of wild-type mice (A) expressed MK markers (B). Immunostaining of FVIII in MK (C) showed co-staining of FVIII (green) and vWF (red) in the cytoplasm of positive cells. DAPI for nuclei (C). Immunoblot analysis of MK (lane 1) decorated with a polyclonal FVIII antibody showef FVIII expression. Purificated protein (Kogenate, lane 2: 10 ng, lane 3: 40 ng) was used as positive control (D). Cells from hemophilic mice did not express FVIII at RNA (E) and protein level (BM-DM,F).FVIII (green), F4/80 as macrophage marker (red), DAPI (blue) for nuclei.

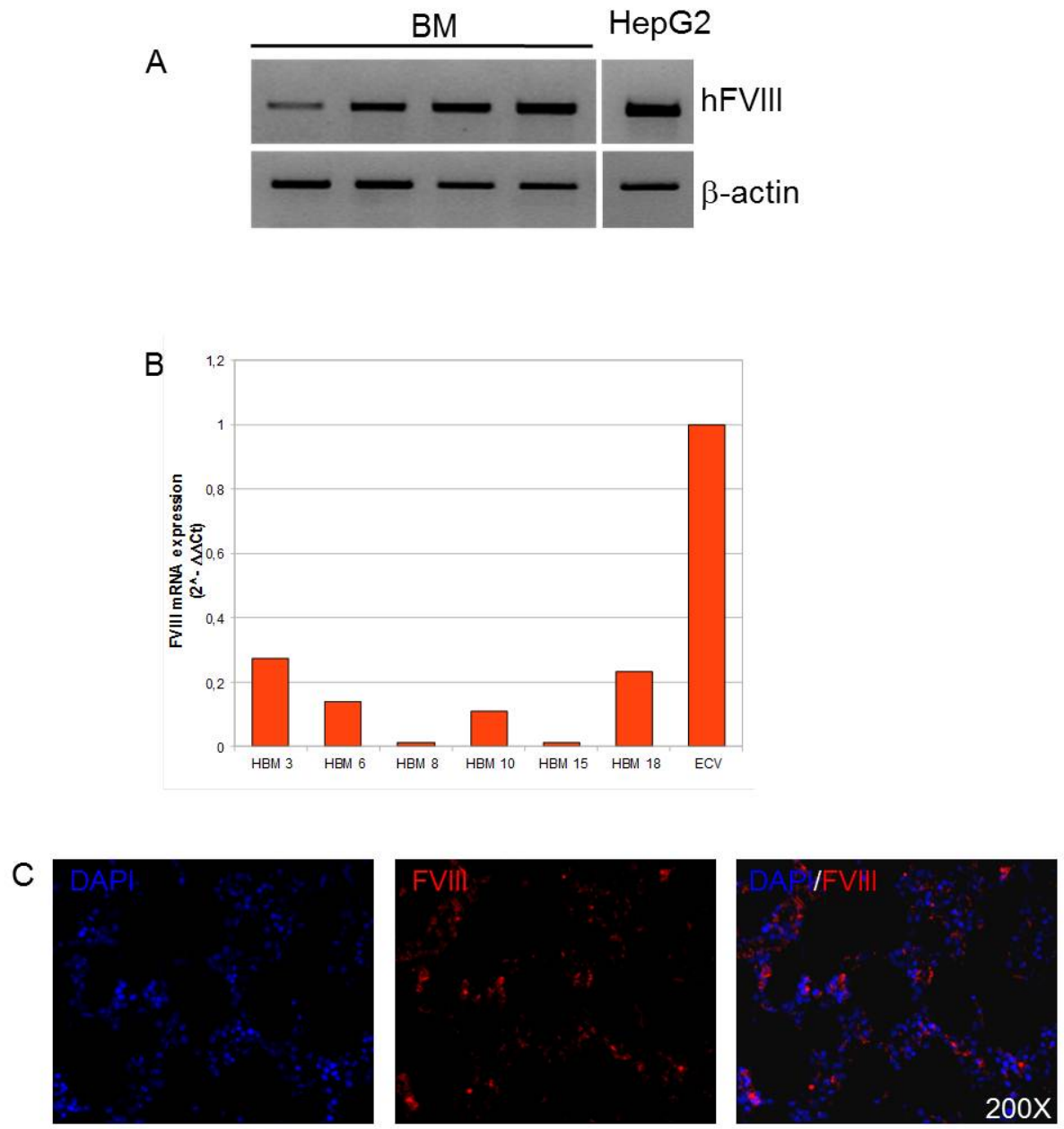

Figure 3. Expression of FVIII in human bone marrow. RT-PCR (A), qPCR (B) and immunofluorescent staining with nuclei in blue (DAPI) and FVIII in red appearance (C) showed expression of FVIII in several human bone marrow samples. Human hepatocarcinoma cell line (HepG2) was used as a positive control (A).

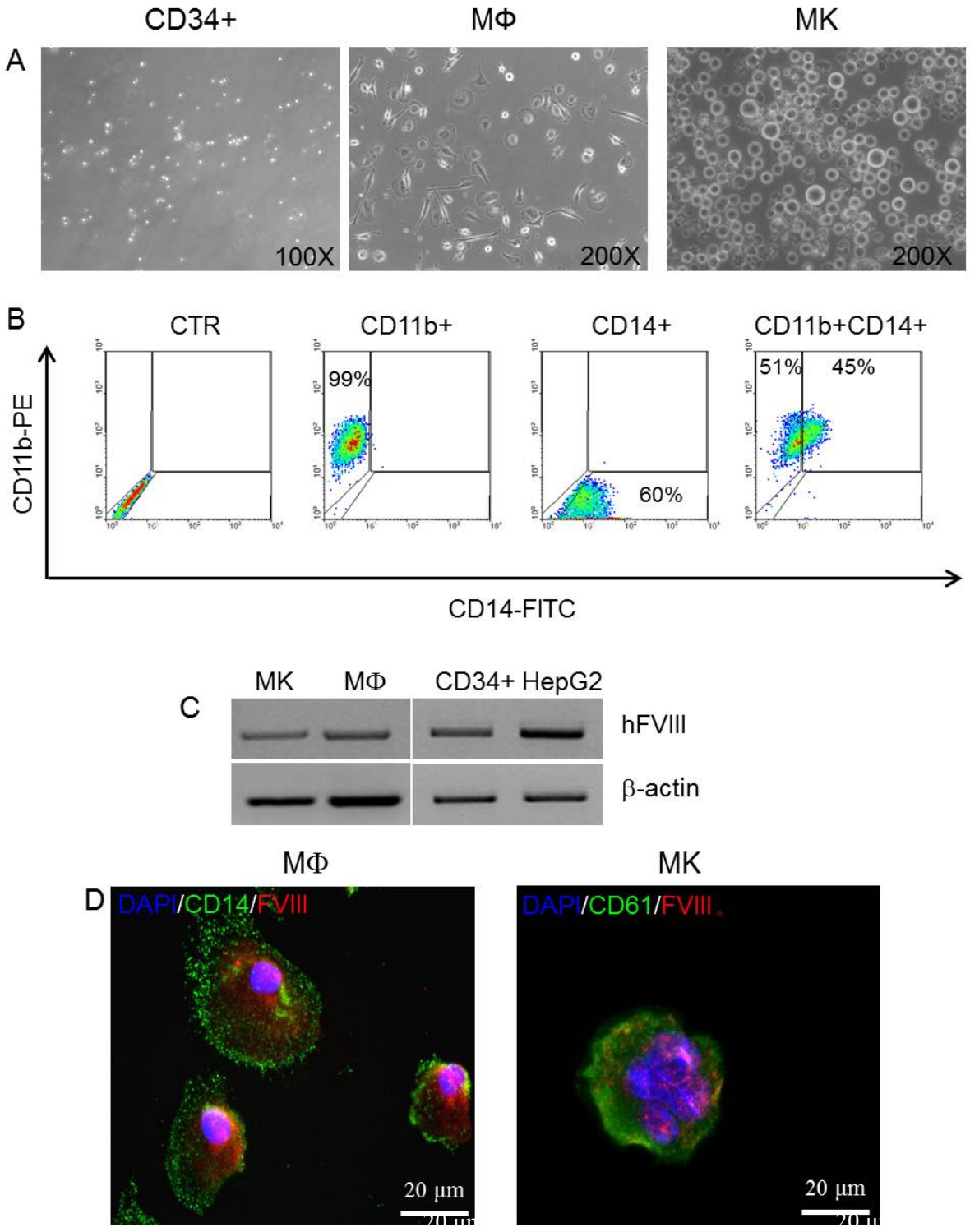

Figure 4. Expression of FVIII in human myeloid cells. CD34+ cells isolated from cord blood were differentiated in macrophages and MK (A). FACS analysis confirmed macrophages differentiation (B). RT-PCR (C) and immunostaining (D) shows FVIII (in red) expression in macrophages (CD14+ in green) and MK (CD61+ in green). DAPI for nuclei. HepG2 as positive control for FVIII expression in C.

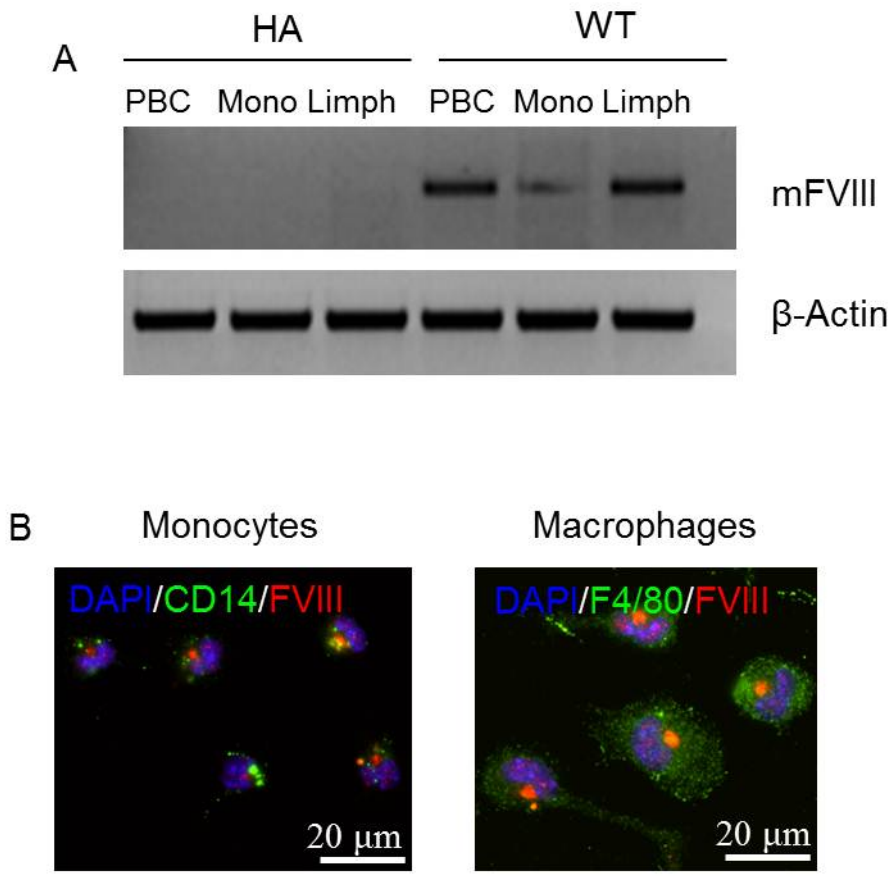

Figure 5. Expression of FVIII mRNA in peripheral blood cells of hemophilia A mice. RT-PCR from PBMC (PBC: peripheral blood cells, Mono: monocytes, Limph: lymphocytes) of hemophilia A and C57BL/6 showed FVIII expression only in wt mice (A). Expression of β-actin shows RNA integrity. Immunostaining for FVIII (red) shows that cells were positive for FVIII in CD14 and F4/80 positive cells (all in green). DAPI for nuclei (B).

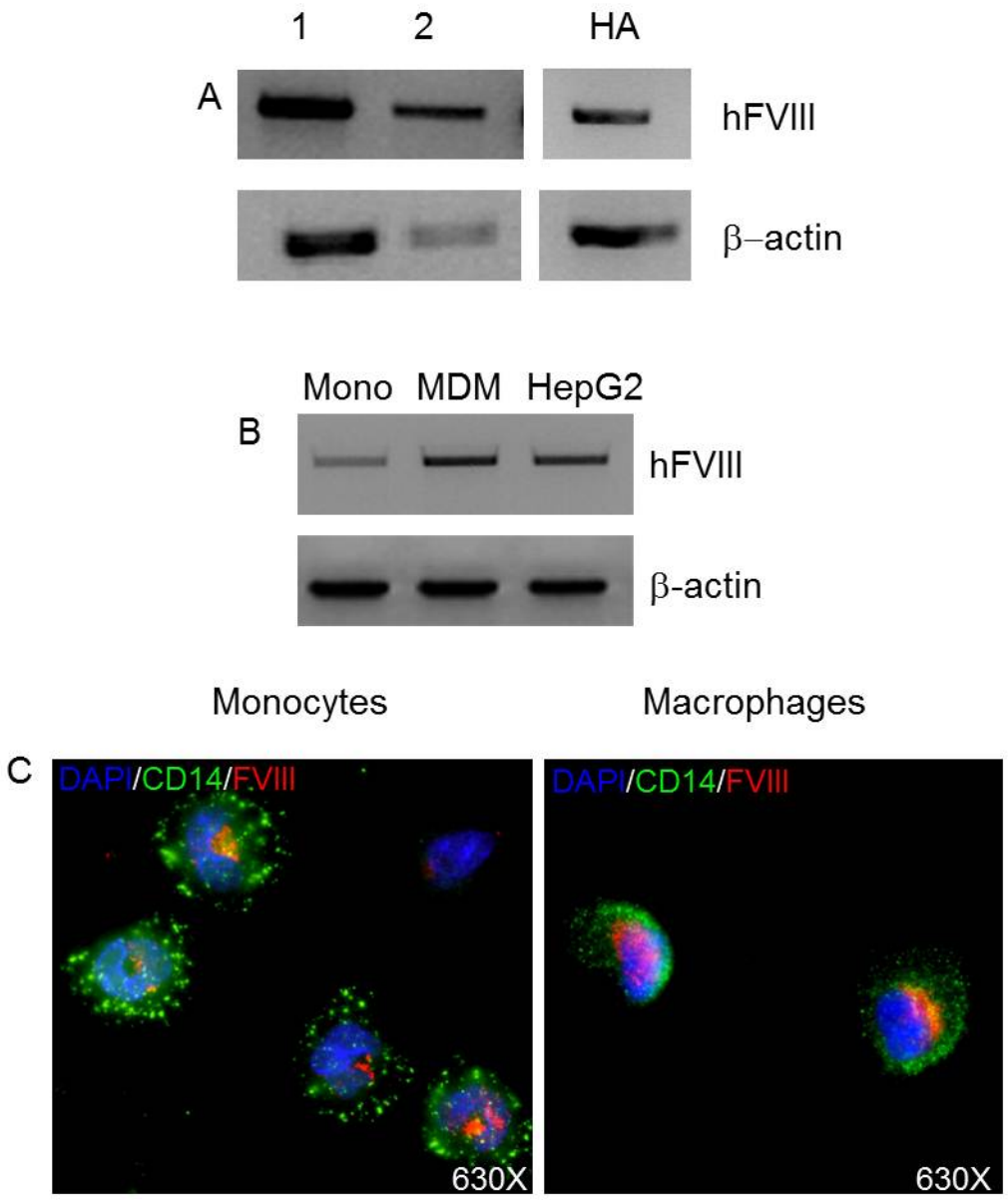

Figure 6. Expression of FVIII in human peripheral blood cells. RT-PCR of cells isolated from peripheral blood showed expression of FVIII in normal donor and a hemophilic patient (A), in particular in freshly isolated monocytes and macrophages (MDM) (B). Same results were demonstrated with an immunostaining for FVIII (red) and CD14 (green). DAPI (blue) for nuclei (C).

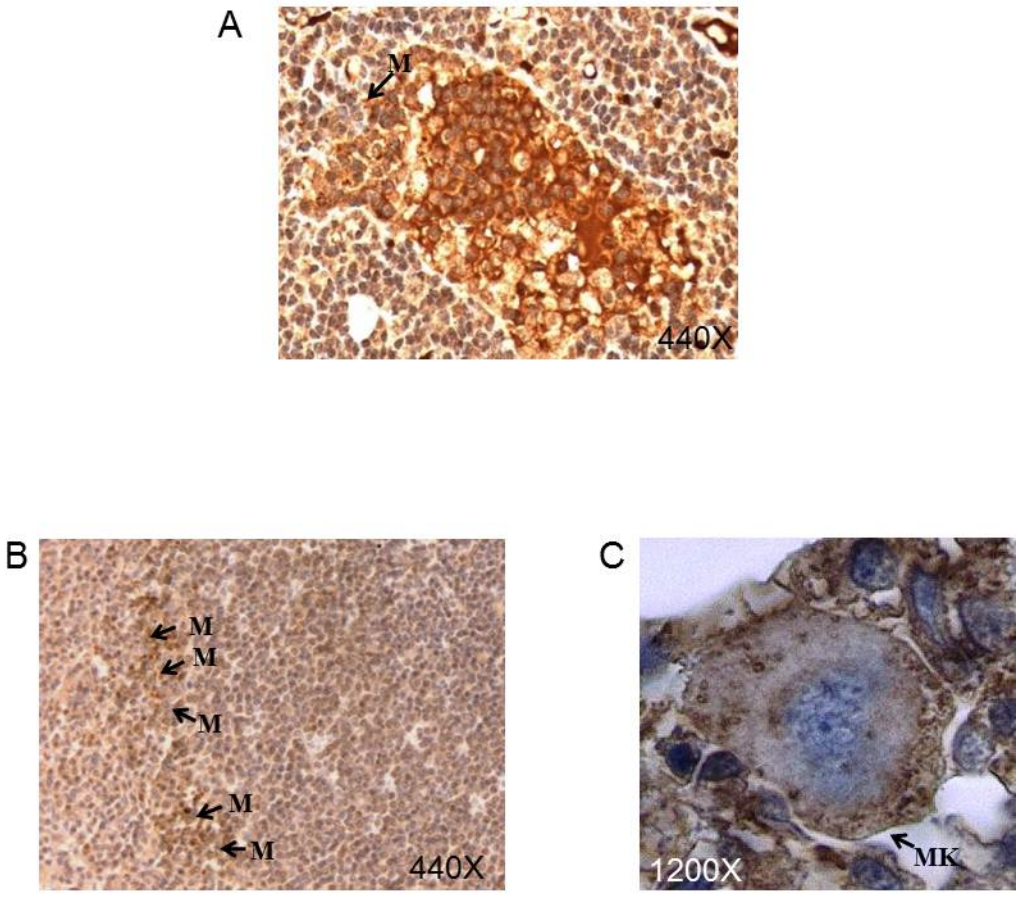

Figure 7. FVIII production in human tissues. Staining on several human tissues confirmed expression of FVIII. IHC showed expression of FVIII in spleen macrophages [arrows] (A) and lymph nodes (B). FVIII positive cells were detected in MK and in BM myeloid cells of BM (C). M=macrophages, MK=megakaryocytes.

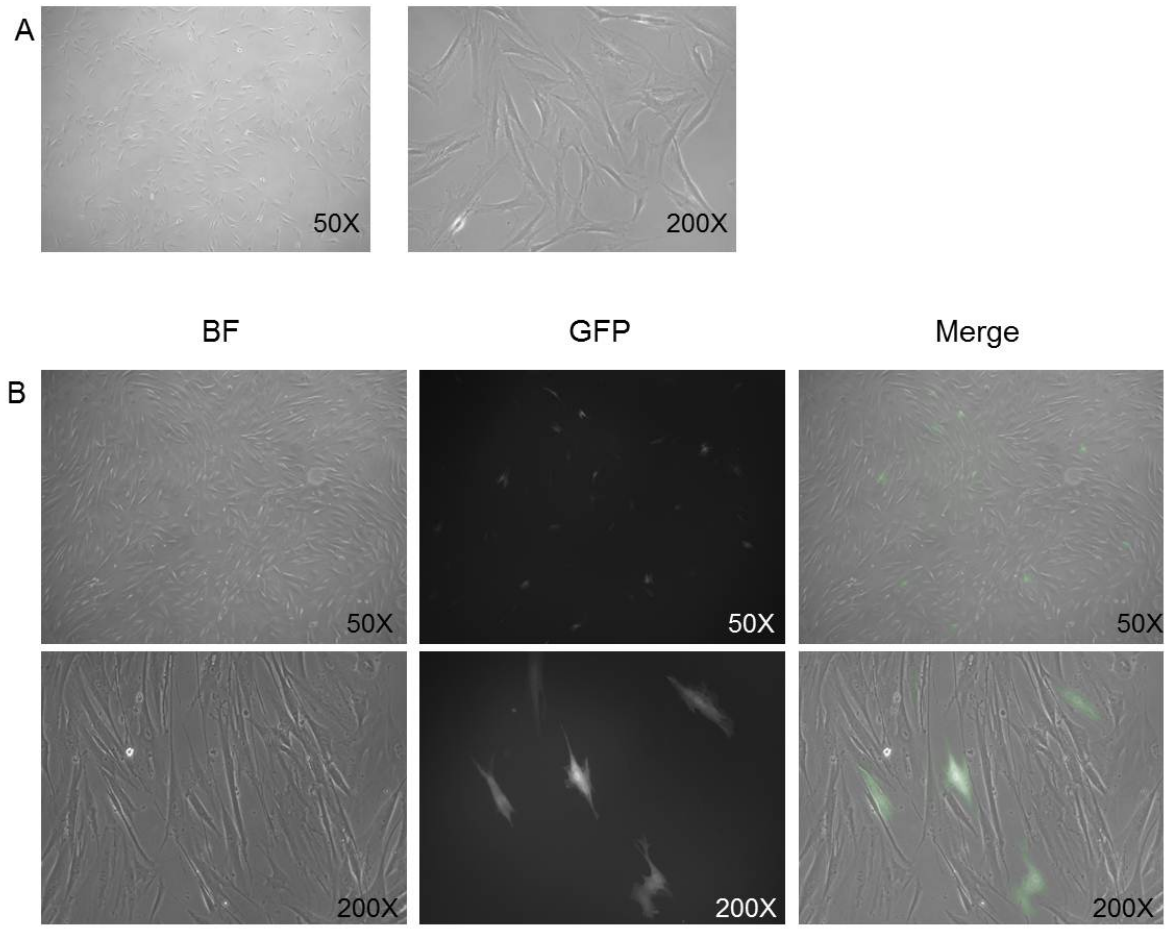

Figure 8. Generation of fibroblast-derived iPS cells. Human fibroblasts (A) were transduced by retroviral vectors carrying Oct4, Sox2,c-Myc and Klf4. Cells transduced with GFP and Oct4, Sox2 and Klf4 were used as transduction control . BF=bright field (B) .

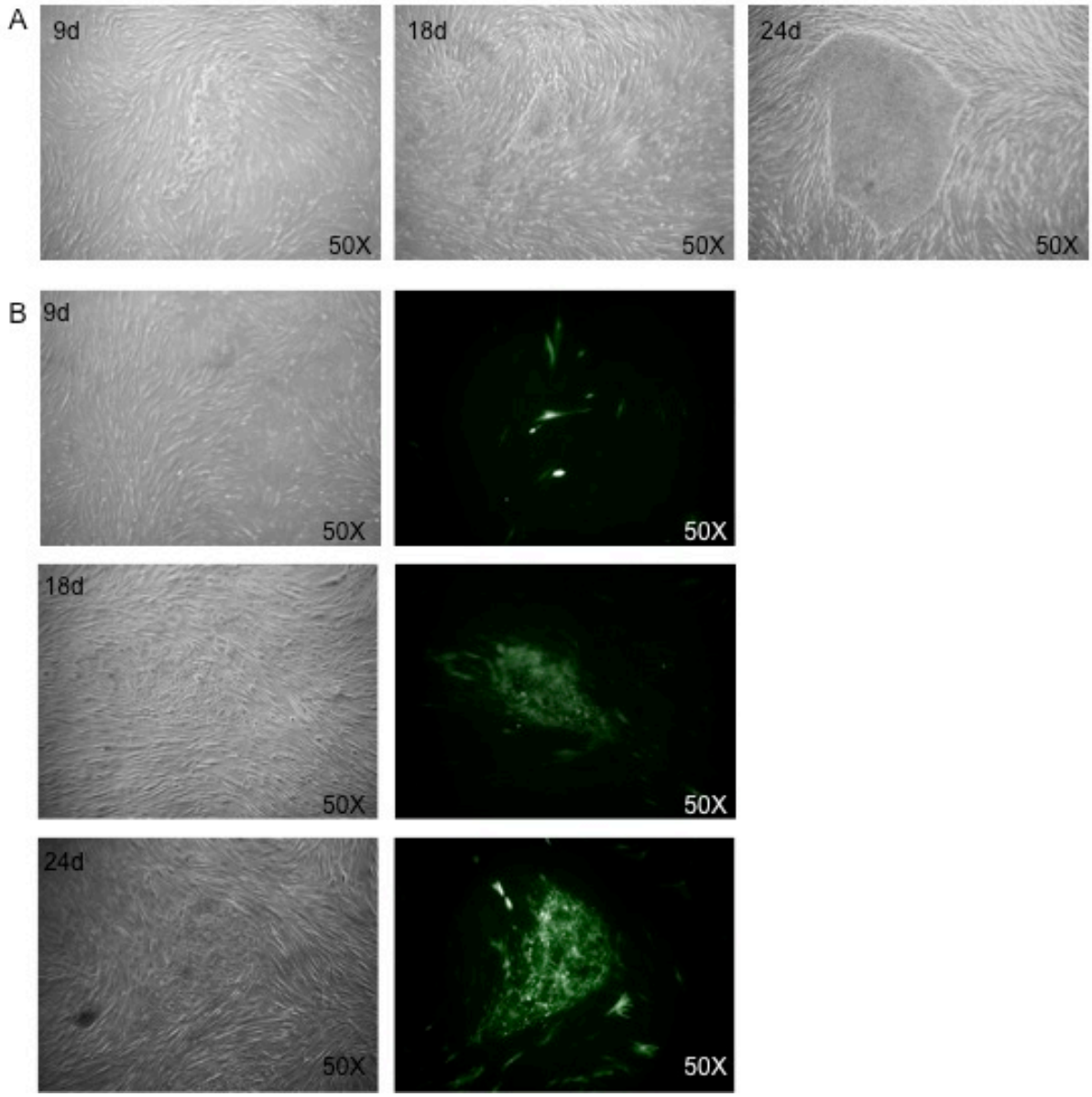

Figure 9. Generation of fibroblast-iPS cells. Typical example of ES cell–like colony 9,18 and 24 d post-infection with RV containing the four factors (A). Typical example of a ES cell–like colony 9,18 and 24 d post-infection with GFP (B).

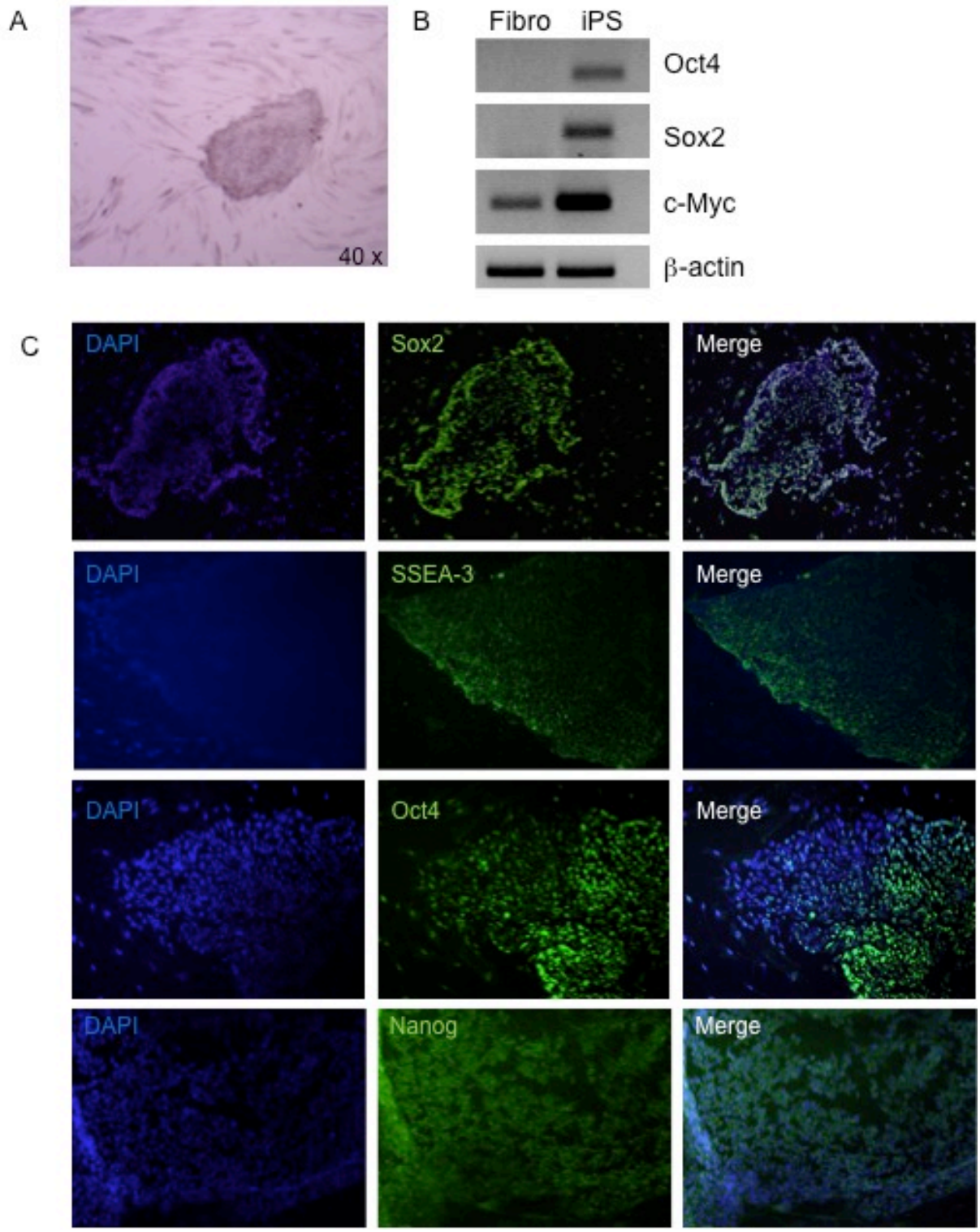

Figure 10. Characterization of iPS cells. Example of AP+ staining iPS colony (A). RT-PCR for reprogramming factors in fibroblasts before transduction (FIBRO) and in iPS cells (B). Immunofluorescence of iPS colony positive for SSEA-3,Sox 2,Oct4,Nanog (all in green). DAPI for nuclei. Enlargement 100X (C).

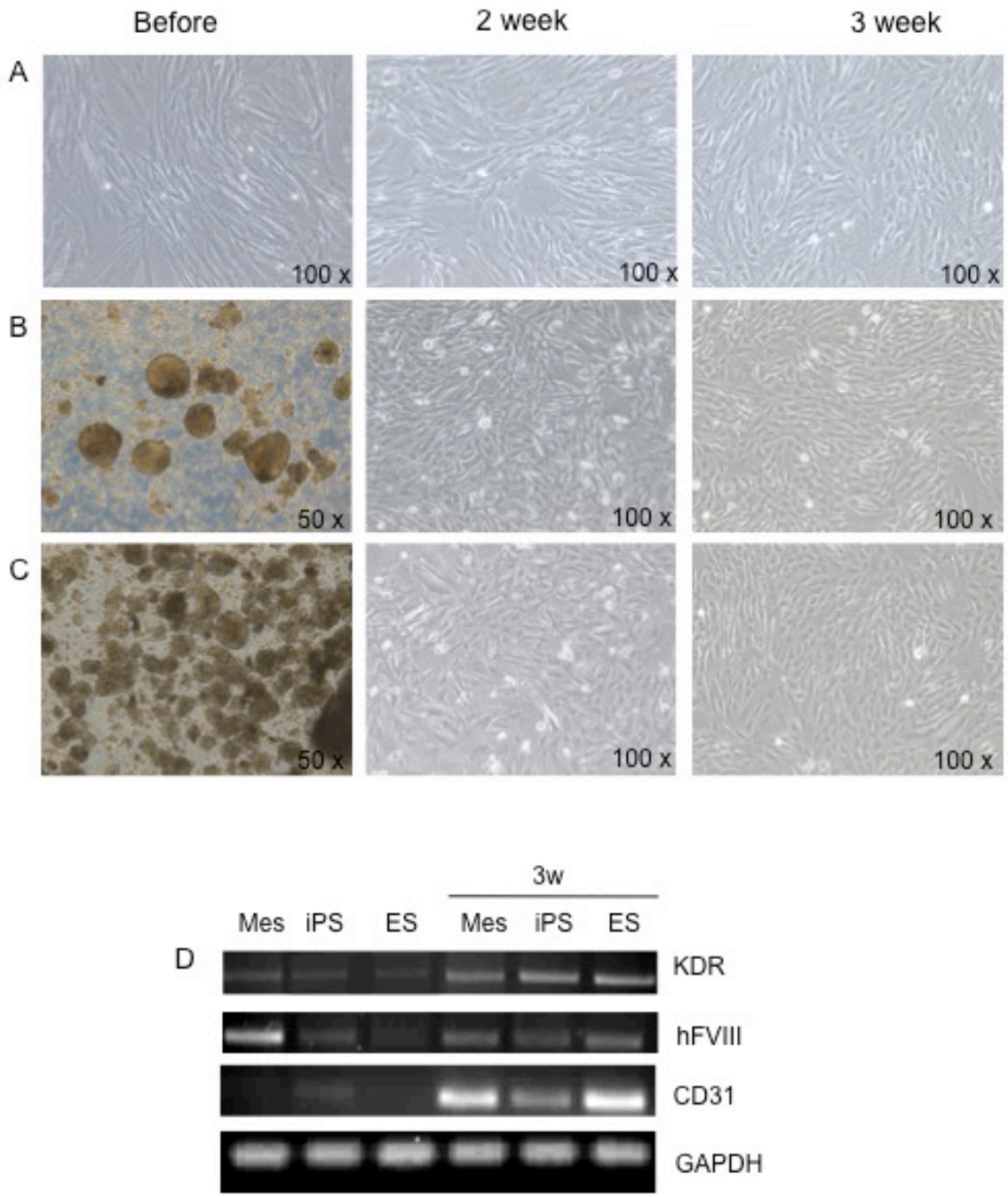

Figure 11. Endothelial differentiation of fibroblast–derived iPS cells. Morphological change in mesenchymal-like cells (A), iPS (B) and ES cells (C) during 3 weeks protocol for endothelial differentiation. Differentiated cells assumed the characteristic “cobblestone” morphological features. Up-regulation of endothelial markers in differentiated cells is shown by RT-PCR (D).

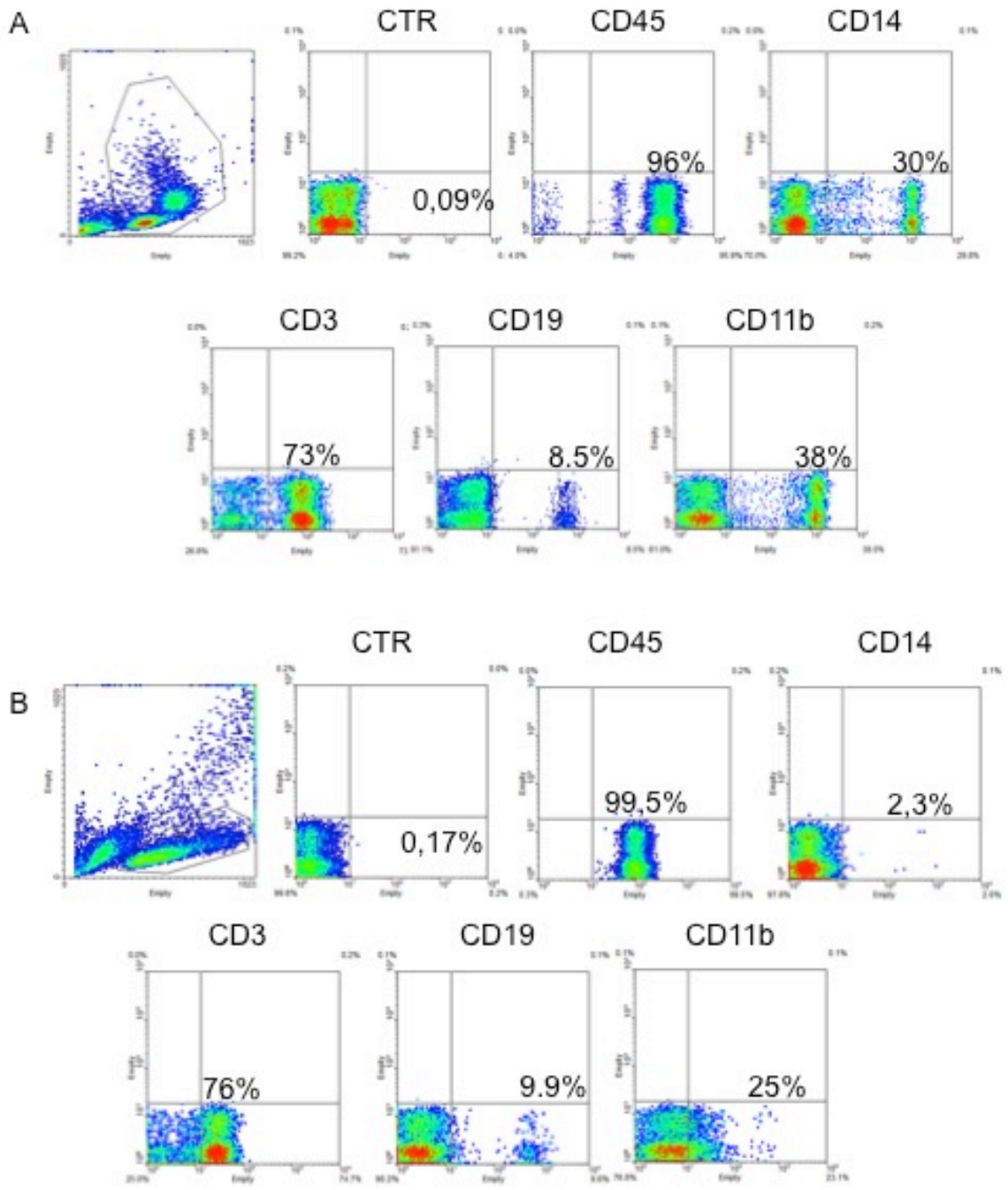

Figure 12. Characterization of MNC. FACS analysis of surface markers typically expressed by MNC after isolation (A) and on the day of reprogramming (B).

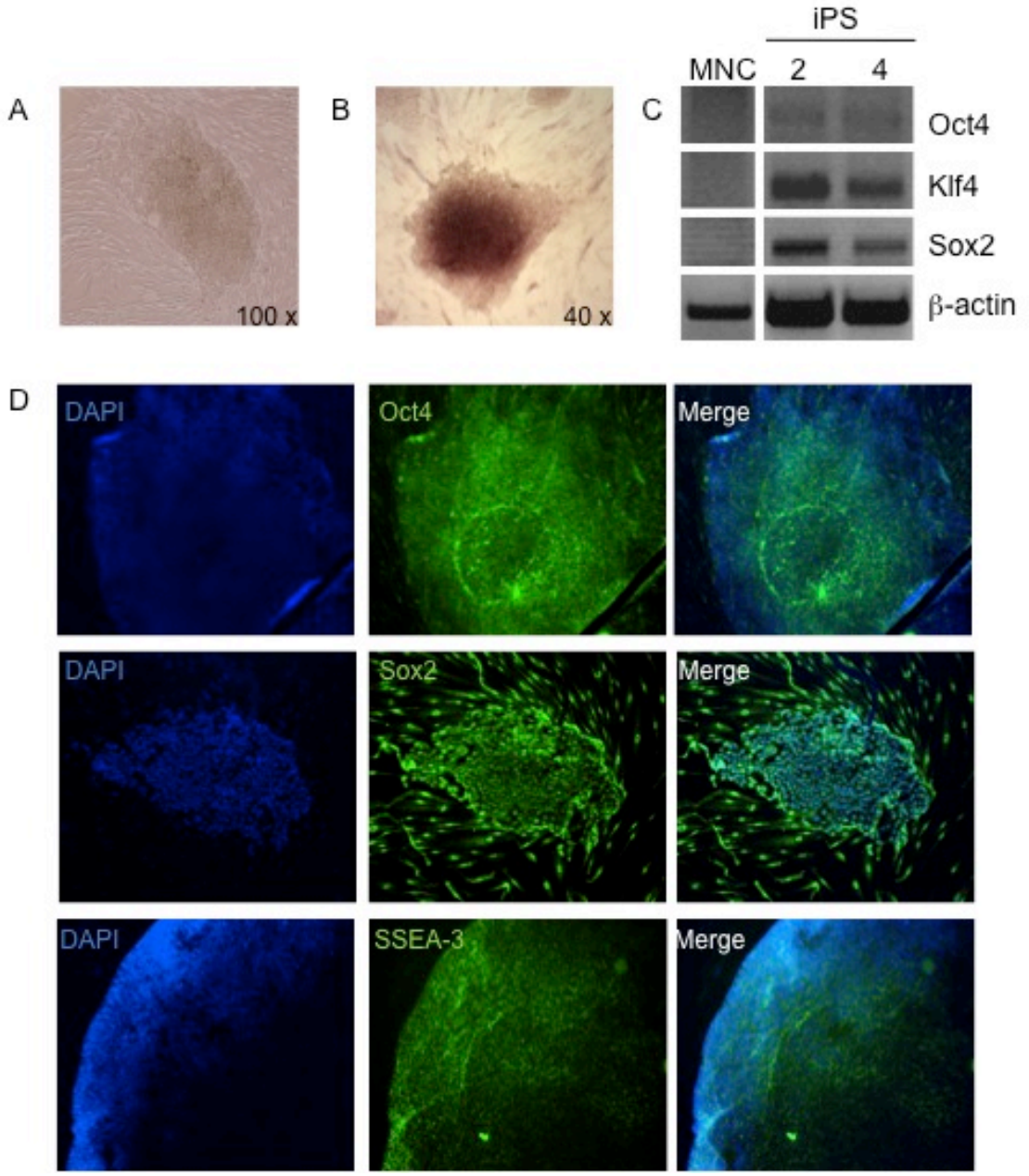

Figure 13. Generation and characterization of MNC derived-iPS cells.
iPS colonies grown on HFF (A). Example of AP+ staining of iPS colony (B). RT-PCR showed expression of reprogramming factors only iPS cells (C). Immunofluorescence of iPS colonies positive for the surface marker SSEA-3 and the nuclear markers Sox 2 and Oct4 (all in green). DAPI for nuclei. Enlargement 100X (D).

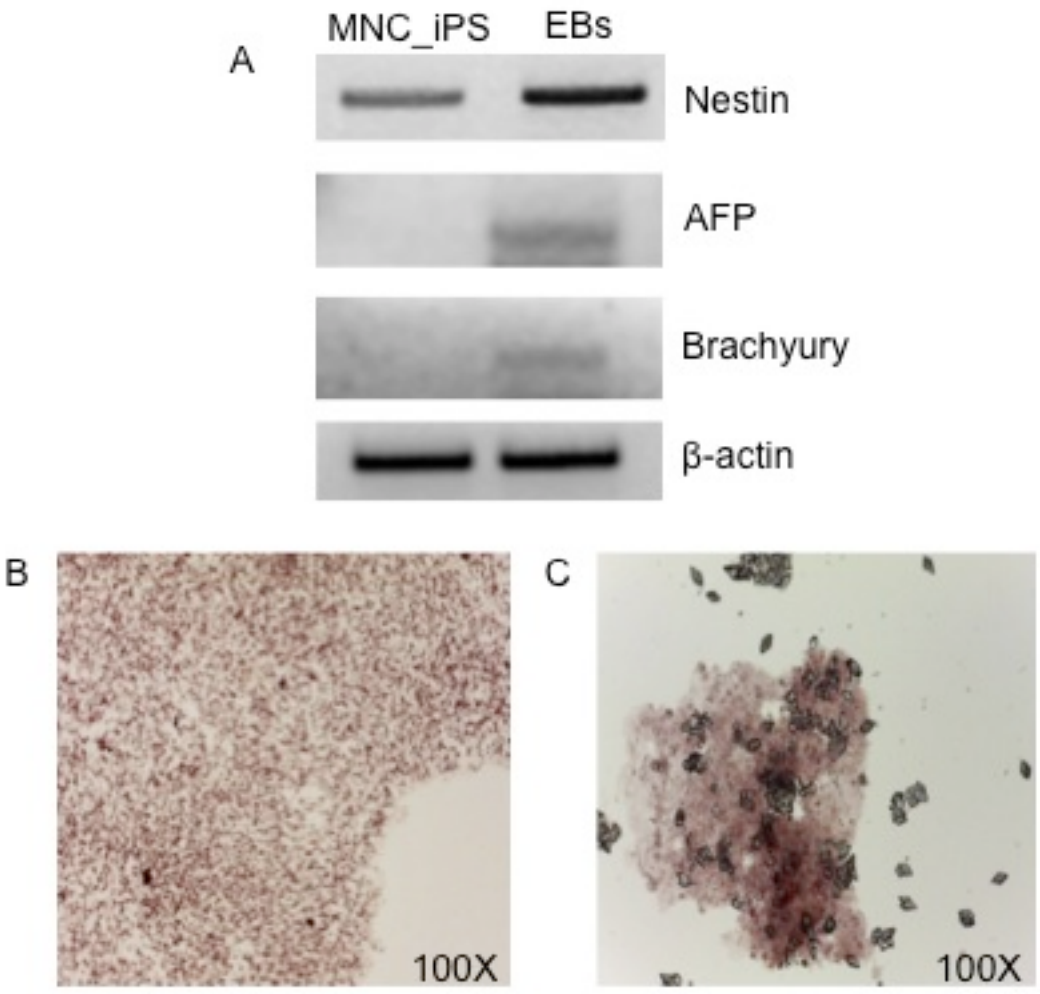

Figure 14. Pluripotency of MNC derived-iPS cells. Expression of three germ lay
specific markers by EBs (A). iPS differentiationn in adipogenic and osteogenic tis
(B,C.)

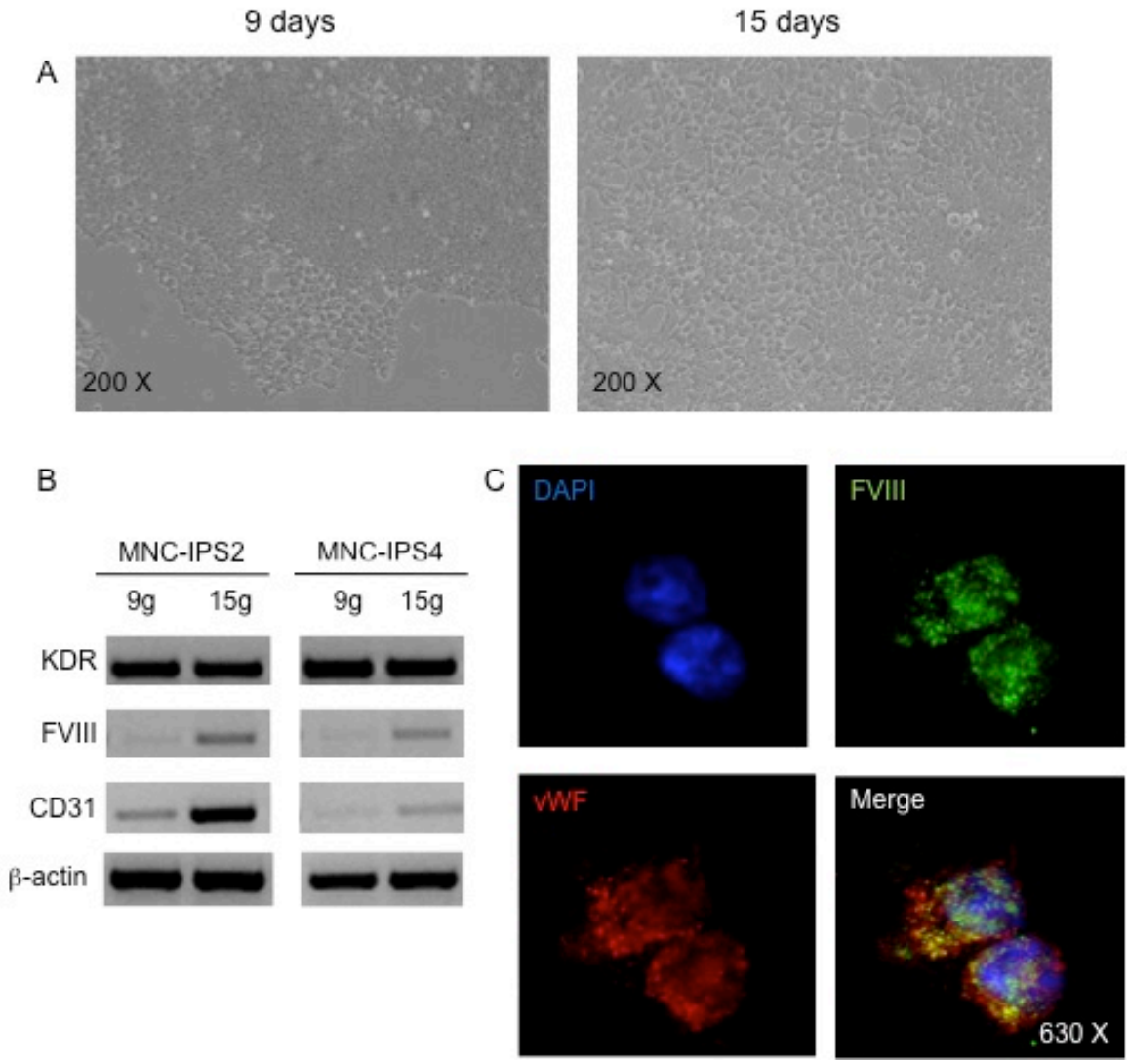

Figure 15. Endothelial differentiation of MNC-derived iPS cells.
iPS cells differentiated through EBs formation. EBs after endothelial differentiation acquired a typical endothelial-like morphology (A) with increased expression of CD31 and FVIII (B). Differentiated cells showed co-staining of FVIII (green) with vWF

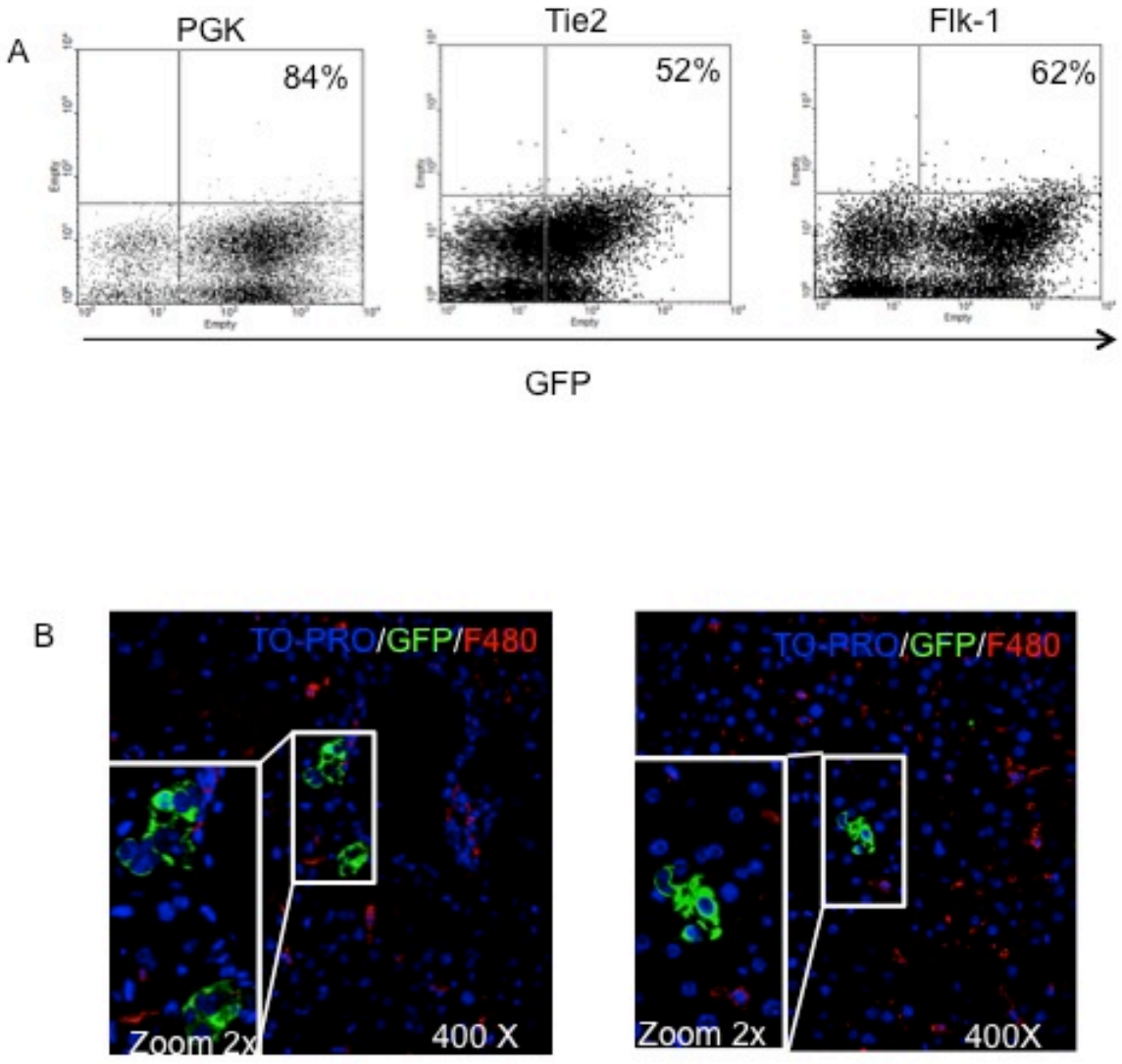

Figure 16.Transplantation of Endothelial Differentiated-iPS cells.
FACS analysis confirmed the efficient endothelial differentiation by GFP expression of transduced cells using LV carrying the GFP under the control of endothelial-specific promoters (Flk-1 and Tie-2) (A). Confocal microscopy of immuno-stained liver sections 96 h after transplantation with Flk-1-GFP+ cells (green) in NOD/SCID HA mice sowed engrafment of cells F4/80 (red), To-pro for nuclei. (B).

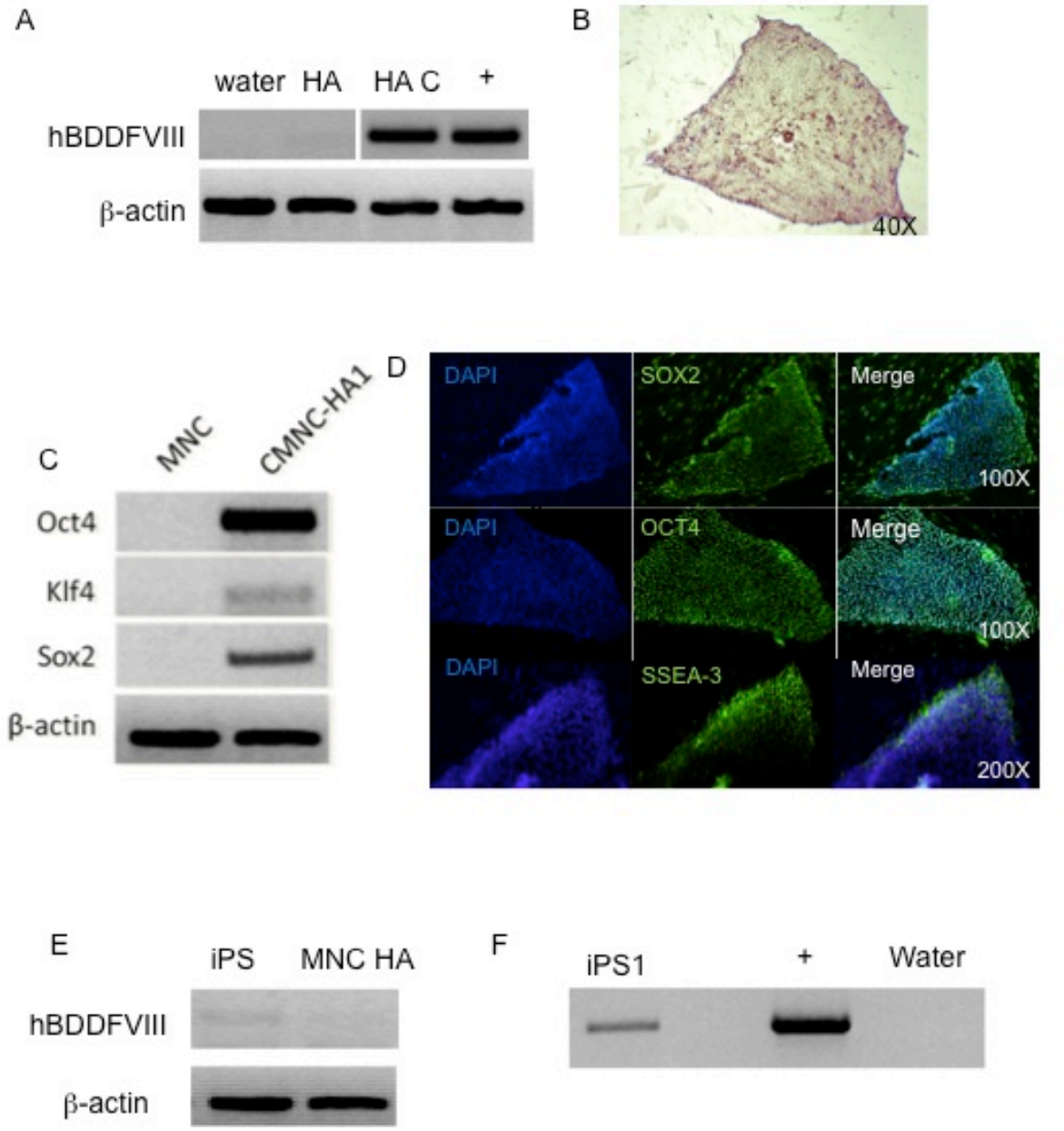

Figure 17.Characterization of MNC HA derived-iPS cells.
MNC from hemophilic patient were corrected with a LV.PGK-hBDD-FVIII on day 3. Only corrected cells (HA C) expressed hFVIII (A). iPS cells from hemophilic donor were positive for AP staining (B) , expressed reprogramming factors at RNA (C) and protein (D) level and showed a reduced FVIII expression (E). PCR analysis demonstrated integration of LV.PGK-hBDDFVIII in corrected iPS cells. cDNA and DNA of other cells previously correcte were used as positive control in A and F.

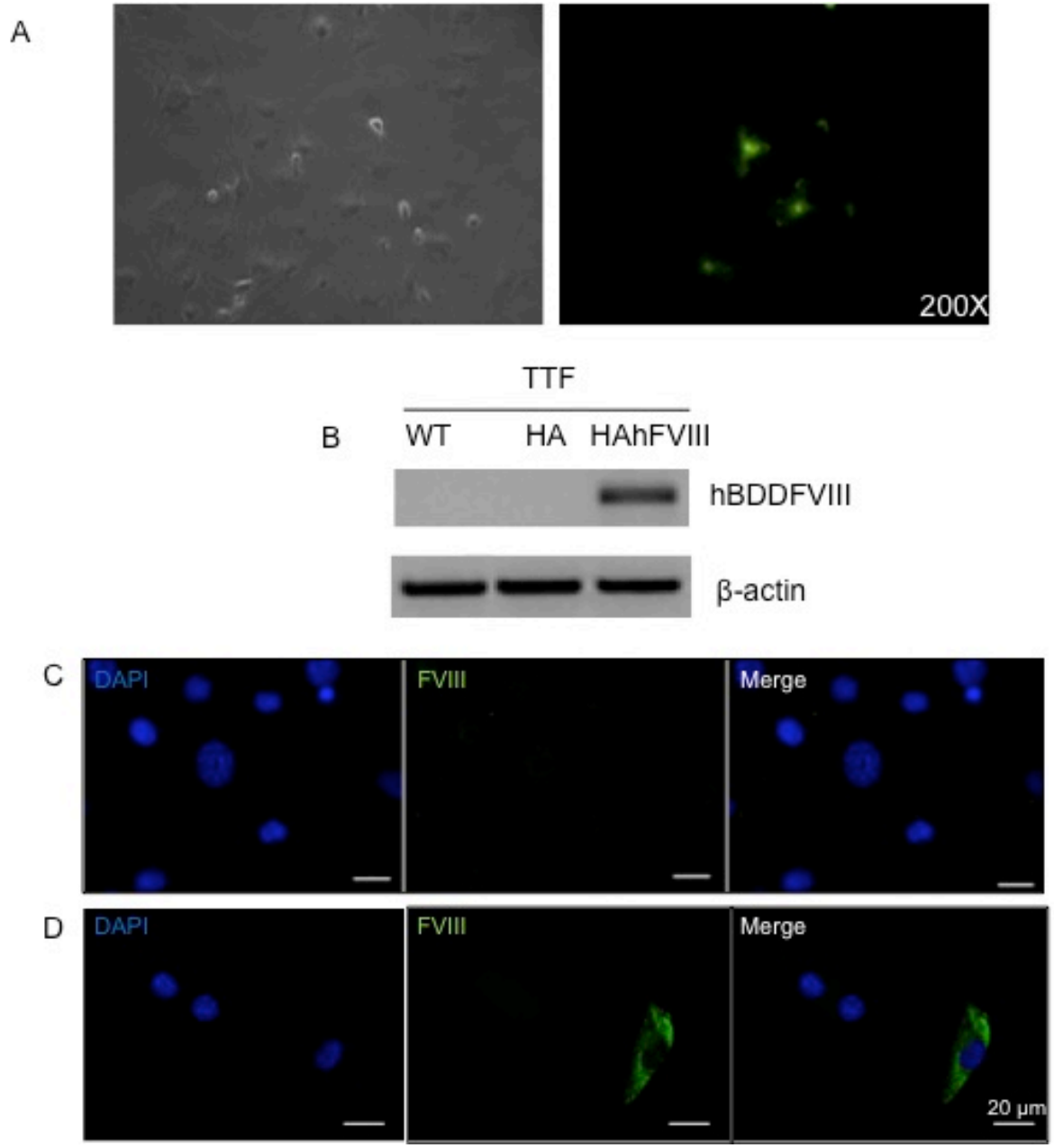

Figure 18. Hemophilic fibroblasts correction with LV.PGK-hBDDhFVIII. Hemophilic TTFs were transduced with LV expressing BDD-hFVIII under the control of PGK promoter and with LV.PGK-GFP as control (A). After two days cells expressed human FVIII as shown by RT-PCR (B) and immunofluorescence (FVIII in green) (D). Hemophilic TTFs did not express hFVIII (B,C).DAPI for nuclei.

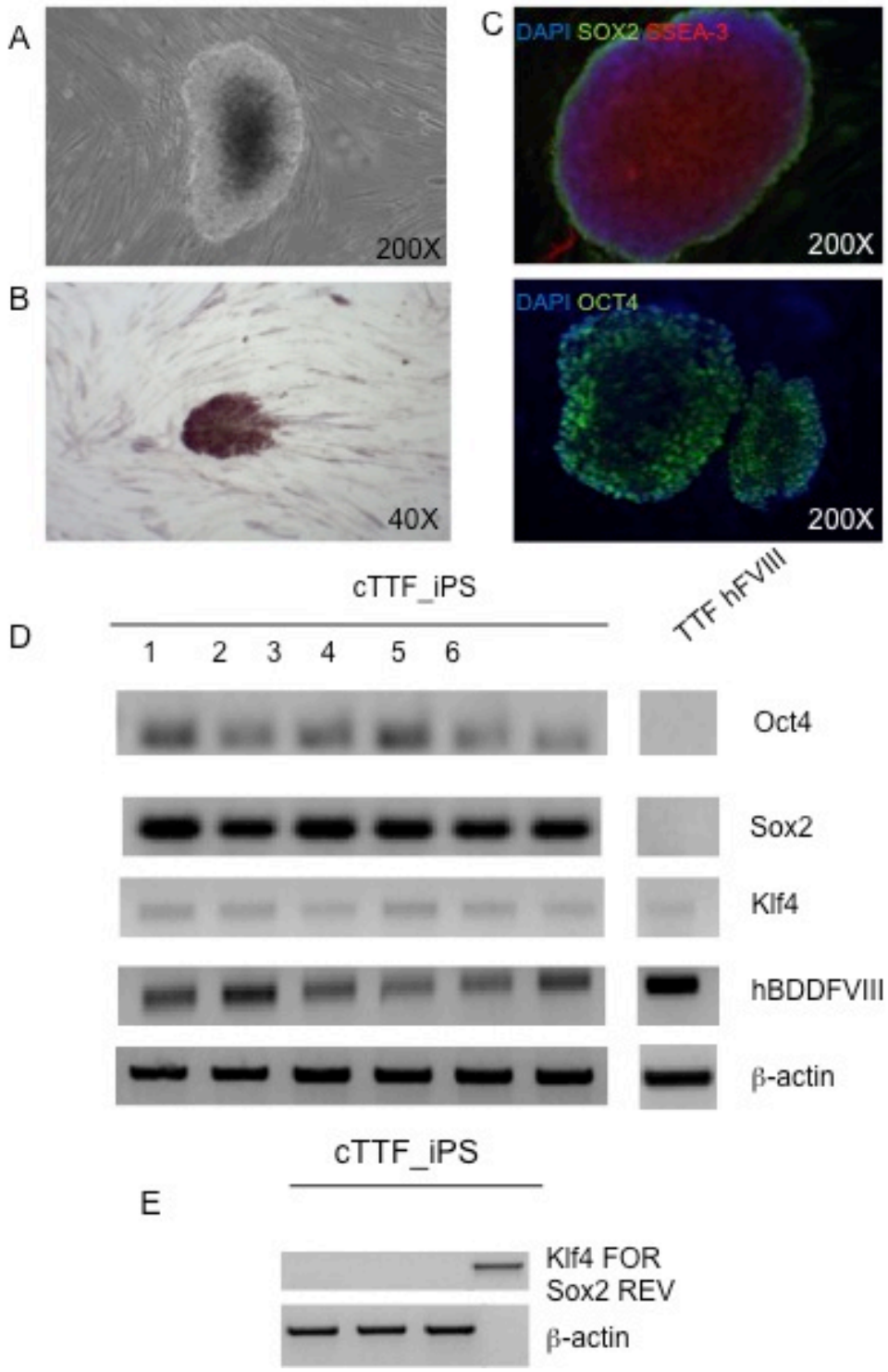

Figure 19. Mouse fibroblast-derived iPS cells. Typical ES-like morphology of reprogrammed colonies (A) positive for AP staining (B) and for reprogramming factors at protein (C) and RNA level (D). RT-PCR showed that all colonies were corrected for FVIII expression (D). PCR with primers specific for polycistronic RNA showed reactivation of endogenous factors in iPS cells. Reprogramming plasmid (1477) was used a control (E).

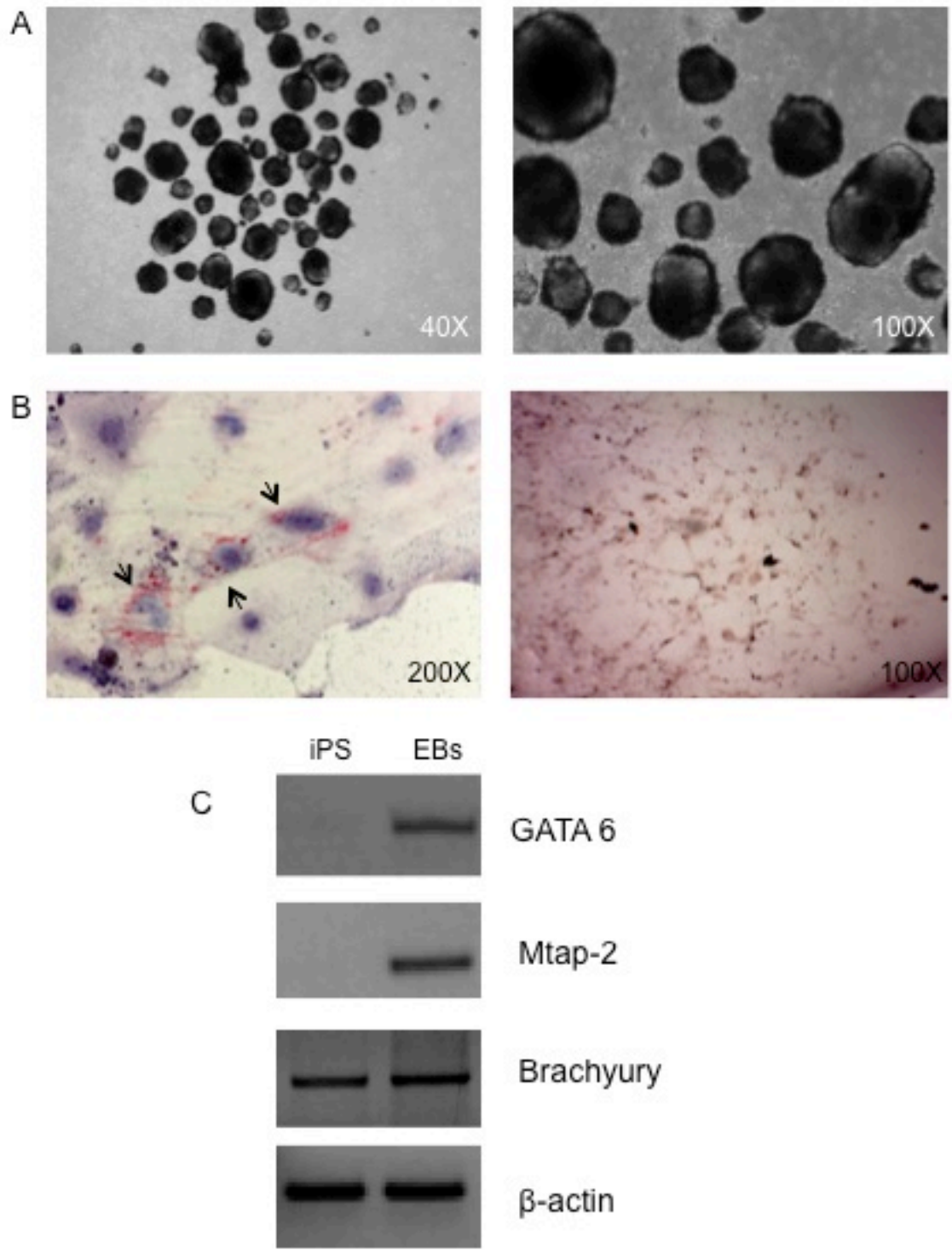

Figure 20. Pluripotency of mouse fibroblast-derived iPS cells. iPS-derived EBs at lower (left) and high enlargement (right) (A). EBs differentiated in adipogenic (left) and osteogenic cells (right) (B). RT-PCR analysis showed expression of the three germ layers markers (C).

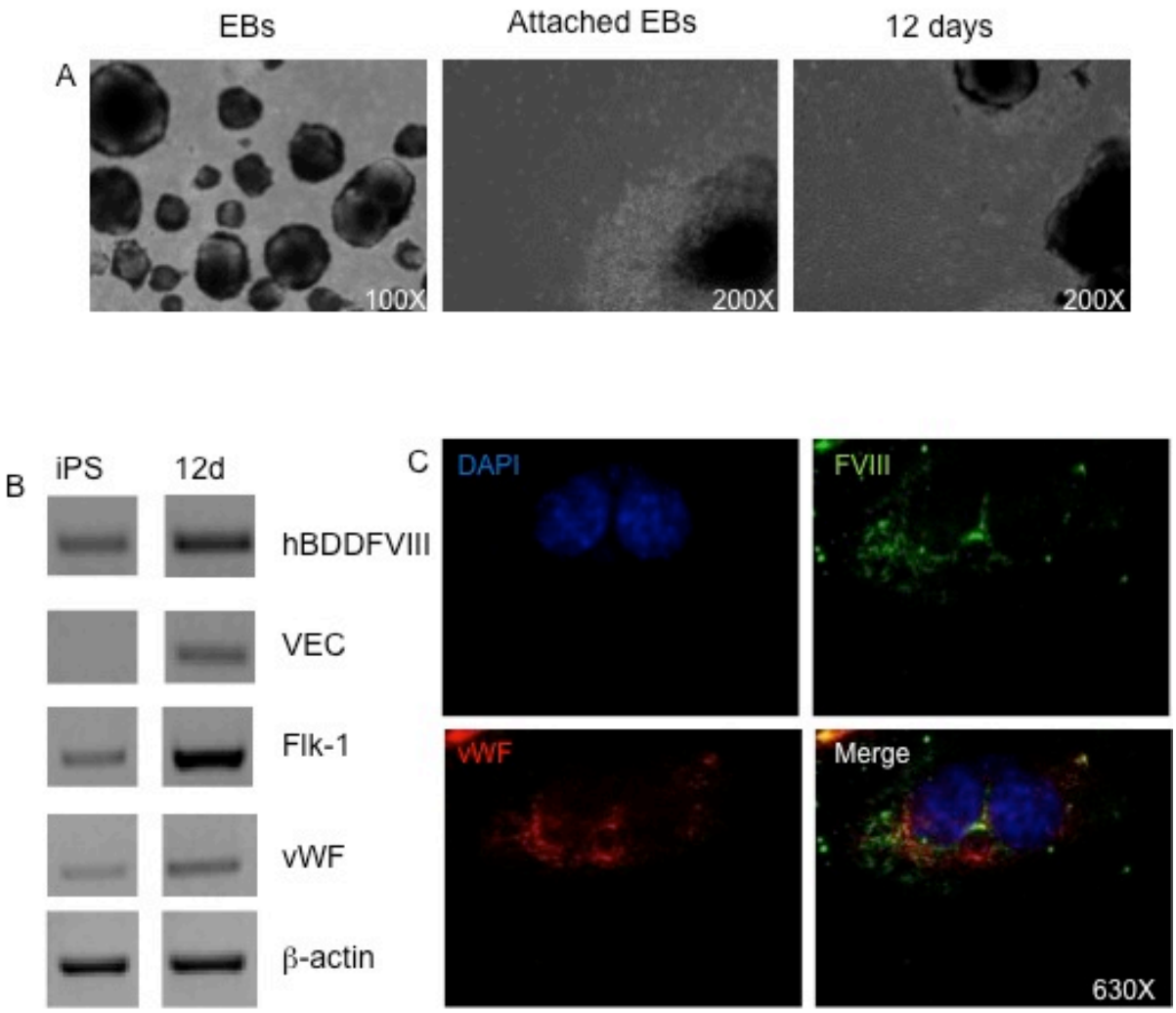

Figure 21. Endothelial differentiation of mouse fibroblast-derived iPS cells. EBs plated on gelatin in EB medium with mVEGF attached and in two weeks cells assumed the typical "cobblestone" morphology (A) and expressed endothelial-specific markers (B). Differentiated endothelial cells were positive for FVIII (green) and vWF (red) co-staining at the immunofluorescent microscope. DAPI for nuclei (C).

Discussion

The current therapy for hemophilia A is the administration of plasma-derived or recombinant FVIII. This procedure is expensive for the community and 20-30% of patients develop inhibitory antibodies against the delivered factors. Therefore, research is trying to find new strategies for a definitive cure. Since hemophilia A is a monogenic disease, it is a good candidate for cell and gene therapy. In particular, recent studies demonstrated that transplantation of bone marrow cells can correct hemophilia A phenotype in mice and in several studies hematopoietic cells were successfully used as target for hemophilia A gene therapy.[58,69,119,120] In this work we investigated which cell population was the origin of FVIII correction. We focused our attention on myeloid lineage and specifically on megakaryocytes, from which platelets originate, and monocytes/macrophages. Megakaryocytes together with endothelial cell synthesized von Willebrand factor, that plays an essential role in haemostasis and it also serves as the carrier protein of FVIII. Therefore targeting expression of FVIII in megakaryocytes using platelet-specific promoter will establish a pool of FVIII together with vWF. FVIII will be released only in the site of injury overcoming the presence of inhibitors and limiting exposure of exogenous FVIII to immune response. Studies of Shi and colleagues demonstrated that lentiviral vector-mediated gene transfer of FVIII under the control of platelet-specific human integrin αIIb (GPIIb) gene promoter in hematopoietic stem cells maintained expression of FVIII for up to 5 months. This strategy improved haemostasis in hemophilic mice with pre-existing immunity as well.[63,69] Notwithstanding these data, there are no evidences showing FVIII synthesis in these type of cells in normal mice. In our study, we showed that megakaryocytes and other population from bone marrow expressed FVIII mRNA and synthesized the coagulation factor according with the idea that there are extra hepatic sources of FVIII both in mice and human.[117] It is difficult to obtain bone marrow from healthy people and for this reason we obtained BM from patients with an initial diagnosis of no malignancy. Allogenic BM transplant has been rarely reported in hemophilia patients. In one case, a hemophilic boy afflicted by aplastic anemia, was transplanted with the BM of his unaffected brother. Four months after BM transplant, the child showed 100% donor engraftment, although no changes in clotting parameters were detected. Moreover, no inhibitors were present before and after BM transplant and this boy continued his replacement therapy and there was no possibility to verify if FVIII was produced.[246] This is the only report where BM transplant was done in hemophilia A patient. Later, we analyzed MK and

macrophages differentiated from human cord blood. We demonstrated that these cells were able to produced FVIII at comparable levels to HepG2, human hepatocarcinoma cell lines. Once obtained these data, we wanted to analyze if also myeloid cells differentiated from peripheral blood mononuclear cells expressed FVIII. We found FVIII both in mouse and in human PBMC. Interestingly, unlike hematopoietic cells originated from hemophilic transgenic mice that do not express mutated FVIII mRNA, blood from hemophilic patients showed FVIII expression at the mRNA level because most of the described mutations are missense and only in rare cases lack of mRNA has been described.[247] Due to the insertion of a transgenic cassette between exon 16 and 17 causing a stop codon in hemophilic mice, differences between wt and haemophilic mice was easier to detect than in humancells.[77] In the latter case,cells were differentiated *in vitro* and results were validated by histochemistry in human tissues. In this case, spleen, lymph nodes and bone marrow macrophages were positive for FVIII. For the first time we determined that human BM, CB and peripheral blood contained cell populations capable of FVIII expression. The identification of additional non-endothelial cell types capable of supporting FVIII synthesis and release provides new targets for cell/gene therapy. Finally with these experiments we validate a rabbit anti-FVIII polyclonal antibodies produced in our laboratory.

Besides exploring new sources of FVIII producing cells, an alternative approach to cure hemophilia A could be the use of autologous stem cells and in particular iPS cells. In this study, we showed that iPS cells can be generated from human fibroblasts by retroviral transduction of four transcription factors, namely Oct3/4, Sox2, Klf4 and c-Myc. The first and critical step during selection of reprogrammed cells is to observe the modification in cell morphology at the microscope. These cells are similar to human embryonic stem cells: colonies are compact, uniform and with defined borders when grown on feeder. It is important to discern these cells from other cells that are not or partially reprogrammed showing absence of borders and the presence of dark centre that are differentiated cells. Sometimes during passages even the right colonies begin to differentiate in the centre of the colony, but this is not a problem because with mechanically picking it is possible to recover only the right cells. The second phase was the characterization of the selected colonies by stem

cell marker analysis. In this studies pluripotent stem cell were positive for alkaline phosphatase and markers like NANOG, Oct3/4, Sox2, SSEA-3.

Generated iPS cells were differentiated to endothelial cells because recently it has been shown that FVIII is expressed by liver sinusoidal endothelial cells and intraportal injection of LSEC correct haemophilia A phenotype.[118] It has been demonstrated that in transgenic mice with FVIII expression under the control of the endothelial cell-specific Tie2 promoter/enhancer normalized plasma FVIII and re-established a releasable pool of FVIII.[63] Finally, endothelial cells in liver sinusoids play roles in immunoregulation, which could potentially be harnessed to avoid deleterious immune responses against FVIII.[248] iPS cells can differentiate in endothelial cells if cultured in a differentiating medium containing IGF-1, ECGS, VEGF and bFGF. After 3 weeks, cells acquired typical endothelial morphology with increased expression of CD31, KDR and FVIII except for mesenchymal-like cells where a high basal level of FVIII was already present. With this method we obtained iPS cells in a faster way, but these cells cannot be used in therapy because they were obtained with c-Myc, a well-known oncogenic factor.[249] For this reason, we decided to use a particular LV (kindly provided by Prof. Luigi Naldini, HSR-TIGET, Milano) containing Oct4, Klf4 and Sox2 without c-Myc and with LoxP sites to have a safer tool for future therapeutic use. We reprogrammed again human fibroblasts from healthy donors and we obtained iPS cells (data not shown). However, fibroblasts were isolated from skin biopsies, a risky procedure for hemophilic patients. Therefore, we used peripheral blood cells as an easy cell source, despite the probability of reprogramming is less efficient than fibroblasts.[250]

In this study, we demonstrated that we can obtain iPS cells from peripheral blood cells. Mononuclear cells were isolated from healthy donors and were reprogrammed at different MOI with a LV. Flow cytometry analysis revealed that at the day of reprogramming, cells present in our culture were mainly CD3, CD19 and CD11b. We obtained colonies with ES cells-like morphology, positive for AP staining and stem cells markers, such as Oct4, Sox2 and SSEA-3. These cells also expressed reprogramming factors that we did not detect in MNC demonstrating a reactivation of these genes during the reprogramming process. Our cells were capable of forming embryo bodies and able to differentiate in vitro, expressing markers of the three germ layer Nestin (ectoderm), AFP (endoderm) and Brachyury (mesoderm).

Then, we differentiated iPS cells from MNC in endothelial cells plating EBs in EB medium with rhVEGF. Cells acquired endothelial morphology and expressed endothelial markers at RNA and protein level, but as it shown by transduction with Tie2 and Flk-1 there was not a total differentiation. For this reason, we are improving the differentiation protocol using different combination of factors and selecting cells for an endothelial surface markers, for example CD31, to obtain a pure population to be further expanded. Nevertheless, these iPS-derived EC were able to engraft when transplanted in NOD-SCID HA and we found these cells in the liver up to 1 week after transplantation. Once optimized the protocol with healthy cells, we isolated MNC from several hemophilic patients. Before reprogramming, we corrected them with a LV expressing the hFVIII-B domain-deleted under the control of PGK promoter. Interestingly, after reprogramming we obtained two colonies and only from FVIII-corrected cells. Now, we have to understand if this bias was due to a technical problem in reprogramming hemophilic cells or if the double transduction for FVIII correction and reprogramming could have harmed the potential of hemophilic cells to be reprogrammed. Moreover, we are also thinking if the patient age and the absence of FVIII in cells can have a role in the loss of potential reprogramming capacity. Actually, iPS cells obtained from healthy donors were from younger people and far more colonies were generated. After characterization only one colony resulted reprogrammed from the hemophilic cells. Unfortunately, in this colony FVIII expression decreased overtime, probably due to the silencing of the PGK promoter. In the future we will correct cells with a LV with FVIII under the control of Vascular Endothelial-cadherine (VEC) promoter to induce FVIII expression only after endothelial differentiation and to understand if cell specific expression can spare FVIII by silencing in reprogrammed cells. To complete the characterization we will further analyze the differentiation of iPS cells in vivo by teratoma's formation, promoter methylation and telomerase activity, to confirm the acquisition of pluripotency by reprogrammed cells. We performed a preliminary experiment to measure telomeres length (data not shown) and we did find no differences between original and reprogrammed cells indicating regular activity of telomerase in our iPS cells. This result confirms that there was no telomere shortening in iPS cells as reported for staminal cells. Moreover, once differentiated, we will transplant iPS-derived EC in gamma-null hemophilia A mice to analyze engraftment, proliferation and phenotypic correction for FVIII activity.

Simultaneously to human experiments, in this work we reprogrammed fibroblasts from hemophilia A mice. Earlier, it has been demonstrated that progenitor cells, differentiated from iPS cells isolated from wild-type mice, engrafted in the liver, produced functional FVIII and corrected hemophilia A phenotype.[251] Here, we first corrected cells with a human BDD-FVIII carried by LV and then we reprogrammed with the same LV used for human cells, but with a different sequential order of the factors that promote reprogramming of mouse cells. We generated iPS cells that expressed pluripotent stem cell markers and markers of the three gem layers, differentiated in several cell types in particular in endothelial cells, the one of our interest. Since the aim of our study is to reach long term correction for durable therapy, we demonstrated that corrected fibroblasts expressed human FVIII and the expression was maintained in reprogrammed cells also after endothelial differentiation. The maintenance of FVIII expression in mouse iPS cells could be due to a different epigenetic control compared to human cells. Indeed, we used the same vector to correct human and mouse cells but we observed promoter silencing only in human iPS cells. Therefore, we demonstrated that iPS cells can be generated from corrected hemophilia A fibroblasts providing an autologous model for further studies.
Moreover, we tried to reprogram hemophilic fibroblasts without correction, but did get no reprogrammed colonies after several experiments. We are evaluating if the correction of cells is necessary for the following reprogramming as showed for Fanconi anemia even though the mutation in FVIII is not crucial in the early stage of life such as FANCA gene.[237]

In this study we confirmed that iPS cells could be generate from human and mouse cells. In particular MNC could be a good source for iPS cells generation for patient-autologous stem cell therapy even if both the reprogramming and differentiation protocol need further optimization. Our results are important for future cell reprogramming approaches of hemophilic patients. Actually, this procedure is a safer and faster alternative to skin biopsies to obtain capable cells for reprogramming. However, we used integrating vectors both for correction and reprogramming therefore further analysis have to be done to evaluate the safety of integration sites of both the correcting transgene and the reprogramming cassette even if silenced. In this way it could be possible to decrease the amount of vector used for

reprogramming. For future applications it is important to avoid the risk of insertional mutagenesis for example promoting a site-specific integration of vectors using zinc fingers or Talen systems that are able to harbor expression cassette in secure genomic sites such as AAVS1, the integration site of adeno-associated virus on chromosome 19.[252,253]

Bibliography

1. Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. *Lancet*. 2003;361(9371):1801-1809.

2. Mannucci PM, Mendolicchio L, Gringeri A. Use of prophylaxis to prevent complications of hemophilia. *Adv Exp Med Biol*. 2001;489:59-64.

3. Oldenburg J, Zeitler H, Pavlova A. Genetic markers in acquired haemophilia. *Haemophilia*. 2010;16 Suppl 3:41-45.

4. Hoyer LW. Hemophilia A. *N Engl J Med*. 1994;330(1):38-47.

5. Otto JC. An account of an hemorrhagic disposition existing in certain families. *Clin Orthop Relat Res*. 1996(328):4-6.

6. Biggs R, Douglas AS, Macfarlane RG, Dacie JV, Pitney WR, Merskey. Christmas disease: a condition previously mistaken for haemophilia. *Br Med J*. 1952;2(4799):1378-1382.

7. Pool JG, Gershgold EJ, Pappenhagen AR. High-Potency Antihaemophilic Factor Concentrate Prepared from Cryoglobulin Precipitate. *Nature*. 1964;203:312.

8. Mannucci PM. Hemophilia and related bleeding disorders: a story of dismay and success. *Hematology Am Soc Hematol Educ Program*. 2002:1-9.

9. Toole JJ, Knopf JL, Wozney JM, et al. Molecular cloning of a cDNA encoding human antihaemophilic factor. *Nature*. 1984;312(5992):342-347.

10. Marchioro TL, Hougie C, Ragde H, Epstein RB, Thomas ED. Hemophilia: role of organ homografts. *Science*. 1969;163(3863):188-190.

11. Storb R, Marchioro TL, Graham TC, Willemin M, Hougie C, Thomas ED. Canine hemophilia and hemopoietic grafting. *Blood*. 1972;40(2):234-238.

12. Hollestelle MJ, Poyck PP, Hollestelle JM, Marsman HA, Mourik JA, Gulik TM. Extra-hepatic factor VIII expression in porcine fulminant hepatic failure. *J Thromb Haemost*. 2005;3(10):2274-2280.

13. Hollestelle MJ, Thinnes T, Crain K, et al. Tissue distribution of factor VIII gene expression in vivo--a closer look. *Thromb Haemost*. 2001;86(3):855-861.

14. Wion KL, Kelly D, Summerfield JA, Tuddenham EG, Lawn RM. Distribution of factor VIII mRNA and antigen in human liver and other tissues. *Nature*. 1985;317(6039):726-729.

15. Gitschier J, Wood WI, Goralka TM, et al. Characterization of the human factor VIII gene. *Nature*. 1984;312(5992):326-330.

16. Wood WI, Capon DJ, Simonsen CC, et al. Expression of active human factor VIII from recombinant DNA clones. *Nature*. 1984;312(5992):330-337.

17. Bovenschen N, Rijken DC, Havekes LM, van Vlijmen BJ, Mertens K. The B domain of coagulation factor VIII interacts with the asialoglycoprotein receptor. *J Thromb Haemost*. 2005;3(6):1257-1265.

18. Kaufman RJ, Pipe SW, Tagliavacca L, Swaroop M, Moussalli M. Biosynthesis, assembly and secretion of coagulation factor VIII. *Blood Coagul Fibrinolysis*. 1997;8 Suppl 2:S3-14.

19. Fang H, Wang L, Wang H. The protein structure and effect of factor VIII. *Thromb Res*. 2007;119(1):1-13.

20. Stubbs JD, Lekutis C, Singer KL, et al. cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. *Proc Natl Acad Sci U S A*. 1990;87(21):8417-8421.

21. Dorner AJ, Bole DG, Kaufman RJ. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. *J Cell Biol*. 1987;105(6 Pt 1):2665-2674.

22. Kaufman RJ. Genetic engineering of factor VIII. *Nature*. 1989;342(6246):207-208.

23. Thompson AR. Structure and function of the factor VIII gene and protein. *Semin Thromb Hemost*. 2003;29(1):11-22.

24. Saenko EL, Scandella D. The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von willebrand factor. *J Biol Chem*. 1997;272(29):18007-18014.

25. Terraube V, O'Donnell JS, Jenkins PV. Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance. *Haemophilia*. 2010;16(1):3-13.

26. Borchiellini A, Fijnvandraat K, ten Cate JW, et al. Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. *Blood*. 1996;88(8):2951-2958.

27. Vlot AJ, Koppelman SJ, van den Berg MH, Bouma BN, Sixma JJ. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood*. 1995;85(11):3150-3157.

28. Camire RM, Bos MH. The molecular basis of factor V and VIII procofactor activation. *J Thromb Haemost*. 2009;7(12):1951-1961.

29. Fay PJ. Factor VIII structure and function. *Int J Hematol*. 2006;83(2):103-108.

30. Pipe SW. Functional roles of the factor VIII B domain. *Haemophilia*. 2009;15(6):1187-1196.

31. Kitchen S, Hayward C, Negrier C, Dargaud Y. New developments in laboratory diagnosis and monitoring. *Haemophilia*. 2010;16 Suppl 5:61-66.

32. Mannucci PM. Back to the future: a recent history of haemophilia treatment. *Haemophilia*. 2008;14 Suppl 3:10-18.

33. Franchini M, Tagliaferri A, Mannucci PM. The management of hemophilia in elderly patients. *Clin Interv Aging*. 2007;2(3):361-368.

34. Aledort LM. Is the incidence and prevalence of inhibitors greater with recombinant products? Yes. *J Thromb Haemost*. 2004;2(6):861-862.

35. Ettingshausen CE, Kreuz W. Recombinant vs. plasma-derived products, especially those with intact VWF, regarding inhibitor development. *Haemophilia*. 2006;12 Suppl 6:102-106.

36. Franchini M, Lippi G. Von Willebrand factor-containing factor VIII concentrates and inhibitors in haemophilia A. A critical literature review. *Thromb Haemost*. 2010;104(5):931-940.

37. Goudemand J. Inhibitor development in haemophilia A: the role of von Willebrand factor/factor VIII concentrates. *Haemophilia*. 2007;13 Suppl 5:47-51.

38. Franchini M, Mannucci PM. Past, present and future of hemophilia: a narrative review. *Orphanet J Rare Dis*. 2012;7:24.

39. Di Minno G, Cerbone AM, Coppola A, et al. Longer-acting factor VIII to overcome limitations in haemophilia management: the PEGylated liposomes formulation issue. *Haemophilia*. 2010;16 Suppl 1:2-6.

40. Sen P, Ghosh S, Ezban M, Pendurthi UR, Vijaya Mohan Rao L. Effect of glycoPEGylation on factor VIIa binding and internalization. *Haemophilia*. 2010;16(2):339-348.

41. Spira J, Plyushch O, Zozulya N, et al. Safety, pharmacokinetics and efficacy of factor VIIa formulated with PEGylated liposomes in haemophilia A patients with inhibitors to factor VIII--an open label, exploratory, cross-over, phase I/II study. *Haemophilia*. 2010;16(6):910-918.

42. Metzner HJ, Weimer T, Kronthaler U, Lang W, Schulte S. Genetic fusion to albumin improves the pharmacokinetic properties of factor IX. *Thromb Haemost*. 2009;102(4):634-644.

43. Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med*. 2008;358(21):2231-2239.

44. Blaese RM, Culver KW, Miller AD, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science*. 1995;270(5235):475-480.

45. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 2000;288(5466):669-672.

46. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature*. 2010;467(7313):318-322.

47. Hauswirth WW, Aleman TS, Kaushal S, et al. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. *Hum Gene Ther*. 2008;19(10):979-990.

48. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet*. 2000;24(3):257-261.

49. Maguire AM, High KA, Auricchio A, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet*. 2009;374(9701):1597-1605.

50. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med*. 2008;358(21):2240-2248.

51. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*. 2011;365(25):2357-2365.

52. Deichmann A, Hacein-Bey-Abina S, Schmidt M, et al. Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-X1 gene therapy. *J Clin Invest*. 2007;117(8):2225-2232.

53. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2003;348(3):255-256.

54. Aiuti A, Cattaneo F, Galimberti S, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med*. 2009;360(5):447-458.

55. Ishiwata A, Mimuro J, Kashiwakura Y, et al. Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene. *Thromb Res*. 2006;118(5):627-635.

56. Van Damme A, Chuah MK, Collen D, VandenDriessche T. Onco-retroviral and lentiviral vector-based gene therapy for hemophilia: preclinical studies. *Semin Thromb Hemost*. 2004;30(2):185-195.

57. Dwarki VJ, Belloni P, Nijjar T, et al. Gene therapy for hemophilia A: production of therapeutic levels of human factor VIII in vivo in mice. *Proc Natl Acad Sci U S A*. 1995;92(4):1023-1027.

58. Gangadharan B, Parker ET, Ide LM, Spencer HT, Doering CB. High-level expression of porcine factor VIII from genetically modified bone marrow-derived stem cells. *Blood*. 2006;107(10):3859-3864.

59. Moayeri M, Hawley TS, Hawley RG. Correction of murine hemophilia A by hematopoietic stem cell gene therapy. *Mol Ther*. 2005;12(6):1034-1042.

60. Moayeri M, Ramezani A, Morgan RA, Hawley TS, Hawley RG. Sustained phenotypic correction of hemophilia a mice following oncoretroviral-mediated expression of a bioengineered human factor VIII gene in long-term hematopoietic repopulating cells. *Mol Ther*. 2004;10(5):892-902.

61. Van Damme A, Chuah MK, Dell'accio F, et al. Bone marrow mesenchymal cells for haemophilia A gene therapy using retroviral vectors with modified long-terminal repeats. *Haemophilia*. 2003;9(1):94-103.

62. Wilcox DA, Shi Q, Nurden P, et al. Induction of megakaryocytes to synthesize and store a releasable pool of human factor VIII. *J Thromb Haemost*. 2003;1(12):2477-2489.

63. Shi Q, Montgomery RR. Platelets as delivery systems for disease treatments. *Adv Drug Deliv Rev*. 2010;62(12):1196-1203.

64. Ide LM, Iwakoshi NN, Gangadharan B, et al. Functional aspects of factor VIII expression after transplantation of genetically-modified hematopoietic stem cells for hemophilia A. *J Gene Med*. 2010;12(4):333-344.

65. Herder C, Tonn T, Oostendorp R, et al. Sustained expansion and transgene expression of coagulation factor VIII-transduced cord blood-derived endothelial progenitor cells. *Arterioscler Thromb Vasc Biol*. 2003;23(12):2266-2272.

66. Matsui H, Hegadorn C, Ozelo M, et al. A microRNA-regulated and GP64-pseudotyped lentiviral vector mediates stable expression of FVIII in a murine model of Hemophilia A. *Mol Ther*. 2011;19(4):723-730.

67. Ohmori T, Mimuro J, Takano K, et al. Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibalpha promoter: in vivo model for platelet-targeting gene therapy. *FASEB J*. 2006;20(9):1522-1524.

68. Ohmori T, Ishiwata A, Kashiwakura Y, et al. Phenotypic correction of hemophilia A by ectopic expression of activated factor VII in platelets. *Mol Ther*. 2008;16(8):1359-1365.

69. Shi Q, Wilcox DA, Fahs SA, et al. Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost*. 2007;5(2):352-361.

70. Greene TK, Wang C, Hirsch JD, et al. In vivo efficacy of platelet-delivered, high specific activity factor VIII variants. *Blood*. 2010;116(26):6114-6122.

71. Ramezani A, Zweier-Renn LA, Hawley RG. Factor VIII delivered by haematopoietic stem cell-derived B cells corrects the phenotype of haemophilia A mice. *Thromb Haemost*. 2011;105(4):676-687.

72. Doering CB, Denning G, Dooriss K, et al. Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A. *Mol Ther*. 2009;17(7):1145-1154.

73. Xu L, Nichols TC, Sarkar R, McCorquodale S, Bellinger DA, Ponder KP. Absence of a desmopressin response after therapeutic expression of factor VIII in hemophilia A dogs with liver-directed neonatal gene therapy. *Proc Natl Acad Sci U S A*. 2005;102(17):6080-6085.

74. Ward NJ, Buckley SM, Waddington SN, et al. Codon optimization of human factor VIII cDNAs leads to high-level expression. *Blood*. 2011;117(3):798-807.

75. Connelly S, Gardner JM, Lyons RM, McClelland A, Kaleko M. Sustained expression of therapeutic levels of human factor VIII in mice. *Blood*. 1996;87(11):4671-4677.

76. Connelly S, Mount J, Mauser A, et al. Complete short-term correction of canine hemophilia A by in vivo gene therapy. *Blood*. 1996;88(10):3846-3853.

77. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH, Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet*. 1995;10(1):119-121.

78. Connelly S, Andrews JL, Gallo AM, et al. Sustained phenotypic correction of murine hemophilia A by in vivo gene therapy. *Blood*. 1998;91(9):3273-3281.

79. Balague C, Zhou J, Dai Y, et al. Sustained high-level expression of full-length human factor VIII and restoration of clotting activity in hemophilic mice using a minimal adenovirus vector. *Blood*. 2000;95(3):820-828.

80. Dormond E, Kamen AA. Manufacturing of adenovirus vectors: production and purification of helper dependent adenovirus. *Methods Mol Biol*. 2011;737:139-156.

81. Chuah MK, Schiedner G, Thorrez L, et al. Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. *Blood*. 2003;101(5):1734-1743.

82. Brown BD, Shi CX, Powell S, Hurlbut D, Graham FL, Lillicrap D. Helper-dependent adenoviral vectors mediate therapeutic factor VIII expression for several months with minimal accompanying toxicity in a canine model of severe hemophilia A. *Blood*. 2004;103(3):804-810.

83. McCormack WM, Jr., Seiler MP, Bertin TK, et al. Helper-dependent adenoviral gene therapy mediates long-term correction of the clotting defect in the canine hemophilia A model. *J Thromb Haemost*. 2006;4(6):1218-1225.

84. Cerullo V, Seiler MP, Mane V, et al. Correction of murine hemophilia A and immunological differences of factor VIII variants delivered by helper-dependent adenoviral vectors. *Mol Ther*. 2007;15(12):2080-2087.

85. Hu C, Cela RG, Suzuki M, Lee B, Lipshutz GS. Neonatal helper-dependent adenoviral vector gene therapy mediates correction of hemophilia A and tolerance to human factor VIII. *Proc Natl Acad Sci U S A*. 2011;108(5):2082-2087.

86. Burton M, Nakai H, Colosi P, Cunningham J, Mitchell R, Couto L. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc Natl Acad Sci U S A*. 1999;96(22):12725-12730.

87. Scallan CD, Liu T, Parker AE, et al. Phenotypic correction of a mouse model of hemophilia A using AAV2 vectors encoding the heavy and light chains of FVIII. *Blood*. 2003;102(12):3919-3926.

88. Chao H, Mao L, Bruce AT, Walsh CE. Sustained expression of human factor VIII in mice using a parvovirus-based vector. *Blood*. 2000;95(5):1594-1599.

89. Jiang H, Lillicrap D, Patarroyo-White S, et al. Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood*. 2006;108(1):107-115.

90. Sarkar R, Tetreault R, Gao G, et al. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood*. 2004;103(4):1253-1260.

91. Sabatino DE, Lange AM, Altynova ES, et al. Efficacy and safety of long-term prophylaxis in severe hemophilia A dogs following liver gene therapy using AAV vectors. *Mol Ther*. 2011;19(3):442-449.

92. Hu C, Lipshutz GS. AAV-based neonatal gene therapy for hemophilia A: long-term correction and avoidance of immune responses in mice. *Gene Ther*. 2012;19(12):1166-1176.

93. Liras A, Olmedillas S. Gene therapy for haemophilia...yes, but...with non-viral vectors? *Haemophilia*. 2009;15(3):811-816.

94. Fernando S, Fletcher BS. Sleeping beauty transposon-mediated nonviral gene therapy. *BioDrugs*. 2006;20(4):219-229.

95. Ye P, Thompson AR, Sarkar R, et al. Naked DNA transfer of Factor VIII induced transgene-specific, species-independent immune response in hemophilia A mice. *Mol Ther*. 2004;10(1):117-126.

96. Miao CH, Ye P, Thompson AR, Rawlings DJ, Ochs HD. Immunomodulation of transgene responses following naked DNA transfer of human factor VIII into hemophilia A mice. *Blood*. 2006;108(1):19-27.

97. Ohlfest JR, Frandsen JL, Fritz S, et al. Phenotypic correction and long-term expression of factor VIII in hemophilic mice by immunotolerization and nonviral gene transfer using the Sleeping Beauty transposon system. *Blood*. 2005;105(7):2691-2698.

98. Kren BT, Unger GM, Sjeklocha L, et al. Nanocapsule-delivered Sleeping Beauty mediates therapeutic Factor VIII expression in liver sinusoidal endothelial cells of hemophilia A mice. *J Clin Invest*. 2009;119(7):2086-2099.

99. Liu L, Liu H, Mah C, Fletcher BS. Indoleamine 2,3-dioxygenase attenuates inhibitor development in gene-therapy-treated hemophilia A mice. *Gene Ther*. 2009;16(6):724-733.

100. Bowman K, Sarkar R, Raut S, Leong KW. Gene transfer to hemophilia A mice via oral delivery of FVIII-chitosan nanoparticles. *J Control Release*. 2008;132(3):252-259.

101. Chao H, Mansfield SG, Bartel RC, et al. Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. *Nat Med*. 2003;9(8):1015-1019.

102. Murphy SL, High KA. Gene therapy for haemophilia. *Br J Haematol*. 2008;140(5):479-487.

103. Webster WP, Zukoski CF, Hutchin P, Reddick RL, Mandel SR, Penick GD. Plasma factor VIII synthesis and control as revealed by canine organ transplantation. *Am J Physiol*. 1971;220(5):1147-1154.

104. Aronovich A, Tchorsh D, Katchman H, et al. Correction of hemophilia as a proof of concept for treatment of monogenic diseases by fetal spleen transplantation. *Proc Natl Acad Sci U S A*. 2006;103(50):19075-19080.

105. Jiang HC, Gao Y, Dai WJ, et al. Ten-year experience with living related donated splenic transplantation for the treatment of hemophilia A. *Transplant Proc*. 2006;38(5):1483-1490.

106. Liu L, Xia S, Seifert J. Transplantation of spleen cells in patients with hemophilia A. A report of 20 cases. *Transpl Int*. 1994;7(3):201-206.

107. Darwish AA, Sokal E, Stephenne X, Najimi M, de Goyet Jde V, Reding R. Permanent access to the portal system for cellular transplantation using an implantable port device. *Liver Transpl*. 2004;10(9):1213-1215.

108. Dhawan A, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. *J Inherit Metab Dis*. 2006;29(2-3):431-435.

109. Fox IJ, Chowdhury JR, Kaufman SS, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med*. 1998;338(20):1422-1426.

110. Horslen SP, McCowan TC, Goertzen TC, et al. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. *Pediatrics*. 2003;111(6 Pt 1):1262-1267.

111. Lee KW, Lee JH, Shin SW, et al. Hepatocyte transplantation for glycogen storage disease type Ib. *Cell Transplant*. 2007;16(6):629-637.

112. Meyburg J, Das AM, Hoerster F, et al. One liver for four children: first clinical series of liver cell transplantation for severe neonatal urea cycle defects. *Transplantation*. 2009;87(5):636-641.

113. Mitry RR, Dhawan A, Hughes RD, et al. One liver, three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. *Transplantation*. 2004;77(10):1614-1616.

114. Muraca M, Gerunda G, Neri D, et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet*. 2002;359(9303):317-318.

115. Sokal EM, Smets F, Bourgois A, et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation*. 2003;76(4):735-738.

116. Stephenne X, Najimi M, Smets F, Reding R, de Ville de Goyet J, Sokal EM. Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. *Am J Transplant*. 2005;5(8):2058-2061.

117. Madeira CL, Layman ME, de Vera RE, Fontes PA, Ragni MV. Extrahepatic factor VIII production in transplant recipient of hemophilia donor liver. *Blood*. 2009;113(21):5364-5365.

118. Follenzi A, Benten D, Novikoff P, Faulkner L, Raut S, Gupta S. Transplanted endothelial cells repopulate the liver endothelium and correct the phenotype of hemophilia A mice. *J Clin Invest*. 2008;118(3):935-945.

119. Follenzi A, Raut S, Merlin S, Sarkar R, Gupta S. Role of bone marrow transplantation for correcting hemophilia A in mice. *Blood*. 2012;119(23):5532-5542.

120. Yadav N, Kanjirakkuzhiyil S, Kumar S, et al. The therapeutic effect of bone marrow-derived liver cells in the phenotypic correction of murine hemophilia A. *Blood*. 2009;114(20):4552-4561.

121. Oertel M, Shafritz DA. Stem cells, cell transplantation and liver repopulation. *Biochim Biophys Acta*. 2008;1782(2):61-74.

122. Walia B, Satija N, Tripathi RP, Gangenahalli GU. Induced pluripotent stem cells: fundamentals and applications of the reprogramming process and its ramifications on regenerative medicine. *Stem Cell Rev*. 2012;8(1):100-115.

123. Oh J, Kang H, Chung JH, Jang Y. Autologous bone-marrow stem cells for myocardial infarction. *Lancet*. 2006;368(9529):27.

124. Ringden O, Labopin M, Bacigalupo A, et al. Transplantation of peripheral blood stem cells as compared with bone marrow from HLA-identical siblings in adult patients with acute myeloid leukemia and acute lymphoblastic leukemia. *J Clin Oncol*. 2002;20(24):4655-4664.

125. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat Med*. 2000;6(3):278-282.

126. Lu B, Malcuit C, Wang S, et al. Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. *Stem Cells*. 2009;27(9):2126-2135.

127. Mayor S. First patient enters trial to test safety of stem cells in spinal injury. *BMJ*. 2010;341:c5724.

128. Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. *Stem Cells*. 2010;28(1):152-163.

129. Briggs R, King TJ. Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc Natl Acad Sci U S A*. 1952;38(5):455-463.

130. Egli D, Rosains J, Birkhoff G, Eggan K. Developmental reprogramming after chromosome transfer into mitotic mouse zygotes. *Nature*. 2007;447(7145):679-685.

131. Li J, Liu X, Wang H, et al. Human embryos derived by somatic cell nuclear transfer using an alternative enucleation approach. *Cloning Stem Cells*. 2009;11(1):39-50.

132. Cowan CA, Atienza J, Melton DA, Eggan K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. *Science*. 2005;309(5739):1369-1373.

133. Yu J, Vodyanik MA, He P, Slukvin, II, Thomson JA. Human embryonic stem cells reprogram myeloid precursors following cell-cell fusion. *Stem Cells*. 2006;24(1):168-176.

134. Nichols J, Zevnik B, Anastassiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998;95(3):379-391.

135. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*. 2000;24(4):372-376.

136. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev*. 2003;17(1):126-140.

137. Chambers I, Colby D, Robertson M, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*. 2003;113(5):643-655.

138. Mitsui K, Tokuzawa Y, Itoh H, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*. 2003;113(5):631-642.

139. Matsuda T, Nakamura T, Nakao K, et al. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J*. 1999;18(15):4261-4269.

140. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev*. 1998;12(13):2048-2060.

141. Takahashi K, Mitsui K, Yamanaka S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. *Nature*. 2003;423(6939):541-545.

142. Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development*. 2005;132(5):885-896.

143. Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC, Chan RJ. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood*. 2005;105(2):635-637.

144. Kielman MF, Rindapaa M, Gaspar C, et al. Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. *Nat Genet*. 2002;32(4):594-605.

145. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med*. 2004;10(1):55-63.

146. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861-872.

147. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126(4):663-676.

148. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;318(5858):1917-1920.

149. Wang X, Dai J. Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology. *Stem Cells*. 2010;28(5):885-893.

150. Gu P, Goodwin B, Chung AC, et al. Orphan nuclear receptor LRH-1 is required to maintain Oct4 expression at the epiblast stage of embryonic development. *Mol Cell Biol*. 2005;25(9):3492-3505.

151. Yang HM, Do HJ, Kim DK, et al. Transcriptional regulation of human Oct4 by steroidogenic factor-1. *J Cell Biochem*. 2007;101(5):1198-1209.

152. Zhang X, Zhang J, Wang T, Esteban MA, Pei D. Esrrb activates Oct4 transcription and sustains self-renewal and pluripotency in embryonic stem cells. *J Biol Chem*. 2008;283(51):35825-35833.

153. Graham V, Khudyakov J, Ellis P, Pevny L. SOX2 functions to maintain neural progenitor identity. *Neuron*. 2003;39(5):749-765.

154. Kim JB, Greber B, Arauzo-Bravo MJ, et al. Direct reprogramming of human neural stem cells by OCT4. *Nature*. 2009;461(7264):649-643.

155. Huangfu D, Osafune K, Maehr R, et al. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol*. 2008;26(11):1269-1275.

156. Chan KK, Zhang J, Chia NY, et al. KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. *Stem Cells*. 2009;27(9):2114-2125.

157. Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat Cell Biol*. 2005;7(11):1074-1082.

158. Wernig M, Meissner A, Cassady JP, Jaenisch R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell*. 2008;2(1):10-12.

159. Suzuki A, Raya A, Kawakami Y, et al. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *Proc Natl Acad Sci U S A*. 2006;103(27):10294-10299.

160. Maherali N, Hochedlinger K. Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell Stem Cell*. 2008;3(6):595-605.

161. Daley GQ, Lensch MW, Jaenisch R, Meissner A, Plath K, Yamanaka S. Broader implications of defining standards for the pluripotency of iPSCs. *Cell Stem Cell*. 2009;4(3):200-201; author reply 202.

162. Ellis J, Bruneau BG, Keller G, et al. Alternative induced pluripotent stem cell characterization criteria for in vitro applications. *Cell Stem Cell*. 2009;4(3):198-199; author reply 202.

163. Aasen T, Raya A, Barrero MJ, et al. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol*. 2008;26(11):1276-1284.

164. Kim JB, Sebastiano V, Wu G, et al. Oct4-induced pluripotency in adult neural stem cells. *Cell*. 2009;136(3):411-419.

165. Hanna J, Markoulaki S, Schorderet P, et al. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell*. 2008;133(2):250-264.

166. Sugii S, Kida Y, Kawamura T, et al. Human and mouse adipose-derived cells support feeder-independent induction of pluripotent stem cells. *Proc Natl Acad Sci U S A*. 2010;107(8):3558-3563.

167. Sun N, Panetta NJ, Gupta DM, et al. Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci U S A*. 2009;106(37):15720-15725.

168. Giorgetti A, Montserrat N, Aasen T, et al. Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. *Cell Stem Cell*. 2009;5(4):353-357.

169. Haase A, Olmer R, Schwanke K, et al. Generation of induced pluripotent stem cells from human cord blood. *Cell Stem Cell*. 2009;5(4):434-441.

170. Loh YH, Agarwal S, Park IH, et al. Generation of induced pluripotent stem cells from human blood. *Blood*. 2009;113(22):5476-5479.

171. Takenaka C, Nishishita N, Takada N, Jakt LM, Kawamata S. Effective generation of iPS cells from CD34+ cord blood cells by inhibition of p53. *Exp Hematol*. 2010;38(2):154-162.

172. Utikal J, Maherali N, Kulalert W, Hochedlinger K. Sox2 is dispensable for the reprogramming of melanocytes and melanoma cells into induced pluripotent stem cells. *J Cell Sci*. 2009;122(Pt 19):3502-3510.

173. Ye Z, Zhan H, Mali P, et al. Human-induced pluripotent stem cells from blood cells of healthy donors and patients with acquired blood disorders. *Blood*. 2009;114(27):5473-5480.

174. Nakagawa M, Koyanagi M, Tanabe K, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008;26(1):101-106.

175. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature*. 2007;448(7151):313-317.

176. Sommer CA, Sommer AG, Longmire TA, et al. Excision of reprogramming transgenes improves the differentiation potential of iPS cells generated with a single excisable vector. *Stem Cells*. 2010;28(1):64-74.

177. Soldner F, Hockemeyer D, Beard C, et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell*. 2009;136(5):964-977.

178. Apolonia L, Waddington SN, Fernandes C, et al. Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol Ther*. 2007;15(11):1947-1954.

179. Yanez-Munoz RJ, Balaggan KS, MacNeil A, et al. Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med*. 2006;12(3):348-353.

180. Hockemeyer D, Soldner F, Cook EG, Gao Q, Mitalipova M, Jaenisch R. A drug-inducible system for direct reprogramming of human somatic cells to pluripotency. *Cell Stem Cell*. 2008;3(3):346-353.

181. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science*. 2008;322(5903):945-949.

182. Zhou W, Freed CR. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem Cells*. 2009;27(11):2667-2674.

183. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci*. 2009;85(8):348-362.

184. Sun N, Longaker MT, Wu JC. Human iPS cell-based therapy: considerations before clinical applications. *Cell Cycle*. 2010;9(5):880-885.

185. Gonzalez F, Barragan Monasterio M, Tiscornia G, et al. Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci U S A*. 2009;106(22):8918-8922.

186. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science*. 2008;322(5903):949-953.

187. Elick TA, Bauser CA, Fraser MJ. Excision of the piggyBac transposable element in vitro is a precise event that is enhanced by the expression of its encoded transposase. *Genetica*. 1996;98(1):33-41.

188. Fraser MJ, Ciszczon T, Elick T, Bauser C. Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Mol Biol*. 1996;5(2):141-151.

189. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*. 2009;458(7239):771-775.

190. Woltjen K, Michael IP, Mohseni P, et al. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature*. 2009;458(7239):766-770.

191. Zhou H, Wu S, Joo JY, et al. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell*. 2009;4(5):381-384.

192. Gump JM, Dowdy SF. TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol Med*. 2007;13(10):443-448.

193. Warren L, Manos PD, Ahfeldt T, et al. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell*. 2010;7(5):618-630.

194. Page RL, Ambady S, Holmes WF, et al. Induction of stem cell gene expression in adult human fibroblasts without transgenes. *Cloning Stem Cells*. 2009;11(3):417-426.

195. Esteban MA, Wang T, Qin B, et al. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell*. 2010;6(1):71-79.

196. Li W, Zhou H, Abujarour R, et al. Generation of human-induced pluripotent stem cells in the absence of exogenous Sox2. *Stem Cells*. 2009;27(12):2992-3000.

197. Shi Y, Desponts C, Do JT, Hahm HS, Scholer HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell*. 2008;3(5):568-574.

198. Judson RL, Babiarz JE, Venere M, Blelloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol*. 2009;27(5):459-461.

199. Heo I, Joo C, Cho J, Ha M, Han J, Kim VN. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell*. 2008;32(2):276-284.

200. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. *Cell*. 2009;137(4):647-658.

201. Hemberger M, Dean W, Reik W. Epigenetic dynamics of stem cells and cell lineage commitment: digging Waddington's canal. *Nat Rev Mol Cell Biol*. 2009;10(8):526-537.

202. Reddien PW, Sanchez Alvarado A. Fundamentals of planarian regeneration. *Annu Rev Cell Dev Biol*. 2004;20:725-757.

203. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science*. 1988;241(4861):58-62.

204. Meshorer EM, T. Chromatin in pluripotent

embryonic stem cells and differentiation. *Nature Rev*

Mol Cell Biol. 2006;7:540-546.

205. Hawkins RD, Hon GC, Lee LK, et al. Distinct epigenomic landscapes of pluripotent and lineage-committed human cells. *Cell Stem Cell*. 2010;6(5):479-491.

206. Boyer LA, Plath K, Zeitlinger J, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*. 2006;441(7091):349-353.

207. Chamberlain SJ, Yee D, Magnuson T. Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. *Stem Cells*. 2008;26(6):1496-1505.

208. Feldman N, Gerson A, Fang J, et al. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nat Cell Biol*. 2006;8(2):188-194.

209. Epsztejn-Litman S, Feldman N, Abu-Remaileh M, et al. De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nat Struct Mol Biol*. 2008;15(11):1176-1183.

210. Loh YH, Zhang W, Chen X, George J, Ng HH. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. *Genes Dev*. 2007;21(20):2545-2557.

211. Mikkelsen TS, Hanna J, Zhang X, et al. Dissecting direct reprogramming through integrative genomic analysis. *Nature*. 2008;454(7200):49-55.

212. Sridharan R, Tchieu J, Mason MJ, et al. Role of the murine reprogramming factors in the induction of pluripotency. *Cell*. 2009;136(2):364-377.

213. Meissner A, Mikkelsen TS, Gu H, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature*. 2008;454(7205):766-770.

214. Huangfu D, Maehr, R., Guo, W., Eijkelenboom, A., Snitow, M., Chen, A.E., Melton, D.A. Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. *Nat Biotechnol*. 2008;26(7):795-797.

215. Shi Y, Desponts, C., Do, J.T., Hahm, H.S., Schöler, H.R., Ding, S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. *Cell Stem Cell*. 2008;3(5):568-574.

216. Onder TT, Kara N, Cherry A, et al. Chromatin-modifying enzymes as modulators of reprogramming. *Nature*. 2012;483(7391):598-602.

217. Hajkova P, Ancelin K, Waldmann T, et al. Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature*. 2008;452(7189):877-881.

218. Wernig M, Zhao JP, Pruszak J, et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A*. 2008;105(15):5856-5861.

219. Dimos JT, Rodolfa KT, Niakan KK, et al. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science*. 2008;321(5893):1218-1221.

220. Huang HP, Chen PH, Hwu WL, et al. Human Pompe disease-induced pluripotent stem cells for pathogenesis modeling, drug testing and disease marker identification. *Hum Mol Genet*. 2011;20(24):4851-4864.

221. Lahti AL, Kujala VJ, Chapman H, et al. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. *Dis Model Mech*. 2012;5(2):220-230.

222. Maclean GA, Menne TF, Guo G, et al. Altered hematopoiesis in trisomy 21 as revealed through in vitro differentiation of isogenic human pluripotent cells. *Proc Natl Acad Sci U S A*. 2012;109(43):17567-17572.

223. Yahata N, Asai M, Kitaoka S, et al. Anti-Abeta drug screening platform using human iPS cell-derived neurons for the treatment of Alzheimer's disease. *PLoS One*. 2011;6(9):e25788.

224. Zhang N, An MC, Montoro D, Ellerby LM. Characterization of Human Huntington's Disease Cell Model from Induced Pluripotent Stem Cells. *PLoS Curr*. 2010;2:RRN1193.

225. Brennand KJ, Simone A, Jou J, et al. Modelling schizophrenia using human induced pluripotent stem cells. *Nature*. 2011;473(7346):221-225.

226. Ebert AD, Yu J, Rose FF, Jr., et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature*. 2009;457(7227):277-280.

227. Lee G, Papapetrou EP, Kim H, et al. Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature*. 2009;461(7262):402-406.

228. Marchetto MC, Carromeu C, Acab A, et al. A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. *Cell*. 2010;143(4):527-539.

229. Pedrosa E, Sandler V, Shah A, et al. Development of patient-specific neurons in schizophrenia using induced pluripotent stem cells. *J Neurogenet*. 2011;25(3):88-103.

230. Sanchez-Danes A, Richaud-Patin Y, Carballo-Carbajal I, et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. *EMBO Mol Med*. 2012;4(5):380-395.

231. Alipio Z, Liao W, Roemer EJ, et al. Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic beta-like cells. *Proc Natl Acad Sci U S A*. 2010;107(30):13426-13431.

232. Gomi M, Takagi Y, Morizane A, et al. Functional recovery of the murine brain ischemia model using human induced pluripotent stem cell-derived telencephalic progenitors. *Brain Res*. 2012;1459:52-60.

233. Hargus G, Cooper O, Deleidi M, et al. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. *Proc Natl Acad Sci U S A*. 2010;107(36):15921-15926.

234. Rufaihah AJ, Huang NF, Jame S, et al. Endothelial cells derived from human iPSCS increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. *Arterioscler Thromb Vasc Biol*. 2011;31(11):e72-79.

235. Swistowski A, Peng J, Liu Q, et al. Efficient generation of functional dopaminergic neurons from human induced pluripotent stem cells under defined conditions. *Stem Cells*. 2010;28(10):1893-1904.

236. Hanna J, Wernig M, Markoulaki S, et al. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science*. 2007;318(5858):1920-1923.

237. Raya A, Rodriguez-Piza I, Guenechea G, et al. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature*. 2009;460(7251):53-59.

238. Copray WSS. The Molecular Mechanism of Induced Pluripotency: A Two-Stage Switch. *Stem Cell Rev and Rep*. 2009;5:204-223.

239. Li Y, Tsai YT, Hsu CW, et al. Long-term Safety and Efficacy of Human-Induced Pluripotent Stem Cell (iPS) Grafts in a Preclinical Model of Retinitis Pigmentosa. *Mol Med*. 2012;18:1312-1319.

240. Zhou L, Wang W, Liu Y, et al. Differentiation of induced pluripotent stem cells of swine into rod photoreceptors and their integration into the retina. *Stem Cells*. 2011;29(6):972-980.

241. Lee EX, Lam DH, Wu C, et al. Glioma gene therapy using induced pluripotent stem cell derived neural stem cells. *Mol Pharm*. 2011;8(5):1515-1524.

242. Senju S, Haruta M, Matsumura K, et al. Generation of dendritic cells and macrophages from human induced pluripotent stem cells aiming at cell therapy. *Gene Ther*. 2011;18(9):874-883.

243. Opalinska JB, Bersenev A, Zhang Z, et al. MicroRNA expression in maturing murine megakaryocytes. *Blood*. 2010;116(23):e128-138.

244. O'Connor MD, Kardel MD, Iosfina I, et al. Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. *Stem Cells*. 2008;26(5):1109-1116.

245. James D, Nam HS, Seandel M, et al. Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. *Nat Biotechnol*. 2010;28(2):161-166.

246. Ostronoff M, Ostronoff F, Campos G, et al. Allogeneic bone marrow transplantation in a child with severe aplastic anemia and hemophilia A. *Bone Marrow Transplant*. 2006;37(6):627-628.

247. El-Maarri O, Singer H, Klein C, et al. Lack of F8 mRNA: a novel mechanism leading to hemophilia A. *Blood*. 2006;107(7):2759-2765.

248. Knolle PA, Limmer A. Control of immune responses by savenger liver endothelial cells. *Swiss Med Wkly*. 2003;133(37-38):501-506.

249. Delgado MD, Albajar M, Gomez-Casares MT, Batlle A, Leon J. MYC oncogene in myeloid neoplasias. *Clin Transl Oncol*. 2012.

250. Staerk J, Dawlaty MM, Gao Q, et al. Reprogramming of human peripheral blood cells to induced pluripotent stem cells. *Cell Stem Cell*. 2010;7(1):20-24.

251. Xu D, Alipio Z, Fink LM, et al. Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. *Proc Natl Acad Sci U S A*. 2009;106(3):808-813.

252. Holkers M, Maggio I, Liu J, et al. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res*. 2012.

253. Lombardo A, Genovese P, Beausejour CM, et al. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol*. 2007;25(11):1298-1306.

Acknowledgments

My first “thank you” goes to Dr. Antonia Follenzi for her supervision throughout this period, for her faith in me and for gives me the possibility to work in her lab and to follow a new exciting project at the beginning.

Special thanks to Prof. Angel Raya who gave me the chance to attend his lab, to Antonella and the other members and in particular to Yvonne who guided me with patient and passion in my first steps in the mysterious pluripotency world.

Thanks to Prof. Maria Prat and all Histology Lab: Simone, Diego, Kevin, Stefano, Marta, Sara, Andrea, Francesca and in particular to Maria. I began to know her and I hope this is the start of a new friendship.

A special thanks to wise Stefania who shared with me this course and always had the right words.

I am very grateful to Chantal, an authentic person, who told me when I was wrong teaching me what it means be a true friend.

Thanks to Maria who rendered less stuffy days of work with her happiness. Without her this project would not have been possible.

Special thanks to Matteo, Manuela, Alessandra e Michela for be present always with a word or an embrace and for our special dinners.

I am very grateful to my mother Gina, my father Fernando and my sister Beatrice for their support and especially for their patient.

Finally, a special “thank you” to Simone who subverted my life, help and tolerate me every day and make me happy.