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TITOLO:

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

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INTRODUCTION

Haemophilia A and factor VIII

Haemophilia A is an X-linked bleeding disorder in which the functionally active coagulation factor VIII (FVIII) is partially or totally deficient. FVIII is a complex plasma glycoprotein that is synthesized primarily by hepatocytes, although extrahepatic FVIII production has been confirmed by mRNA detection in spleen, kidney, sinusoidal endothelial cells (EC), pulmonary endothelial cells, lymphatic tissues and by the transplantation of a haemophilia A liver donor into a non hemophilic recipient with alcoholic cirrhosis [1].

The FVIII gene comprises 26 exons, which encode a polypeptide chain for a signal peptide of 19 amino acids and a mature protein of 2332 amino acids. FVIII is synthesized as an inactive single chain with the discrete domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2. The protein contains a large B domain of unknown function that is not required for clotting activity, encoded by exon 14. It is one of the largest and least stable coagulation factors, circulating 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. Activated FVIII (FVIIIa) functions as a non-enzymatic cofactor for factor IXa in the activation of factor X (FXa). FVIII has a half-life of about 12 h in adults (shorter in children) [2].

The most common mutation in patients with haemophilia 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. It has an incidence of 1-2 per 10000 males. It is characterized by frequent spontaneous bleeding episode, mostly into joints. Haemophilia A is typically divided into three classes, which are defined by factor plasma activity: severe, when the activity of the FVIII 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 [3].

However, there is a type of acquired haemophilia (HA), 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 disease, pregnancy, cancer or drug ingestion. Acquired haemophilia occurs in about one person per million, and can affect females [4].

There isn't a cure for haemophilia A, but only a treatment which consists in infusion of plasma-derived or recombinant FVIII (rFVIII) when bleeds occur or regularly in order to prevent bleeds in cases of severe haemophilia A. Unfortunately, current management is not optimal: there is the risk of transfusion-transmitted infections (hepatitis A,B and HIV), development of inhibitory antibodies (30-50% of patients) and high cost for haemophiliacs. Cell and/or gene therapy are alternative to supplemental therapy.

Haemophilia 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 [5].

Actually, gene therapy for haemophilia A consists in the addition of normal FVIII gene. [6],[7]. Despite that it has been demonstrated a phenotypic correction of haemophilia A by RNA repair with spliceosome-mediated RNA *trans*-splicing [8].

Between 1998 and 2001 five different Phase I clinical trials were initiated for the treatment of haemophilia A in human with different gene delivery systems included retroviral vector, an adenoviral vector, two adeno-associated viral vector and non viral gene-delivery methods [9], but this approach have shown worse results compared with those obtained in animals. Moreover there are some problems to be considered: insertional mutagenesis of some viral vectors that randomly insert genes through the genome and immune response against vector's component. An important question remain to whether gene therapy by the production of ectopic FVIII will be a risk for inhibitor development [3].

Therefore, new approaches to cure haemophilia A are required. Early reports showed that in dogs, only orthotropic liver transplantation (OLT) corrected the phenotype of haemophilia A [10].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 haemophilia A without immune responses against the reintroduced FVIII [11]. Similarly, data from a Swedish and Chinese group of investigators, established the safety and efficacy of spleen transplantation in haemophilic patients with remarkable long term therapeutic results [12, 13]. 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 in order to treat diseases with or without the addition of gene transfer. Recent studies have demonstrated that transplanted liver sinusoidal endothelial cell (LSEC) and bone (BM) marrow from healthy donor can correct the phenotype of haemophilia A mice [11, 14, 15]. Other attempt to correct haemophilia A phenotype was target the expression of FVIII in

specific cells. Several types of cells were used for this purpose as hematopoietic stem cells [16, 17] and platelets [18-23].

Platelets are the main players involved in primary haemostasis. Together with endothelial cells they are considered to be the main FVIII storage compartment [24]. Moreover, megakaryocytes (cells responsible for the production of platelets) and endothelial cells synthesized and stored vWF, a large adhesive glycoprotein that perform an essential role in haemostasis and it also serve as the carrier protein of FVIII. Therefore, targeting expression of FVIII in these cells could establishing a pool of FVIII with vWF, which will be released in sites of injury. Thus, the inhibitory activity of antibodies might be circumvented and it could be limited the exposure to immune system[25].

Since it is difficult to obtain cells for human, it is necessary to find new sources of cell. One could be stem cells. The possibility of using genetically-modified autologous stem cells after appropriate expansion in vitro could help avoid allograft-related issues.

Induced pluripotent stem cell (iPSC)

Experiments performed several decades ago showed that dormant gene expression programs can be dominantly awakened in differentiated cells by the fusion of different pairs of cell types. Subsequently, lineage conversions could be effected simply through the introduction of defined transcription factors [26]. 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. Yamanaka showed that retroviral transduction of mouse and human fibroblasts with four transcription factor Oct3/4,Klf-4,c-Myc and Sox2 induced pluripotency in somatic cells. The established iPS cells are similar to embryonic stem (ES) cells in many aspects, including morphology, proliferation, feeder dependence, surface markers (SSEA-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81 and TRA-2-49/6E (alkaline phosphatase, AP), and NANOG, gene expression, promoter activities, telomerase activities, in vitro differentiation, and teratoma formation[27],[28].

Since then more studies have reported the derivation of human iPS cells from different cell types: keratinocytes [29], neural stem cells[30], B lymphocytes[31], adipose stem cells ([32],[33]) peripheral and cord blood ([34],[35],[36],[37],[38],[39]) and melanocytes [40].

There have been several reports of improvements of safe iPS cell generation. First of all, the elimination of c-Myc from the transcription cocktail due to its oncogenic activity and the

use of other transcription factors like Lin28 and Nanog with Oct4 and Sox2 [41]. Another approach is the reduction of integration sites by putting the reprogramming factors into a single vector with IRES or 2A self cleavage peptide [42]. This reprogramming cassette was used with a lentivirus (LV) system containing a loxP sequence in the LTR and produced iPS cells with only single 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 alteration [43]. A transposon system encoding a reprogramming cassette has also been used for iPS induction ([44],[45]).

Several other methods accomplished iPS induction by the transient expression of reprogramming factors. These methods include adenovirus [46], DNA vectors (plasmid, episomal plasmid, and minicircle vector), direct protein delivery and use of small compound (BIX,VPA,5-aza-cytidine). 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 [47].

Because 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 and drug development. As a result, these cells have been regarded as a leading candidate for donor cell source in regenerative medicine [48].

Recent studies demonstrated that iPS cells indeed can be generated directly from human patients suffering from Parkinson's disease, type I diabetes mellitus, severe combined immunodeficiency, muscular dystrophy and Down syndrome. Later these cells can differentiate in other cell type and correct some disease: functionally neurons which integrated in a rat model of Parkinson's disease [49], hematopoietic progenitor for the correction of Fanconi and Sickle Cell Anemia ([50],[51]), cardiomycites [52], hepatocytes [53].

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 and 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.

Specific aims

Identification of cells capable of synthesizing and releasing FVIII is critical for therapeutic development in haemophilia A.

The aim of this project is to find a new approach to cure haemophilia A by iPS-based strategy. Previously we generated iPSC from fibroblasts of healthy donors. However, in hemophilic patients, to harvest fibroblasts from skin biopsies is at risk of bleeding; for this reason, we utilized peripheral blood cells as an easy-to-access source of cells and reprogrammed mononuclear cells (MNC) from healthy donors and hemophilic patients.

a) We will transduce MNC from haemophilic patients with a LV expressing the human B-domain-deleted FVIII.

b) We will generate FVIII corrected-specific iPS cells and characterize them for stem cell potential based on presence of pluripotent-specific markers.

c) We will induce differentiation of iPS cells in endothelial cells with specific growth factors in culture condition.

d) We will perform transplantation of the iPS-derived-EC expressing-FVIII in the liver of NOD/SCID haemophilia A mice to assess engraftment, proliferation and phenotypic correction.

Material and methods

Cell culture.293T cells were cultured in DMEM supplemented with 10% FBS (Hyclone), penicillin/streptomicyn, GlutaMAX (GIBCO) at 37 °C, 5% CO2.

Feeder cells used: HFF, human foreskin fibroblast (ATCC) mitotically inactivated by gamma irradiation (55 Gy).

iPS cells were cultured on top of irradiated mouse or human fibroblasts in ES cell medium (Knockout DMEM supplemented with 20% knockout serum replacement, nonessential aminoacids, 2-mercaptoethanol, penicillin/streptomycin, GlutaMAX (all reagents were from GIBCO),bFGF (Imunotools)) and picked mechanically.

Embryoid bodies (EBs) formation was induced by mechanically pick of colonies and were cultured in low attachments plates in HPGM medium (LONZA).

Lentiviral vector production. 293T cells were co-transfected with four vectors by calcium phosphate precipitation; these vectors were the pMD.Lg/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.

Isolation of peripheral blood mononuclear cells (MNC) and transduction. 30 ml of peripheral blood was isolated from healthy donors or haemophilic 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 IL-7, GM-CSF, IL-3 and IL-6 10 ng/ml 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 IL-7, GM-CSF, IL-3 and IL-6 10 ng/ml for four more days. After, cells were cultured in standard human ES medium for 25-40 days before iPS colonies were picked.

Flow cytometry. For immunophenotypic analysis of MNC freshly isolated or after 5 days of culture were 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).

Immunofluorescence and AP staining. iPSC grown on plastic cover slide chamber were fixed with 4% paraformaldehyde (PAF) for 10 minutes at 4 °C and washed with PBS. The cells were then permeabilized with 0,5% Triton (SIGMA) for about 10 minutes at 4 °C and blocked with 5% goat-serum (SIGMA) for 1 h at room temperature (RT). The following primary antibodies were used: Oct3/4 (Abcam), SSEA3 (Abcam), Sox2 (Abcam), all 1:100. For endothelial differentiation was used anti-FVIII (Green Mountain) 1:150 and anti-vWF (SIGMA) 1:100. Secondary antibodies used were all from Invitrogen (all 1:500).
Direct AP activity was analyzed using an alkaline phosphatase blue membrane substrate solution kit (Sigma, AB0300) according to the manufacturer's guidelines.

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) were 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.

Endothelial differentiation. EBs were plated on gelatin 0,1% in water and left to adhere for 1 week in EB medium (Knockout DMEM, 10% fetal bovine serum,FBS, nonessential aminoacids, 2-mercaptoethanol, penicillin/streptomycin, Glutammine, LONZA). Then, 50 ng/ml of hVEFG (Immunotools) was added in HPGM medium (LONZA) for 20 days, adding fresh VEGF twice a week. Morphology and specific endothelial markers were analyzed over time.

RT-PCR analyses. Total RNA from IPS and EBs was isolated using TRIZOL and cDNA was synthesized using the RevertAidPremiun First Strand cDNA Synthesis KitFermentas. 2 ul of the reaction were used to analyze gene expression by PCR (F:forward; R:reverse). Primers for human BACT were BACT F 5'-gagaaaatctggcaccacacc-3'; R 5'-cgacgtagcacagcttctc-3', with 25cycles 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 OCT4, SOX2 and

KLF4 were OCT4 F 5'-cgtaagcagaagaggatc acc-3', R 5'-gcttcctccacccacttctgc-3';SOX2 F 5'-gcagctacagcatgatgcagg-3', R 5'-agctggtcatggagttgtactgc-3';KLF4 F 5'-ccagaggagccc aagcca a-3', R 5'-5'-cgcaggtgtgccttgagatg-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 hFVIII-BDD were: A2 F 5'-tgccacacctcagactttcg-3', A3 R 5'-gac ggcgtttcaagactg 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: FVIII F 5'-ggagagtaaagcaatatcaga tgc-3', R 5'-ggtgaattcgaaggtagc gac-3'; KDR F 5'-tgcaaggaccaaggagactatgt-3', R 5'-taggatgatgacaagaagtag cc-3'; CD31 F 5'-aggtcagcagcatcgtggtcaacat-3', R 5'-gtggggttgtctttgaataccgcag-3' with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 60°C (FVIII); 55°C (KDR); 65 °C (CD31) for 30 seconds, 72°C for 30 seconds and 72°C for 7 minutes. Primers for pluripotency [38] were: b3-tubulin F 5'-gaacagcacggccatccagg-3', R 5'-tgcgaggcccagggcccaag-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, 59°C (β3-tubulin); 52°C (Brachyury); 54 °C (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: BACT, 400bp; OCT4, 179bp; SOX2, 134bp; KLF4, 130bp; hBDDFVIII, 180 bp; FVIII, 400 bp; KDR, 457 bp; CD31, 450 bp; β3-tubulin, 242 bp; Brachyury, 357 bp; AFP, 255 bp.

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- conjugated for immuno-staining of liver sections.

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

Results

Generation and characterization of MNC-derived iPSC

Previously, we generated iPSC from fibroblast both with retroviral and LVs transduction. However, in hemophilic patients, to harvest fibroblasts from skin biopsies is a risky procedure for bleeding. It has been demonstrated that iPSC could be generated from blood cells ([34],[35],[36],[37],[38],[39]) so we utilized peripheral blood cells as an easy-to-access source of cells and reprogrammed mononuclear cells from donors and hemophilic patients.

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 colture to know the cell population present at the day of reprogramming. As shown in Fig.1A all cells were positive for CD45, a marker of hematopoietic lineage, and CD3 and CD11b. It is interesting to note that after 5 days, cells remained were mainly CD3+ and CD19+ (Fig.1B).For reprogramming, cells were transduced with a LV containing the three factors (OCT4, KLF4 and SOX2) at different MOI. These vector contained LoxP sites to remove after reprogramming the expression cassette with Cre-recombinase and decrease alterations in the genome. After two days cells were seeded onto a layer of irradiated human foreskin fibroblasts (HFFs) in α–MEM. After 4 days medium was changed with ES medium.

After about one month from transduction we picked cells from colonies displaying a typical human cell-like morphology (Fig.2A) and seeded them onto HFF in order to expand and characterize them.

These colonies stained positive for AP activity (Fig. 2B), proposed as the most reliable pluripotency marker in hES cells [54]. RT-PCR of iPS mRNA showed expression of reprogramming factors unlike MNC before reprogramming (Fig.2C). Human iPS cells expressed protein Oct4, Sox2 and the typical surface antigens of ES cells including SSEA3 (Fig. 2D).

To assess the in vitro differentiation capacity of iPSC, the cells were differentiated into EBs. To trigger EBs formation, colonies were mechanically detached and cultured in low-attached plates in HPGM medium. After 1 week RNA was isolated and RT-PCR of mesodermal (Brachyury), endodermal (AFP) and ectodermal (Nestin) markers was performed. As shown in Fig. 3A the three lineage markers were upregulated in the

differentiated EBs as compared to the undifferentiated iPSC. Moreover, EBs were differentiated in adipogenic and osteogenic tissues (Fig. 3 B-C).

Endothelial differentiation of iPS cells

It has been demonstrated that mouse mesenchymal cell can differentiate in endothelial cells using a particular differentiation medium used in our laboratory [55]. To induce endothelial differentiation of iPS, 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. Cells were collected and analysed for expression of endothelial markers at different time point during differentiation. Cells changed morphology and assumed cuboidal shape typical of endothelial cells (Fig. 4A). Analysis of gene expression showed an increase in endothelial markers such as CD31 and FVIII, instead KDR remained the same (Fig. 4B). The expression of FVIII was also analysed by immunofluorescence. iPS-derived EC expressed FVIII and vWF (Fig. 4C), the FVIII-carrier protein in the plasma, which is a protein typically expressed by endothelial cells and megakaryocytes [25].

As further demonstration of 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. 5A). Moreover, we analyzed transduction efficiency of differentiated cells by using an LV containing GFP under the control of an 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. 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 section. By confocal analysis we detected cells near blood vessel without a significant inflammatory response to transplanted cells (Fig. 5B).

Generation and characterization of MNC HA-derived iPSC

Once optimized the protocol with healthy cells, we isolated MNC from several haemophilic patients. At day 3 from isolation, we corrected $2x\,10^6$MNC with a LV expressing the hFVIII B-domain-deleted (hBDDFVIII) 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. 6A). After 2 days we reprogrammed corrected and non-corrected

cells and after about 5 weeks colonies appeared in corrected cells. Also iPSC from haemophilic patients were correctly reprogrammed, indeed stained positive for AP activity (Fig. 6B) and expressed reprogramming factors (Fig. 6C-D). Unfortunately, expression of FVIII decreased in IPSC overtime (Fig. 6E). 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 but expression of FVIII driven by PGK was absent (Fig. 6F). We used PGK forward and hFVIII 5' reverse primers to discern integration of LV-PGK.hBDDFVIII from the reprogramming LV.
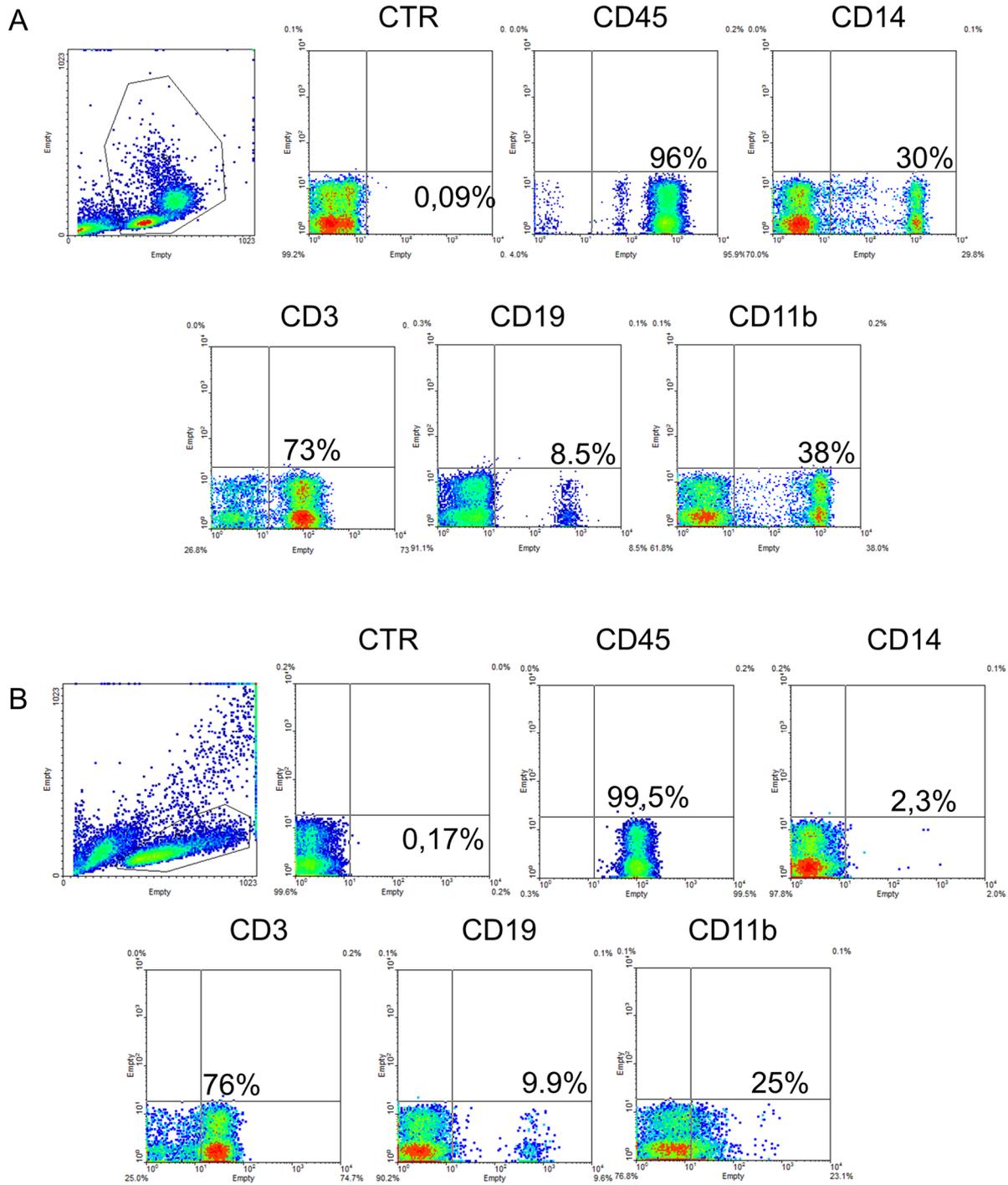
Figure 1. Characterization of MNC.
MNC were characterized for typical blood leucocytes markers after isolation (A) and on the day of reprogramming (B).
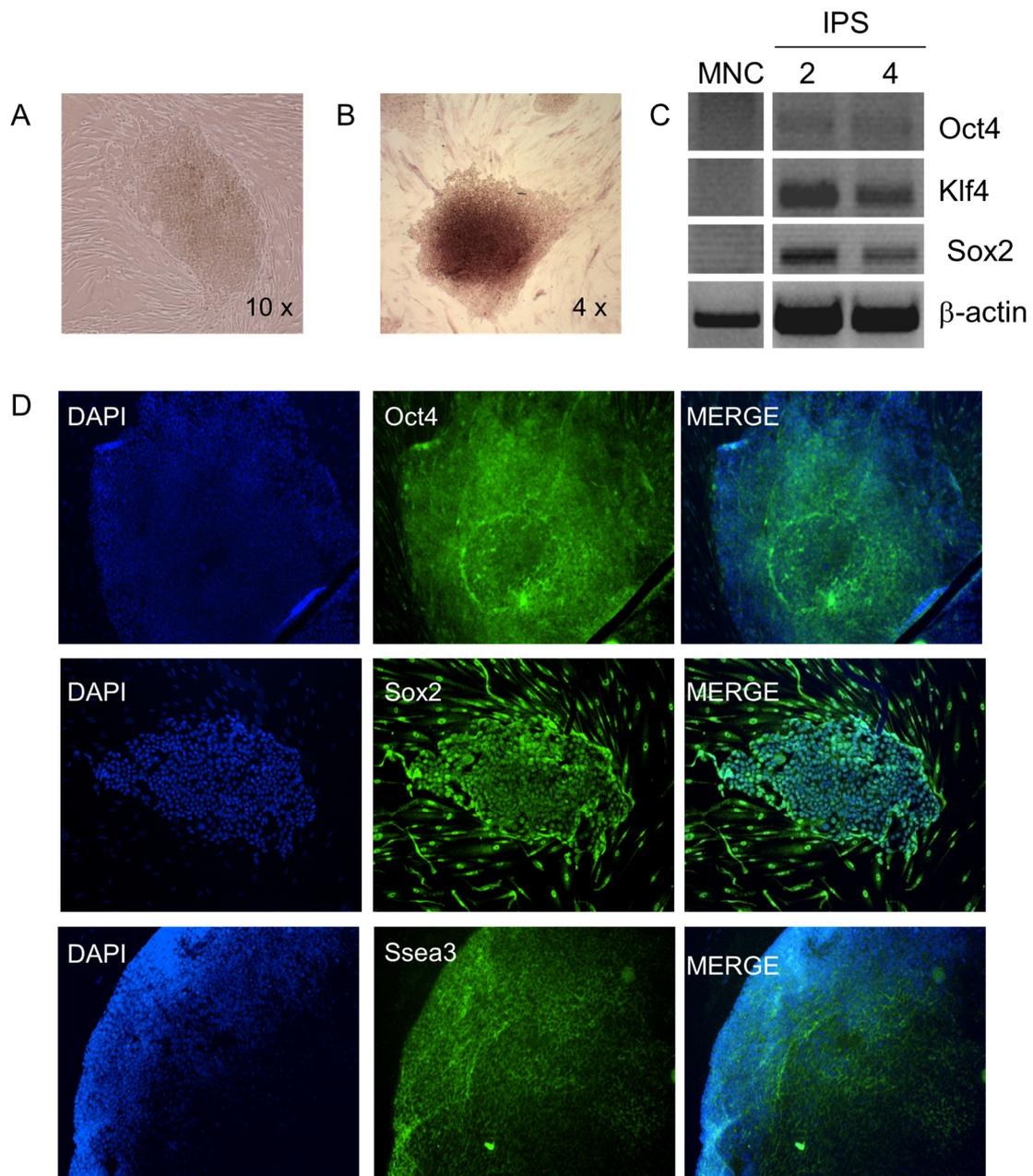
Figure 2. Generation and characterization of MNC derived-iPS cells.
A) Example of iPS colonies grown on HFF B) Example of AP+ iPS colony. C) RT-PCR showed expression of reprogramming factors only in iPS cells. D)Immunofluorescence of iPS colonies positive for the surface marker SSEA-3 and the nuclear markers Sox 2 and Oct4.
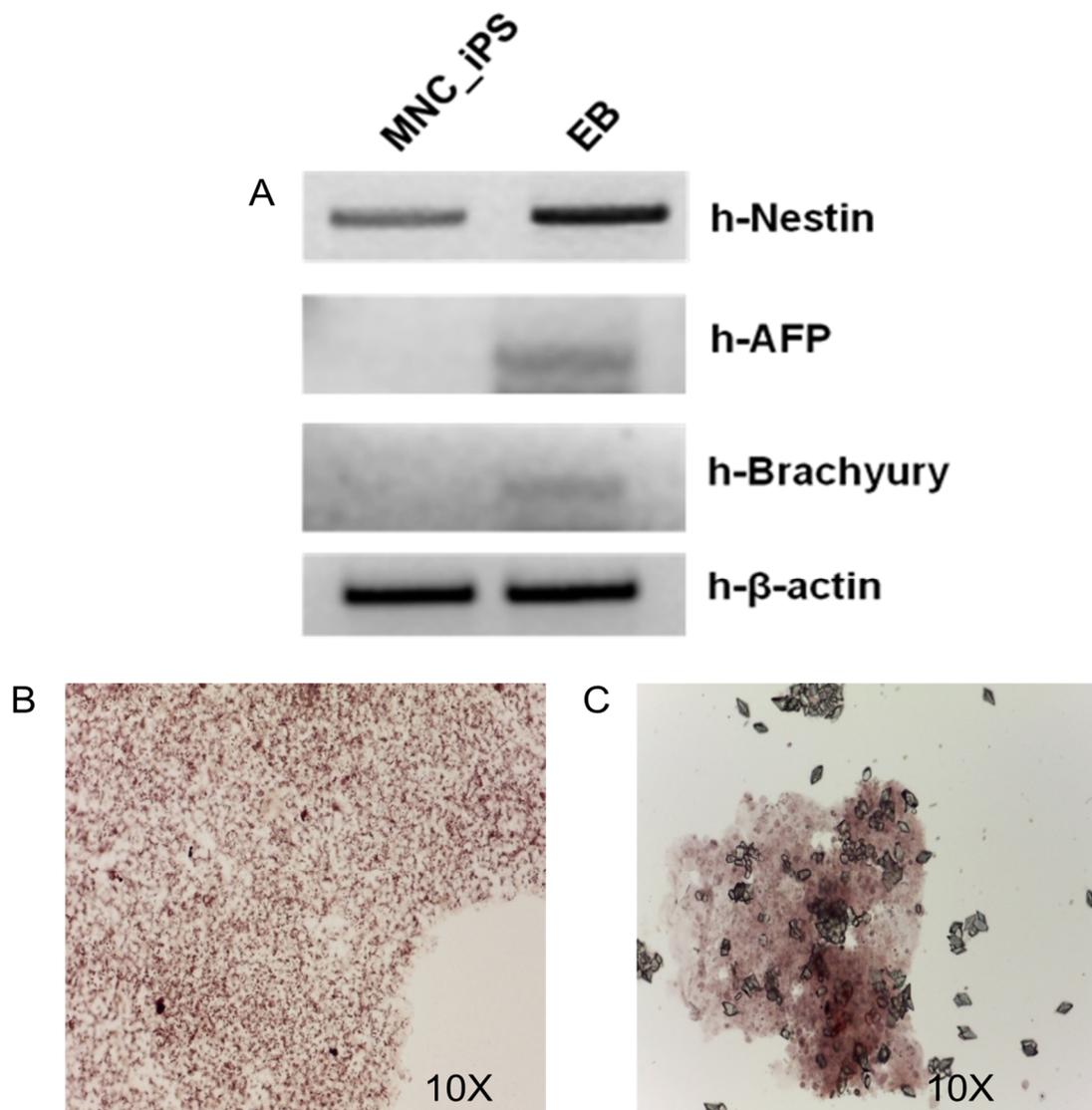
Figure 3. Pluripotency of MNC derived-iPS cells.
A) EBs express specific markers of three germ layers. iPS were able to differentiate in adipogenic and osteogenic tissue (B,C.)

Figure 4. Endothelial differentiation of MNC-derived iPSCs.
IPSCs were differentiated through EBs formation. EBs were plated on gelatin-coated dishes in EB medium with rhVEGF. During differentiation cells acquired a typical endothelial morphology (A) with increased expression of CD31 and FVIII (B). Cells showed costaining of FVIII with vWF, the carrier protein of FVIII in the plasma (C).

Figure 5. Differentiated cells transplantation.
EC-differentiation was demonstrated by transducing cells with LV-expressing GFP under the control of endothelial-specific promoters (Flk-1 and Tie-2, A). Flk-1-GFP+ cells were transplanted in NOD SCID HA mice and were detected by confocal microscopy of immuno-stained liver sections (B).
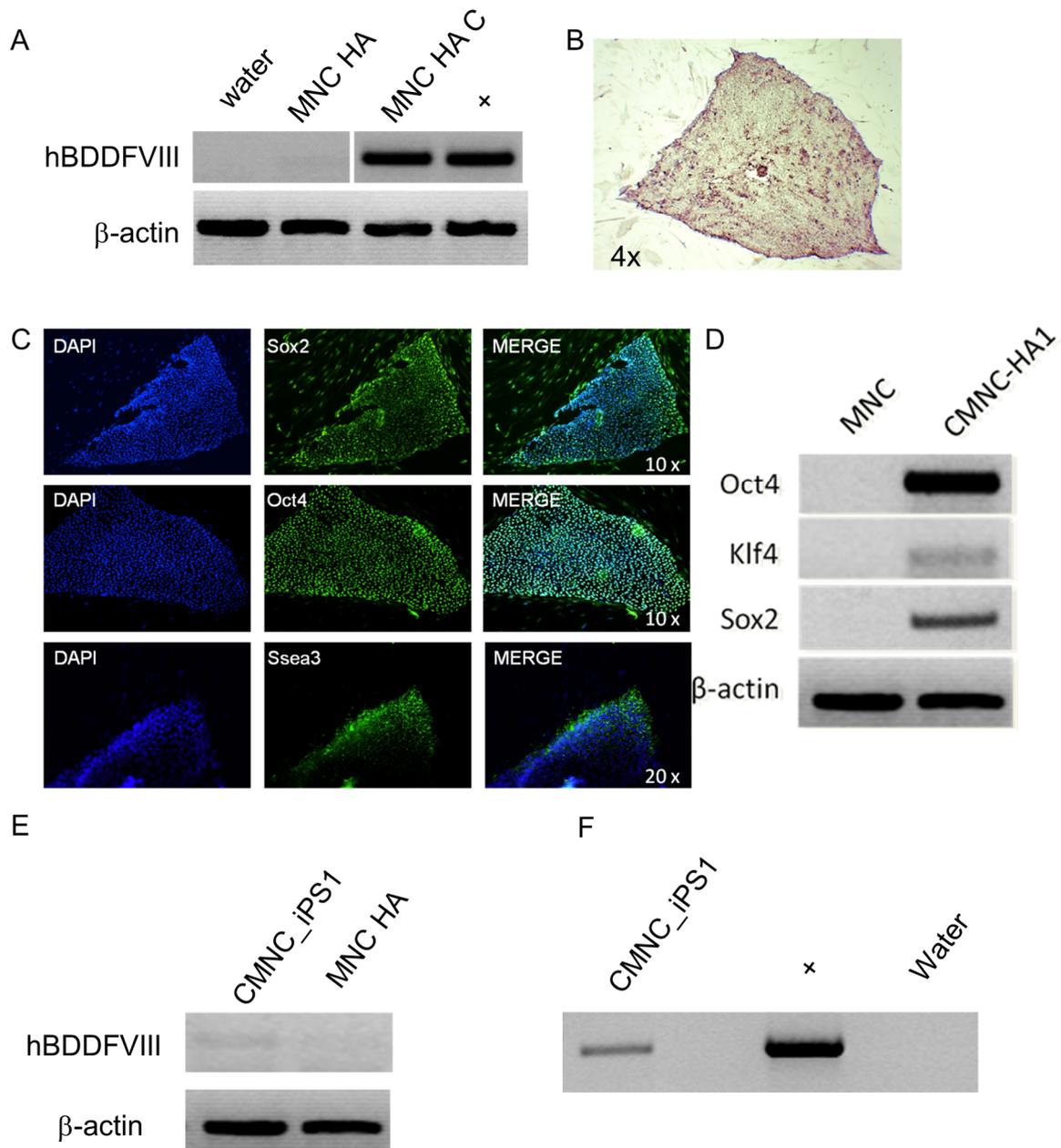
Figure 6. Characterization of MNC HA derived-iPS cells.
A) MNC from hemophilic patient were corrected with a LV-PGKhBDDFVIII on day 3. Only correctec cells (MNC HA C) expressed hFVIII. iPS from hemophilic were positive for AP (B) , express reprogramming factors at RNA (C) and protein (D) level and showed a decrease in FVIII expression (E). Integration of LV-PGK.hBDDFVIII in corrected iPS (F).

Discussion

Previously in our lab we generated iPSC from fibroblasts by retroviral vectors transduction. By this method we obtained iPS cells in a faster way, but these cells cannot be used in therapy because they are obtained with c-Myc, a well known oncogenic factor. 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 iPSC. However, fibroblasts were isolated from skin biopsies, a risky procedure for haemophilic patients. Therefore, we used peripheral blood cells as an easy cell source, despite the probability of reprogramming is less efficient than fibroblast [39].

In this study, we demonstrated that we can obtain iPSC from peripheral blood cells. MNC 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 colture were mainly CD3, CD19 and CD11b. We obtained colonies with ES cells-like morphology, positive for AP and stem cells markers, such as Oct4, Sox2 and SSEA3. In these cells there was also expression of 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, indeed they expressed markers of the three germ layer Nestin (ectoderm), AFP (endoderm) and Brachyury (mesoderm). The following step was to differentiate iPSC in endothelial cells. We decided to differentiate iPS cells to endothelial cells because recently it has been shown that FVIII is expressed by liver sinusoidal endothelial cells (LSEC) and intraportal injection of LSEC correct haemophilia A phenotype [14]. Moreover, endothelial cells are the only cells along with megakaryocytes that produce and store vWF. 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 [56]. So targeted expression of FVIII in cell that synthesize vWF will provide a pool of FVIII immediately stabilized by vWF. Finally, endothelial cells in liver sinusoids play roles in immunoregulation, which could potentially be harnessed to avoid deleterious immune responses against FVIII [57].We differentiated iPSC in endothelial cells using 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 markers, for example CD31, to obtain a pure population to further expand. 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 haemophilic patients. Before reprogramming, we corrected cells 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 haemophilic cells or if the double transduction for FVIII correction and reprogramming could have harmed the potential of haemophilic cells to be reprogrammed. Moreover, we are also thinking if the patient age of our cells and the absence of FVIII in cells can have a role in the loss of potential reprogramming capacity. Actually, iPSC obtained from healthy donors were from young people and far more colonies were generated. After characterization only one colony resulted reprogrammed from the haemophilic cells. Unfortunately, in this colony FVIII expression decreased in overtime, probably for 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 iPSC in vivo by teratoma's formation, promoter methylation and telomerase activity, to confirm the pluripotency of 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. This result confirm that there was no telomere shortening in iPS as reported for staminal cells.

Moreover, once differentiated, we will transplant iPS-derived EC in gamma-null haemophilia A mice not only to analyzed engraftment, but also proliferation and phenotypic correction for FVIII activity.

This study demonstrated that iPSC could be generate from MNC and could be differentiate in endothelial cells even if both the reprogramming and differentiation protocol need further optimization. Our results are important for future reprogramming approaches of

cells derived from haemophilic patients. Actually, this procedure is a safer and faster alternative to skin biopsies to obtain cells for reprogramming.

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I.   Seminari interni al Dipartimento di Scienze Mediche AA 2011-2012

New trends in allergy and immunology
Prof. Joseph A. BELLANTI
10/10/2012

The resolution of inflammation: players and targets
Prof. Mauro Perretti
07/11/11

Alpha-MSH and the melanocortin system in inflammation
Prof. Mauro Perretti
19/12/11

Galectins-carbohydrate binding protein: sweet or sour?
Prof. Mauro Perretti
20/12/2011

Hepatocellular Carcinoma, novel advances from genomics to treatment
Dr. Rohini SHARMA
13/01/2012

Next-generation DNA sequencing and target arrays in the clinics
Dr Paolo Fortina
25/01/12

Signalling pathways controlling integrin trafficking during invasion
Dr.ssa Elena Rainero
08/03/12

Molecularclassification of multiple myeloma
Prof.Antonino Neri
22/03/2012

Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs
Prof.ssa Isabel MERIDA
23/03/2012

Developing strategies for tissues specific targeting using peptides and antibodies isolated by in vivo phage display selection
Prof. Costantino PITZALIS
28/03/2012

A translational approach to the study of endometriosis
Dott.ssa Paola PANINA-BORDIGNON
10/05/2012

Microparticles as novel effectors in Inflammation
Prof. Mauro Perretti
15/05/2012

Resolvins and Omega-3 in Inflammation
Prof:MauroPerretti
16/05/2012

Association of Merkel cell polyomavirus with human tumors
Prof. Mauro Tognon
14/06/2012

High-throughput Biochemical Target Investigation Unveils a Novel Function of miR-21 as a Negative Modulator of Signal Transduction in T-lymphocytes
Prof. PinoMancino
15/06/12

SEDDA: Scale for Early Detection of Drug Abuse
Dott. Daniel LLORET
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Recent Advances in Hematopoietic Stem Cell Gene Therapy: from microRNA Regulation to Targeted Gene Transfer
Prof. Luigi Naldini
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Next generation sequencing in T-ALL
Dr. Kim De Keersmaecker
10/07/12

UHRF1 protein promotes epigenetic cross-talks and is involved in cancer progression
Dott. Ian Marc BONAPACE
12/07/2012

Molecular control of human fetal globin expression: towards a potential cure for β - thalassemia and sickle cell anemia
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BRAIN, BRAIN QUITE CONTRARY HOW DO YOU NEURONES DIE? PROGRAMMED CELL DEATH
Prof. Philip Beart
19/07/12

OXIDATIVE STRESS AND RECRUITMENT OF AUTOPHAGY TO BRAIN CELL DEATH
Prof. Philip Beart
20/07/12

II.   Attività formativa svolta

- Lezioni frontali (Prof. Ellis) interne al dipartimento

III.   Partecipazioni e comunicazioni a congressi

- 3rd IBEC SYMPOSIUM ON BIOENGINEERING AND NANOMEDICINE, Barcelona, 1-2 June 2010

- EUROPEAN SOCIETY OF GENE AND CELL THERAPY XVIIITH ANNUAL CONGRESS, Milano, 22-25 October 2010

- XVI CONVEGNO TELETHON, Riva del Garda, 7-9 Marzo 2011

  "Therapeutic roles of healthy donor human LSEC, bone marrow or cord blood-derived cells in Haemophilia A"

  Simone Merlin, Gabriella Ranaldo, Diego Zanolini, Maria Prat and Antonia Follenzi

- EUROPEAN SOCIETY OF GENE AND CELL THERAPY XIXTH ANNUAL CONGRESS, Brighton UK, 27-31 October 2011

- ABCD Meeting "Stem Cells, Development and Regenerative Medicine", Turin, 4-6 May 2012

  "A Novel iPSC-based Strategy to Correct the Bleeding Phenotype in Hemophilia A"

  Gabriella Ranaldo, MSc, Yvonne Richaud-Patin, MSc, Chantal Grosso, BSc, Maria Talmon, MSc, Stefania Cannizzo, MSc, Angelo Lombardo, PhD, Angel Raya, MD PhD, Luigi Naldini, MD PhD, PiercarlaSchinco, MD and Antonia Follenzi, MD PhD.

- XV Annual Meeting of the American Society of Gene and Cell Therapy, Philadelphia,Pennsylvania, 15-19 May 2012

  "A Novel iPSC-based Strategy to Correct the Bleeding Phenotype in Hemophilia A"

  Gabriella Ranaldo, MSc, Yvonne Richaud-Patin, MSc, Chantal Grosso, BSc, Maria Talmon, MSc, Stefania Cannizzo, MSc, Angelo Lombardo, PhD, Angel Raya, MD PhD, Luigi Naldini, MD PhD, PiercarlaSchinco, MD and Antonia Follenzi, MD PhD.

- 2nd Interrogations at the Biointerface Advanced Summer School", Barcellona, 25-29 giugno 2012

"A Novel iPSC-based Strategy to Correct the Bleeding Phenotype in Hemophilia A"

Gabriella Ranaldo, MSc, Yvonne Richaud-Patin, MSc, Angelo Lombardo, PhD, Chantal Grosso, BSc, Maria Talmon, MSc, Stefania E. Cannizzo, MSc, Angel Raya, MD PhD, Luigi Naldini, MD PhD[3], PiercarlaSchinco, MD and Antonia Follenzi, MD PhD

IV.   Pubblicazioni

"Transplantation of primary Kupffer cells but not bone marrow-derived macrophages replaces resident hepatic macrophages in the mouse liver"

Simone Merlin [1*], Antonia Follenzi[1,2*], Kuldeep K. Bhargava[3], Gabriella Ranaldo [1], Christopher J. Palestro [3], Maria Prat[1], Laura Santambrogio[2] and SanjeevGupta[2, 4]†

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