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TITOLO:

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

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INTRODUCTION

Hemophilia A and factor VIII

Hemophilia A is an X-linked bleeding disorder in which the functionally active coagulation factor VIII (FVIII) is partially or totally deficient. Factor VIII 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, pulmonary endothelial cells, lymphatic tissues and by the transplantation of a hemophilia A liver donor into a nonhemophilic recipient with alcoholic cirrhosis [1].

Tha 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, 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). Factor VIII has a half-life of about 12 h in adults (shorter in children) [2].

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 factor VIII 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. Hemophilia A is tipically divided into three classes, which are defined by factor plasma activity: severe, when the activity of the FVIII is less then 1%; moderate when the activity is between 1% and 5% and mild, when the activity is greater then 5% but less then normal [3].

However, there is a type of acquired hemophilia (AH), a rare autoimmune bleeding disorder, which arises as a result of the spontaneous production of autoantibodies against endogenous factor VIII. 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 hemophilia occurs in about one person per million, and can affect females [4].

There isn't a cure for hemophilia 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 hemophilia A. Unfortunately, current management is not optimal: there is the risk of transfusion-trasmitted infections (hepatitis A, B and HIV), development of inhibitory antibodies (30-50% of patients) and high cost for hemophiliacs.

Cell and/or gene therapy are alternative to supplemental therapy.

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 [5].

Actually, gene therapy for hemophilia A consists in the addition of normal factor VIII gene. [6,7]. Despite that it has been demonstrated a phenotypic correction of hemophilia 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 hemophilia 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 hemophilia A are required. Early reports showed that in dogs, only orthotropic liver transplantation (OLT) corrected the phenotype of hemophilia 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 hemophilia 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 hemophilic 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 hemophilia A mice [11, 14, 15]. Other attempts to correct hemophilia A phenotype with cells were transplantation of BM after gene transfer of FVIII in hematopoietic stem cells by integrating viral vectors [16, 17] and expression of FVIII in platelets [18]. 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 (iPS)

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 [19]. 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). 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 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), and NANOG), gene expression, promoter activities, telomerase activities, in vitro differentiation, and teratoma formation [20],[21].

Since then more studies have reported the derivation of human iPS cells from different cell types: keratinocytes [22], neural stem cells [23], B lymphocytes [24], adipose stem cells [25,26] peripheral and cord blood [27,28,29,30,31] and melanocytes [32].

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 [33]. Another approach is the reduction of integration sites by putting the reprogramming factors into a single vector with IRES or 2A selfcleavage peptide [34]. This reprogramming cassette was used with a lentivirus 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 [35]. A transposon system encoding a reprogramming cassette has also been used for iPS induction [36,37].

Several other methods accomplished iPS induction by the transient expression of reprogramming factors. These methods include adenovirus [38], DNA vectors (plasmid [39], 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.

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 [40].

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 disesase: functionally neurons which integrated in a rat model of parkinson's disease [41], hematopotietic progenitor for the correction of Fanconi and Sickle Cell Anemia [42,43], cardiomycites [44], hepatocytes [45].

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.

Aim of the work

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

The aim of this project is to find a new approach in the cure of hemophilia A with iPS-based strategy.

Firstly, we will transduce fibroblasts from normal and haemophilic patients with a retroviral vector expressing the human β-domain-deleted FVIII. Then we will generate FVIII correted-specific iPS cells and, after characterization of these cells for stem cell potential based on presence of pluripotent-specific markers, we will induce differentiation in endothelial cells with specific growth factors.

Finally, we will perform cell engraftment and proliferation studies of the transplanted iPS-derived FVIII-expressing-EC in the liver of NOD/SCID hemophilia A mice to assess phenotypic correction.

In this first year I spent 3 months in Barcelona at IBEC (Institute for Bioengineering of Catalunia). I learned how to obtain and characterize iPS cells from human fibroblasts (in collaboration with the laboratory of Prof. Angel Raya in Barcelona) and I began preliminary studies of endothelial differentiation of iPS generated cells.

At the same time, because it has been demonstrated that transplantation of bone marrow (BM) in mice can correct hemophilia A's phenotype, I analyzed the expression of mRNA of human FVIII (hFVIII) in several patient-derived bone marrow and peripheal blood samples (in collaboration with Prof. Gaidano, Hematology division, Novara Hospital).

Material and methods

Cell culture. Patient's fibroblasts, Phoenix (packaging cell line for retroviral production), Mesenchymal-like and ECV (human endothelial cell line) 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); MEFs (mouse embryonic fibroblast) were established from dissociated C57BL/6 mouse embryos (13.5 d gestation). Both HFF-1 and MEF were mitotically inactivated by gamma irradiation (55 Gy).

ES (embryonic stem) cells and iPS cells were cultured on top of irradiated mouse or human fibroblasts in ES cell Knockout DMEM supplemented with 20% knockout serum replacement, nonessential

amino acids, 2-mercaptoethanol, penicillin/streptomycin, GlutaMAX (all reagents from GIBCO), bFGF (Peprotech) and picked mechanically, or on Matrigel by trypsinization (using MEF-conditioned medium).

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, EGFP 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 d cells were trypsinized and seeded into 10-cm dishes containing 4 million irradiated mouse fibroblasts and ES cell medium.

Immunofluorescence and AP staining. Cell were grown on plastic cover slide chamber, fixed with 4% parafolmaldehyde. The following antibodies were used: Oct3/4 (SANTACRUZ), SSEA3 and SSEA4 (Iowa University), TRA-1-60 and TRA-1-81 (Chemicon), NANOG (Red system), Sox2 (Affinity Bioreagent). Secondary antibodies used were all from Jackson (all 1:200). Images were taking using LEICA confocal microscope.

Direct AP activity was analyzed using an alkaline phosphatase blue membrane substrate solution kit (Sigma, AB0300) according to the manufacturer's guidelines.

***In vitro* differentiation.** Embryoid bodies (EBs) formation was induced by mechanically pick of colonies and were cultured in low attachments plates in ES medium. After 3–4 d the embryoid bodies were transferred to 0.1% gelatin-coated glass chamber slides and cultured in differentiation medium (DMEM supplemented with 20% fetal bovine serum, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, nonessential amino acids and penicillin-streptomycin) for 2–3 weeks to allow spontaneous endoderm formation. The medium was changed every other day. For mesoderm/cardiomyocyte differentiation, iPS cells were maintained on gelatin-coated plate in differentiated medium supplemented with 500 µM ascorbic acid (Sigma).

Endothelial differentiation of iPS, hES, mesenchymal-like cells. Embryoid bodies formation was induced by mechanically picking of colonies and were cultured in low attachments plates in ES medium. After 3-4 days the embryoid bodies were transferred to 0.1% gelatin-coated six-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.

RT-PCR analyses. Total RNA was isolated using TRIZOL and 1ug was used to synthesize cDNA using the Invitrogen Super Script III kit. 2 ul of the reaction were used to analyze gene expression by PCR (F:forward; R:reverse).
Primers for human GAPDH were GAPDH F 5'-GCACCGTCAAGGCTGAGAAC-3'; R 5'-AGGGATCTCGCTCCTGGAA-3', with 30 cycles at 94° C for 10 minutes, 94°C for 30 seconds, 60°C for 30 seconds 72°C for 30 seconds and 72°C for 10 minutes. Primers for CD31, KDR and hFVIII were CD31 F 5'- AGGTCAGCAGCATCGTGGTCAACAT-3'; R 5'-GTGGGGTTGTCTTTGAATACCGCAG-3', KDR F 5'-TGCAGGACCAAGGAGACTATGT-3'; R 5'-TAGGATGATGACAAGAAGTAGCC-3', hFVIIILC2 F 5'-GGAGAGTAAAGCAATATCAGATGC-3'; R 5'-GGTGAATTCGAAGGTAGCGAG-3', with 30 cycles at 94° C for 5 minutes, 94°C for 30 seconds, 60°C for 30 seconds for CD31, 55 °C for 30 seconds for KDR and FVIII, 72°C for 30 seconds and 72°C for 10 minutes. Primers for vWF were vWF F 5'-

GTTCGTCCTGGAAGGATCGG-3'; R 5'-CACTGACACCTGAGTGAGAC-3' with 30 cycles at 94° C for 10 minutes, 94°C for 30 seconds, 60°C for 30 seconds 72°C for 1 minute and 72°C for 10 minutes. PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: GAPDH, 75 bp; CD31, 450 bp; KDR, 457 bp; FVIII, 400bp; vWF, 696 bp.

RNA isolation and quantitative PCR (qPCR) analyses. 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. 2ul of the reaction were used to analyze the FVIII expression levels by PCR (F:forward; R:reverse). Primers for hβ-Actin were F 5'-GAGAAAATCTGGCACCACACC-3'; R 5'-CGACGTAGCACAGCTTCTC-3', with 25 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute and 72°C for 10 minutes. Primers for FVIII were hFVIIILC2 F 5'-GGAGAGTAAAGCAATATCAGATGC-3'; R 5'-GGTGAATTCGAAGGTAGCGAG-3', with 30 cycles at 94° C for 3 minutes, 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute and 72°C for 10 minutes. PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: hβ-Actin, 400 bp and FVIII, 400bp.

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.

Results

Generation and characterization of fibroblast-derived cells

Fibroblast from human skin (Fig. 1a) were seeded (day 0) and infected on day 1 and 2 with a 1:1:1:1 (Oct3/4, Sox2, Klf4 and c-Myc) and a 1:1:1 (Oct3/4, Sox2 and Klf4) mixture of retroviruses. Control of transduction was done with the infection of Sox2, Klf4 and Oct4 and GFP (Fig. 1c). Cells were trypsinized on day 4 and seeded onto a layer of irradiated mouse embryonic fibroblasts (MEFs) in embryonic stem (ES) cell medium (Fig. 1b).

Within 7 days post infection we detected several cell colonies (Fig. 1d). After one month from transduction we picked four-factor-induced cells (Fig. 1e) displayed typical human (h) ES cell-like morphology (Fig. 1g) and seeded them onto fresh irradiated human foreskin fibroblast (HFF) in order to expand and characterize them (Fig. 1f).

These colonies stained positive for alkaline phosphatase (AP) activity (Fig. 2a), proposed as the most reliable pluripotency marker in hES cells with correct morphology.

Human iPS cells expressed typical surface antigens of ES cells including SSEA4 and SSEA3, TRA-1-60, TRA-1-81 and the protein Oct4, Sox2 and NANOG (Fig. 2 b, c, d).

The isolated clones were plated in single wells and considered cell lines. The cells were plated and cultured in ES medium (without bFGF) to trigger embryoid bodies (EB) formation. After 3-4 days embryoid bodies were plated on gelatine 0,1% and cultured with ascorbic acid to differentiate in mesoderm. Cells differentiates in contractile cells (Fig. 2e).

In Fig. 1h there is an example of a typical non-ES cell-like colony: the colony is granulated, cells aren't compact and are differentiated and there aren't defined borders.

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 [46]. To induce endothelial differentiation of iPS and ES cells, we first induced EB formation. After 3 days EBs were seeded on 0,1% gelatine coating plate in differentiation medium. Cells were collected and analyzed for gene expression 3 weeks after differentiation. Mesenchymal-like cells, obtain from differentiation of iPS, were used as a control.

During differentiation cells changed morphology and assumed cuboidal shape typical of endothelial cells (Fig. 3). 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

basal levels of FVIII. RT-PCR showed vWF expression in non differentiated cells and very low expression in differentiated cells (Fig. 4). As vWF is an important endothelial marker, we have to repeat this experiment to rule out a possible technical mistake in the PCR.

FVIII expression in human bone marrow and peripheral blood

It has been demonstrated that bone marrow transplantation in mice correct hemophilia A phenotype [15]. So, we investigated if human bone marrow express FVIII mRNA as well. In collaboration with the haematology division of Prof. Gaidano, we collected 21 bone marrows from “healthy” patients. We considered “healthy” a patient that received BM biopsy with non malignant diagnosis. After red blood cell lysis, mRNA was isolated with Isol-RNA Lysis Reagent (5 PRIME) and analyzed for FVIII expression by RT-PCR. All patients expressed human FVIII with exception for patients 2 and 4 because the mRNA quality was not good (Fig. 5). To verify mRNA integrity after isolation and retrotranscription β-Actin PCR was used as a control. Although we retrotrascribed 2 ug of RNA for all patients the quality of cDNA was not consistently of high quality. To normalize the expression of FVIII for some patients, we perfomed a qPCR. We used as a positive control the endothelial cell line ECV. The expression of FVIII was lower than in ECV, but it was present at different level in all patients (Fig. 5).

Then we analyzed the expression of human FVIII in peripheral blood of healthy donors and one haemophilic patient (in collaboration of Dott. Schinco, Torino) and we found FVIII expression in all samples (Fig.6).

So we analysed the presence of FVIII in a sub-population of blood cells; monocytes are positive for FVIII expression (monocytes were a gift from the laboratory of Pharmocology in our Department) (Fig. 6).

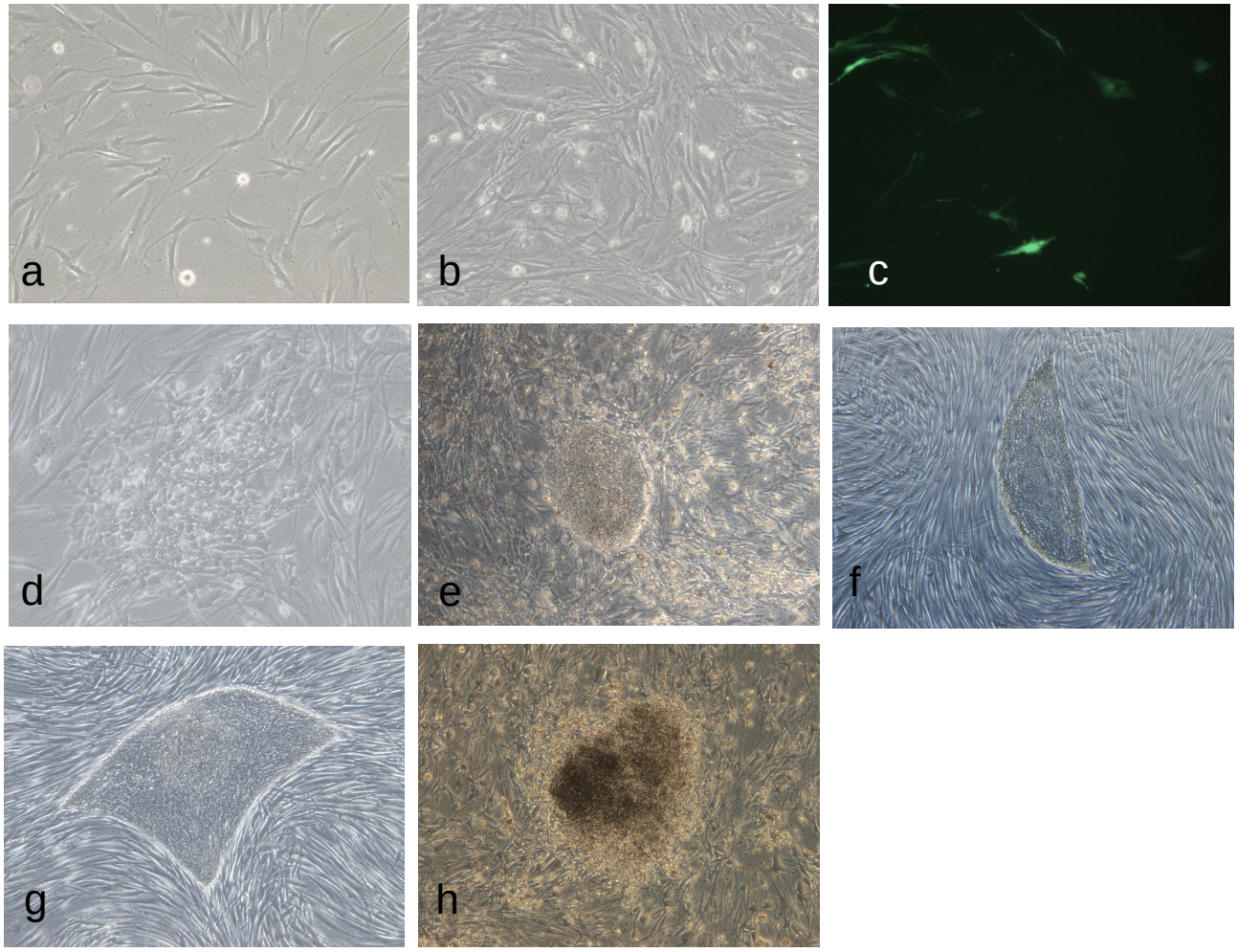

Figure 1. Generation fibroblast derived-iPS cells.
a) Morphology of fibroblasts before infection with retrovirus vector. b,c) Example of fibroblasts 4 days after infection with GFP retrovirus. d) Typical example of a small ES cell–like colony 7 d post-infection. e) Typical example of a small ES cell–like colony 30 d post-infection. f)Example of iPS colonies grown on HFF g). Example of ES colony grown on HFF. h) Typical image of non-ES cell-like colony.

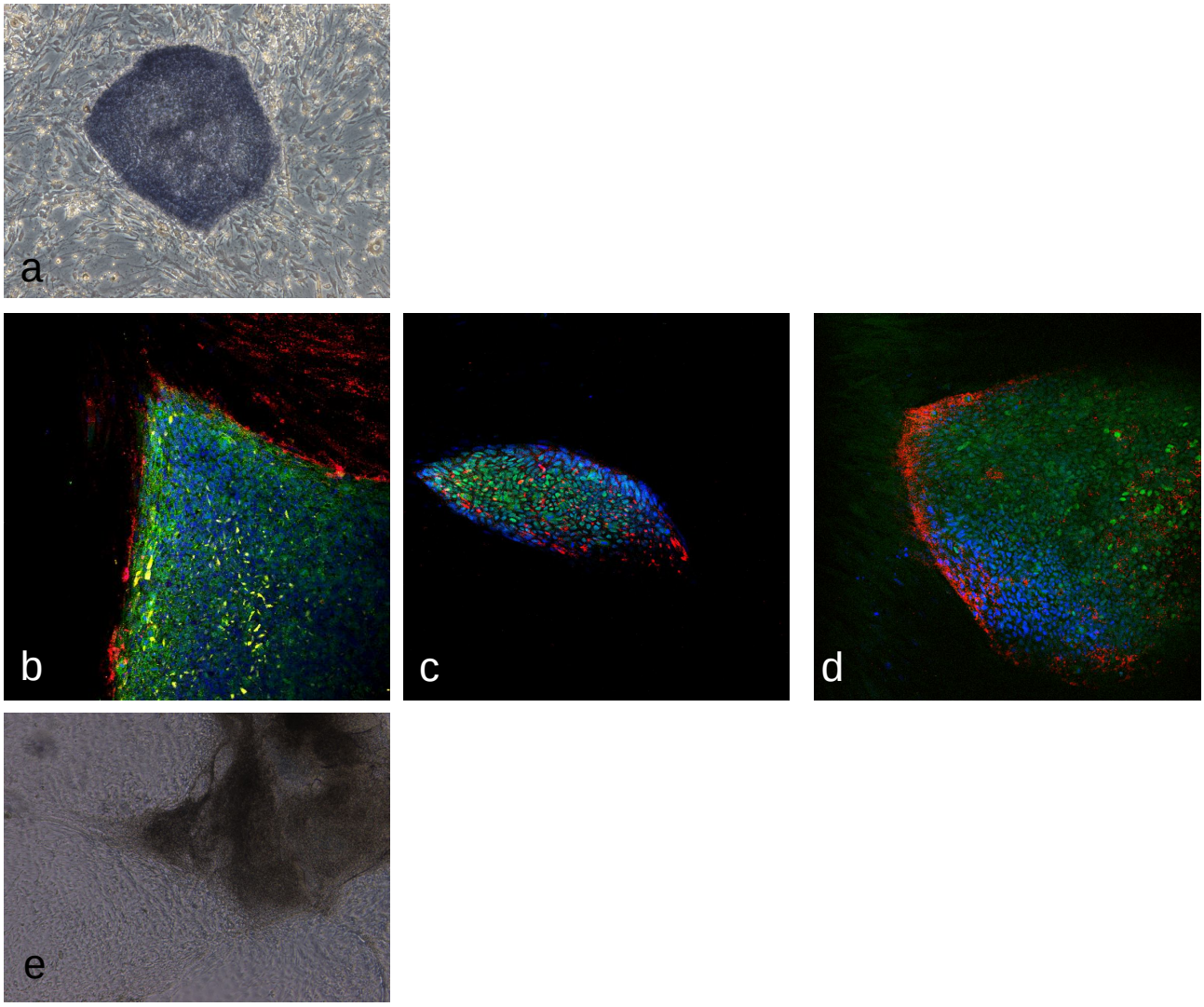

Figura 2. Characterization of iPS. a) Example of AP+ iPS colony. b)Rappresentative immunofluorescence of iPS colony positive for the surface marker SSEA4 (red) and TRA-1-60 (yellow) and the nuclear marker SOX 2 (green). c) Rappresentative immunofluorescence of iPS colony positive for NANOG (green) and TRA-1-81(red). d) Rappresentative immunofluorescence of iPS colony positive for Oct4 (green) and SSEA3 (red). DAPI for nuclei. e) *In vitro* differentiation of iPS cells in mesoderm.

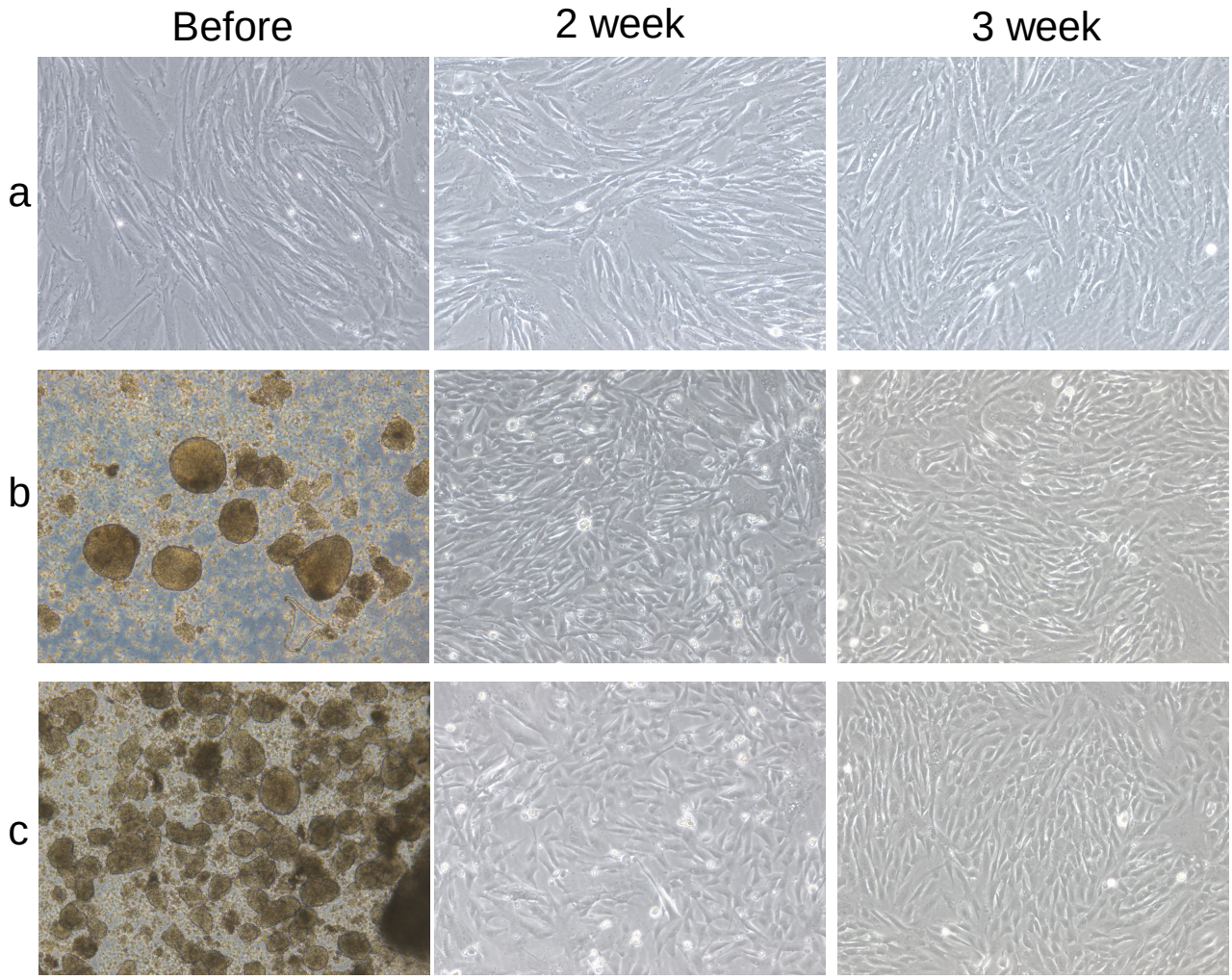

Figure 3. Endothelial differentiation of iPS cells.
Morphology change in mesenchymal-like cells (a), iPS (b) and ES cells (c) during 3 week endothelial differentiation. Differentiated cells assumed the characteristic “cobblestone” morphological features.

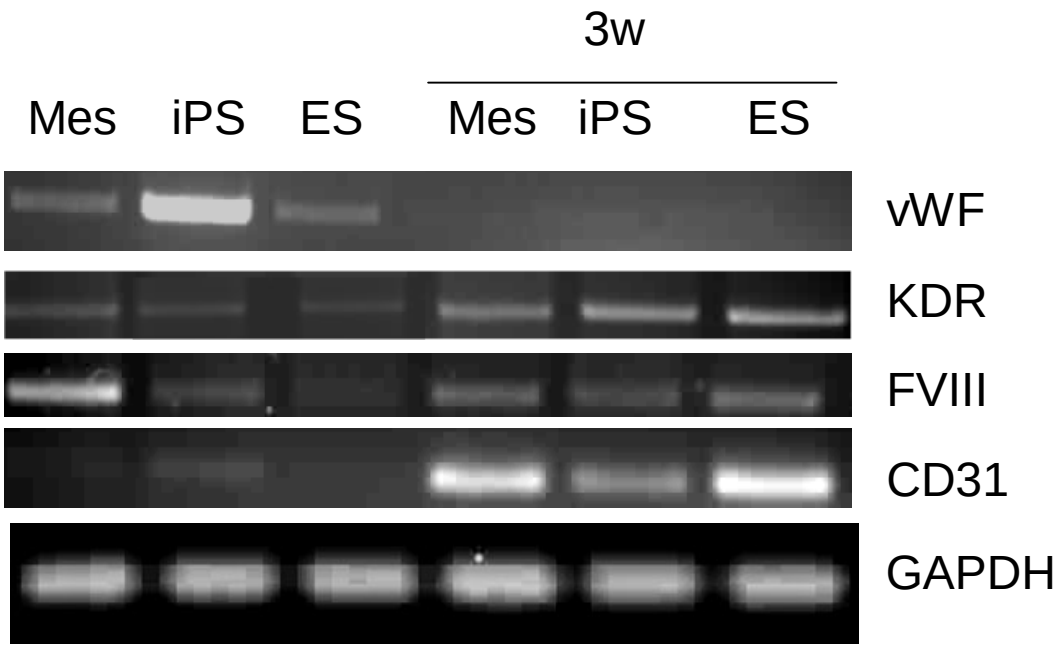

Figure 4. Endothelial gene expression. Endothelial gene expression at 3 weeks of differention in mesenchymal like (Mes), iPS and embryonic stem (ES) cells.

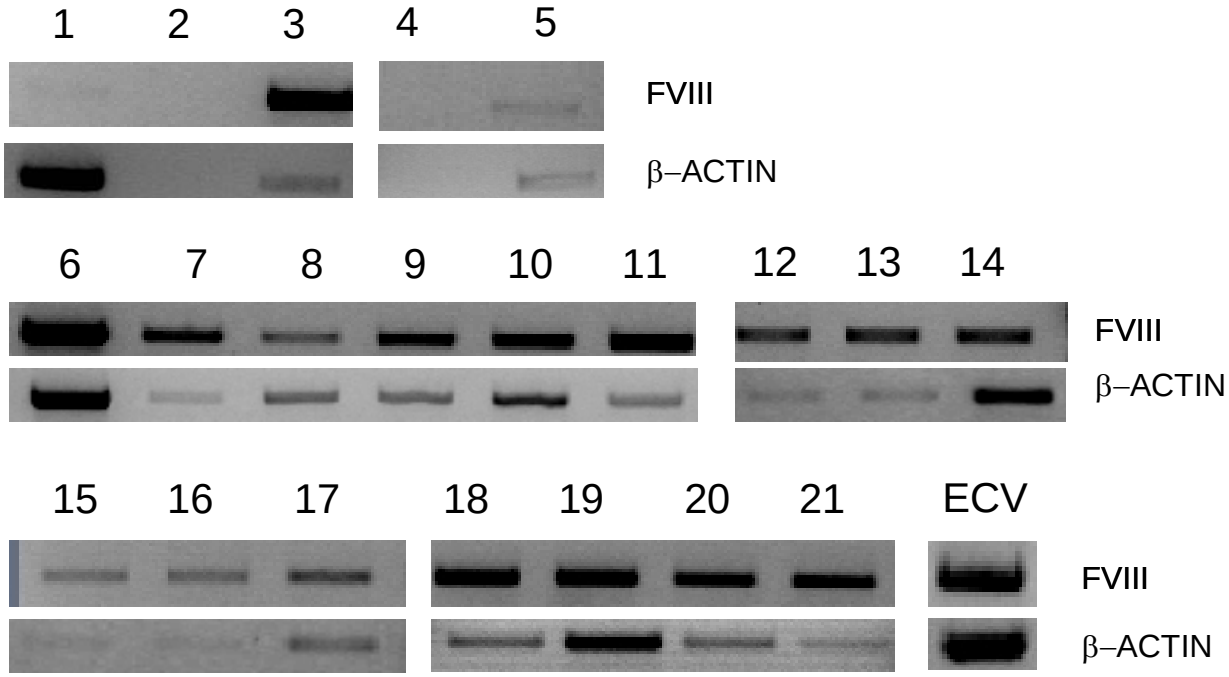

Figure 5. FVIII expression in human bone marrow. FVIII mRNA expression in human bone marrow with RT-PCR (above) and qPCR (below).

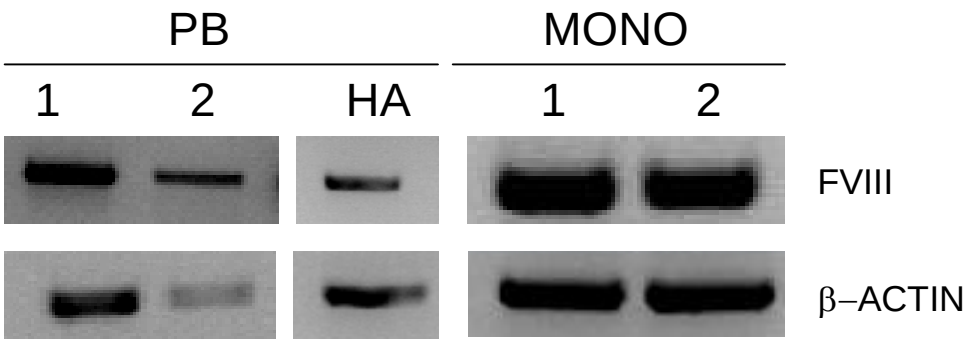

Figure 6. FVIII expression in human peripheal blood. FVIII mRNA is expressed both in healthy people and hemophilic peripheal blood (PB). In particular in monocytes (MONO).

Discussion

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 center 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, TRA-1-60 and TRA-1-81, SSEA 3 and 4. Then these cells should have maintained the capacity of differentiate in several cell types. In our results we tested iPS cells kept in culture with ascorbic acid. After 2 weeks these cells assumed a contractile phenotype resembling muscle cells. The control with GFP showed that the percentage of cell transduction was low and the reprogramming with 3 factors was slower than the 4-factors reprogramming program.

We decided to differentiate iPS cells in endothelial cells because ithas been demonstrated these cells can correct hemophilia A phenotype [13, 14]. Moreover, LSEC play roles in immunoregulation, which could potentially be harnessed to avoid deleterious immune responses against FVIII [47].

Preliminary data suggest that iPS cell can differentiate in endothelial cells if cultured in a differentiating medium containing IGF-1, ECGS, VEGF and bFGF. After 3 weeks, cells acquired a 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. We also performed a flow cytometry analysis for CD31 and an *in vitro* Matrigel assay to test the capacity of tubulogenesis, typical of endothelial cells (data not shown). FACS analysis showed absence of CD31 expression and cells did not assembled in networks of tubular structure. This is in contrast with the results of RT-PCR, so we have to repeat these experiments.

It has been demonstrated that transplantation of bone marrow can correct hemophilia A phenotype [15]. In people, BM transplant in hemophilia has been rarely reported. In one case, a hemophilic boy afflicted by aplastic anemia, was transplanted with the BM of his unaffected brother. Four months after this 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 [48]. This is the only report where BM transplant was done in hemophilia A patient.

Finally, we showed that human bone marrow expressed FVIII mRNA according with the idea that there is an extra-hepatic source of FVIII [1]. It is difficult to obtain bone marrow from healthy people and for this reason for us was easier to obtain BM from we patients with a initial diagnosis of no malignancy. In the analysed samples the oscillation in FVIII expression was dependent on the patient pathology and patient variability. In order to normalize the expression we performed a qPCR on a small group of samples and we used ECV cells as a positive control.

Then we demonstrated that also peripheral blood cells expressed FVIII mRNA and in particular monocytes. It is non surprising that we found FVIII mRNA in the peripheal blood of hemophilic patients. In fact the cause of hemophilia A is due to a partial or total deficiency of protein activity and in literature there is only one patients with severe hemophilia A in whom no mRNA of the F8 gene was detected [49].

Future Perspectives

In this initial studies we reproduced data of fibroblast reprogramming with retroviruses-mediated transduction of four factors (Oct3/4, Sox2, Klf4 and c-Myc). This method is faster then using only three transcription factors (Oct3/4, Sox2 and Klf4) and it is a good positive control. However, cells produced with this system are not safe due to the possibility of oncogenesis induced by the use of retroviruses and c-Myc. So in the future we will reprogram fibroblasts from hemophilic patients and normal people and we will use a strategy based on non integrative lentiviral vectors containing the three reprogramming genes (Oct4, Sox2 and Klf4) linked to the 2A peptides and flanked by loxP sequences in order to excise, by transient Cre expression, once reprogramming has been achieved.

For endothelial differentiation of iPS cells, we will repeat this experiment with the use of a positive control, like HUVEC. We will do a complete characterization of differentiation and

we will repeat FACS analysis for endothelial markers and tubulogenesis on matrigel. Besides, we will try different medium and method in order to improve endothelial differentiation like the use of other coating agents (collagene) and new cocktail of growth factors.

Finally we will analyze the expression of FVIII with an absolute qPCR in order to know how much factor is expressed in the bone marrow and in other blood cell types. To verify the secretion of FVIII protein, we will analyze the production of FVIII in all cell lines and sample supernatants will be tested in ELISA.

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I. ***SEMINARI INTERNI AL DIPARTIMENTO DI SCIENZE MEDICHE (A. A 2009/10)***

LSD1, Lysine Specific Demethylase1: insight into a new epigenetic regulator involved in neuronal gene regulation
DR. Elena Battaglioli
20/01/10

Cellule mesenchimali stromali fetali ed adulte: potenziali applicazioni in patologie ischemiche
Prof. Giulio Alessandri
21/01/10

Dal difetto genetico all'infiammazione
Dott.Luigi Maiuri
28/01/10

R & D of magnetic-nanoparticles and carriers for drugs
Dott.Giovanni Baldi
09/02/10

A novel antiviral mechanism mediated by the chemokine receptor CCR6
Prof. Alfredo GARZINO-DEMO
11/02/10

Computational analysis of protein-protein interactions
Prof. Stefano Pieraccini
17/02/10

Basi genetiche del diabete di tipo 1 e della sclerosi multipla: l'esempio della popolazione sarda.
Prof. Francesco Cucca
03/03/10

Role of membrane lipids in neuronal synapses organization
Dott. Paola Camoletto
05/07/10

Tissue Transglutaminase, a polyhedric enzyme: friend or foe?
Dott. Dario Fortunati
06/07/10

Ruolo delle micro vescicole nella comunicazione tra cellule
Dott. Giovanni Camussi
06/09/10

Le cellule staminali tumorali: dalla biologia alla clinica
Dott. Ruggero De Maria

22/09/10

New aspects of Cathepsin E functions in host defense mechanisms
Prof. Kenji Yamamoto
22/09/10

Preventing Persisters:Targeting Epigenetic Changes in Cancer Chemotherapy
Dr. Steven R. Ellis
23/09/10

Low-dose Bafilomycin is cytoprotective against Lysosome dysfunction, Implication of an Autophagy-associated therapeutic target for Neurodegenerative disease
Dott. John Shacka
24/09/10

A self-antigen peptidome carried by the human lymph
Prof. Laura Santambrogio
24/09/10

II. Journal club interni al Dipartimento di Scienze Mediche

III. Attività formativa

12 Aprile 2010 – 30 giugno 2010: Stage presso il laboratorio del Prof. Angel Raya per imparare la riprogrammazione di cellule somatiche in cellule pluripotenti (induced pluripotent stem cell, iPS) , Institute for Bioengineering of Catalunia, Barcelona Science Park (Baldiri Reixac 15-21 08028 Barcelona, Spain).

IV. Partecipazione 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