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# Cell therapy approaches to cure of haemophilia 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. 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 haemophilia 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 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 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. Haemophilia 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 haemophilia (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 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-trasmitted 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 factor VIII 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 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 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 von Willebrand factor (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 (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 [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), 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] and melanocytes [39].

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 [40]. Another approach is the reduction of integration sites by putting the reprogramming factors into a single vector with IRES or 2A selfcleavage peptide [41]. 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 [42]. A transposon system encoding a reprogramming cassette has also been used for iPS induction [43],[44].

Several other methods accomplished iPS induction by the transient expression of reprogramming factors. These methods include adenovirus [45], DNA vectors (plasmid [46], 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 [47].

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 [48], hematopotietic progenitor for the correction of Fanconi and Sickle Cell Anemia [49],[50], cardiomycites [51], hepatocytes [52].

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 new approaches in the cure of haemophilia A by cell therapy.

1) The first aim is to use a iPS-based strategy.
    a) We will transduce fibroblasts from normal and haemophilic patients with a retroviral vector expressing the human β-domain-deleted FVIII.
    b) We will generate FVIII corrected-specific iPS cells and after we will characterize these cells 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.
    d) We will perform cell engraftment and proliferation studies of the transplanted iPS-derived FVIII-expressing-EC in the liver of NOD/SCID haemophilia A mice to assess phenotypic correction.
2) The second aim is to find addition extra hepatic sources able to express and synthesized FVIII. In particular we concentrated our efforts on the myeloid lineage.

## Material and methods

**Cell culture.** Patient's fibroblasts, Phoenix (packaging cell line for retroviral production), 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 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).

Embryoid bodies (EBs) formation was induced by mechanically pick of colonies and were cultured in low attachments plates in ES medium. After 5 d the embryoid bodies were transferred to 0.1% gelatin-coated glass chamber slides and cultured in EB medium with 500 uM (SIGMA).

**Preparation of megakaryocytes (MK),bone marrow-derived macrophages (BM-DM) and peripheral blood mononuclear cells.** Femuri and tibiae of 8-9 weeks-old wt or haemophilia 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 Span 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 Span 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 p6 wells for 7 days with 5 ng/ml MCSF.

Red blood cells of peripheal blood from wt mice were lysed as above described. Total PBMC were plated 5-6x10^6 cells in p6 wells. The day after monocytes were attached.

**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.** For IPS, cells were grown on plastic cover slide chamber, fixed with 4% parafolmaldehyde. The following antibodies were used: Oct3/4 (Abcam), SSEA3 (Abcam), NANOG (Abcam), Sox2 (Abcam), all 1:100. Direct AP activity was analyzed using an alkaline phosphatase blue membrane substrate solution kit (Sigma, AB0300) according to the manufacturer's guidelines. MK were cytospined at 1000 rpm for 5 minutes, fixed with 4% parafolmaldehyde. The following antibodies were used: FVIII (Abcam) 1:100 and vWF (SIGMA) 1:100.
BM-DM and monocytes were fixed with 4% parafolmaldehyde. The following antibodies were used: anti-hFVIII (produced in our laboratory) 1:150, F4/80 (AB direct) 1:300, CD14 (EBioscience) 1:150 and CD115 (EBioscience) 1:150. Secondary antibodies used were all from Invitrogen (all 1:500).

**RT-PCR analyses.** Total RNA from IPS and fibroblast was isolated using TRIZOL and cDNA was synthesized using 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 human BACT were BACT F 5'- gag aaa atc tgg cac cac acc-3'; R 5'- cga cgt agc aca gct tct c-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 OCT4, SOX2 and cMYC were OCT4 F 5'-cgt aag cag aag agg atc acc-3'; R 5'-gct tcc tcc acc cac ttc tgc-3', SOX2 F 5'-gca gct aca gca tga tgc agg-3'; R 5'-agc tgg tca tgg agt tgt act gc-3', cMYC F 5'-cat cca gga ctg tat gtg gag-3'; R 5'-gcg agc tgc tgt cgt tga g-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. PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: BACT, 400bp; OCT4, 179 bp; SOX2, 134 bp; cMYC, 226 bp.
Total RNA from MK and bone marrow cells was isolated using TRIZOL and cDNA was synthesized using 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 BACT were BACT F 5'-gat gac cca gat cat gtt tga ga-3'; R 5'-gtc tcc gga gtc cat cac aat-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 aIIb, vWF and FVIII were aIIb F 5'-CAG GGC CAA GTG CTG ATA TT-3'; R 5'-TTG AAG CAG CTG ACT GGT GT-3', vWF F 5'-tgt tca tca aat ggt ggg cag c-3'; R 5'-aca gac gcc atc tcc aga ttc a-3' and FVIII F 5'-ggt atc aaa gtg aca atg tac c-3'; R 5'-cca att aat ccc gag tgc ata tc-3', with 30 cycles at 94° C for 3 minutes, 94° C for 30'', 54°C for 30'' for aIIb, 62° for 45'' for vWF and 54°C for 30" for mFVIII. PCR products were resolved in 2% agarose gels. Expected product sizes were as follows: BACT, 550bp; aIIb, 300 bp; vWF, 270 bp; FVIII, 400 bp. In all experiments with this antibody we used pre-immune serum from rabbit as control (data not shown) and there was no reaction.

**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).

# Results

**FVIII expression in mice bone marrow and peripheral blood cells**

Since it has been demonstrated that bone marrow transplantation in mice corrected haemophilia A phenotype [15], we investigated which population of hematopoietic cells expressed FVIII. Bone marrow from wilde type mice of 8-9 weeks was cultured for 7 days with MCSF for differentiation in macrophages. cDNA was prepared from total bone marrow and bone marrow-derived macrophages (BM-DM, Fig.1D) RNA and RT-PCR was used to analyze expression of FVIII. Figure 1A shows that FVIII transcripts were detected in total BM and the expression increase in BM-DM. Since the presence of RNA does not mean presence of protein, we analyzed FVIII protein in BM-DM by immunostaining. We used anti-F4/80, a specific marker of mouse macrophages, and anti-FVIII, produced in our laboratory. As showed in Figure 2, cell positive for F4/80 were also positive for FVIII.

To analyze the presence of FVIII in megakaryocytes (MK), c-Kit+ progenitor cells were isolated from total BM with magnetic separation and cultured for 4 days with TPO, IL-3 and IL-6. MK were identified by their large size (Fig.1B) and expression of vWF and $\alpha$IIb integrin (Fig.1C), specific marker of differentiation of these cells. As shown in Figure 1A MK and c-Kit+ expressed FVIII, instead c-Kit- cells had low level of FVIII mRNA.

Then to investigate the expression of protein, MK were cytospined onto cloverglass and they were stained for vWF and FVIII for immunofluorescence microscopy analysis. FVIII was expressed in MK and co-localized with vWF (Fig.3A). FVIII was also detected with a western blot analysis on MK lysates (Fig.3B). In this experiment 10 and 40 ng of purified recombinant protein (KOGENATE, Bayer) was used as positive control.

Since there are no evidence of FVIII expression in peripheral blood cells, we isolated monocytes and lymphocytes from blood wt mice. RT-PCR analysis showed that FVIII RNA was expressed both in monocytes and lymphocytes (Fig.4B). In particular monocytes expressed FVIII protein as shown in Fig.5, CD14 and CD115 were used as specific monocytes markers.

Then we would analyzed FVIII expression in MK,BM-DM, monocytes and lymphocytes in heamophilic mice. As expected FVIII mRNA (Fig.4A,B) and protein (Fig.6) were not expressed.

**Generation and characterization of fibroblast-derived cells**

Fibroblast from human skin (Fig. 7A) were seeded (day 0) and infected on day 1 and 2 with a 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.7B). Cells were treated with trypsin/EDTA solution on day 4 and seeded onto a layer of irradiated human foreskin fibroblasts (HFFs) in embryonic stem (ES) cell medium.

Within 9 days post transduction we detected several cell colonies (Fig. 8A). After about one month from transduction we picked four-factor-induced cells (Fig. 8A) displayed typical human (h) ES cell-like morphology and seeded them onto HFF in order to expand and characterize them. In Figure 8B was showed a typical ES cell-like colony of cells transducted with GFP.

These colonies stained positive for alkaline phosphatase (AP) activity (Fig. 9A), proposed as the most reliable pluripotency marker in hES cells with correct morphology. RT-PCR showed that iPS expressed RNA of reprogramming factor unlike fibroblasts before reprogramming (Fig.9B). Human iPS cells expressed protein Oct4, Sox2 and NANOG and typical surface antigens of ES cells including SSEA3 (Fig. 9C).

To trigger embryo bodies formation, colonies were mechanically detached and cultured in low-attached plates with ES medium without bFGF (Fig.10A). After 5 days embryo bodies were plated on gelatine 0,1% with ascorbic acid for 1 week to differentiation in mesoderm (Fig.10B).

## Discussion

It has been demonstrated that transplantation of bone marrow $Lin^-$ cells can correct haemophilia A phenotype [15]. In several studies hematopoietic cells were successfully used as target for haemophilia A gene therapy. We focused our attention on myeloid lineage and specifically on megakaryocytes, from which originate platelets, and bone marrow-derived macrophages. Megakaryocytes together with endothelial cells synthesized von Willebrand factor (vWF), that plays an essential role in haemostasis. It also serves as the carrier protein of FVIII, a large and unstable protein that is naturally confined to the circulation. 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 inhibition by antibodies and limiting exposure of exogenous FVIII to immune response [25]. 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 [19]. This strategy can provide improvement of haemostasis also in haemophilic mice with pre-existing immunity [23]. Notwithstanding these data, there are no evidence showing FVIII synthesis in these cells. In our study, we showed that megakaryocytes and other population from bone marrow and peripheral blood expressed FVIII RNA and synthesized protein according with the idea that there are extra hepatic sources of FVIII [1]. All these data were confirmed in our laboratory in human cells. Previously, in our laboratory we demonstrated that also human bone marrow and peripheral blood cells express FVIII mRNA, but when we analyzed blood from haemophilic patient we found FVIII expression. This is due to the fact that haemophilia A is caused by a partial or total deficiency of protein activity and in literature there is only one case of patient with severe haemophilia A with no detection of F8 mRNA [53]. In our experiments investigation of differences between wt and haemophilic mouse is easier than in human. Our transgenic mice have a insertion cassette between exon 16 and 17 [54] so they don't express FVIII RNA.

In the future we will analyzed expression and activity of FVIII protein both in megakaryocytes and monocytes from haemophilic mice and it will be interesting to transplant megakaryocytes to investigate the ability of these cells to correct haemophilia A phenotype.

Finally with these experiments we validate a rabbit anti-FVIII polyclonal antibodies produced in our laboratory by Prof Maria Prat. Polyclonal antibodies were obtained immunizing rabbit with human purified recombinant protein (KOGENATE,Bayer, kindly provided by Dott.ssa Pier Carla Schinco Ospedale Molinette, Torino).

In this study, we demonstrated that we can reproduce data obtained in Raya's laboratory. We reprogrammed human fibroblasts by retroviral transduction with four transcription factors Oct3/4, Sox2, Klf4 and c-Myc. We obtained colonies with ES cells-like morphology, positive for alkaline phosphatase and stem cells markers, such as Nanog, Oct4, Sox2 and SSEA3. In these cells there was also expression of reprogramming factors that we didn't find in fibroblasts demonstrating a reactivation of these genes. Our cells were capable of forming embryo bodies and able to differentiate with ascorbic acid. The next passage is to differentiate these cells in endothelial cells and characterized them. With this method we can obtain iPS cells in a faster way, but these cells cannot be used in therapy because they are obtained with c-Myc, a knowed oncogenic factor, and with retroviruses. For this reason we are reprogramming both human and mouse fibroblasts with lentiviral vectors (kindly provided by Dott. Angelo Lombardo, TIGET, Milano) containing Oct4,Klf4 and Sox2 without c-Myc. We transducted cells with concentrated lentiviral vectors at MOI 3, 5 and 10. After two months we detected colonies in cells transducted with MOI 5. Once we will obtain satisfying results from healthy cells, we will reprogram cells from haemophilic patients and mice to differentiate them in endothelial cells. We decided to differentiate iPS cells to endothelial cells because recently it was 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/enancher normalized plasma FVIII and re-established a releasable pool of FVIII [55]. 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 [56].

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## I. Seminari interni al Dipartimento di Scienze Mediche AA 2010-2011

*Biomineralization and preparation of biomimetic nanoapatites*
Dr. J.G. Morales
11/11/10

*Le cellule NK: dalla biologia alla terapia di leucemie ad alto rischio*
Prof. L. Moretta
29/11/10

*TARGETING LACTATE-FUELED RESPIRATION IN CANCER: A NEW THERAPEUTIC OPPORTUNITY?*
Prof. Pierre Sonveaux

*Fluorescent mesoporus nanoparticles as tracer of the endocytic and exocytic pathways*
Dott.ssa Maneerat Ekkapongpisit
01/03/2011

*Linfomi cutanei primitivi*
Prof. E. Berti
02/03/11

*Ferritins: ancient proteins with novel unexpected features*
Dr.ssa S. Levi
12/04/11

*Metabolic syndrome: the gastroenterologist point of view*
Prof.ssa E. Bugianesi
13/04/11

*Liver fibrosis in children affected by NASH: how to investigate that*
Prof. V. Nobili
29/04/11

*Reverse vaccination in autoimmune diseases*
Prof. G. Filaci
03/05/11

*Innate immunity and the pathogenicity of inhaled microbial particles*
Prof. H. Wolff
09/05/11

*Farmacologia dell'aterosclerosi*
Prof. A. Corsini
13/05/11
*Ion torrent technology for massive parallel sequencing*
Dott. A. Di Nicola
23/05/11

*Iron management in the hepcidin Era*
Dr. S.R. Ellis
25/05/11

*Molecular pathogenesis and biomarkers of Parkinson's disease*
Prof. M. Fasano
17/06/11

*Importance of pathobiology in rheumatoid arthritis*
Prof. Pitzalis
24/06/11

*Genetics and clinical phenotypes of Frontotemporal lobar degeneration*
Dr. J. Rohrer
24/06/11

*Role of PET imaging in early drug development*
Dr.ssa R. Sharma
29/06/11

*Management of high risk chronic lymphocytic leukemia*
Prof. T. Zenz
30/06/11

*Hypoxia, angiogenesis and liver fibrogenesis*
Prof. M. Parola
01/07/11

**II. Attività formativa svolta**

- Lezioni frontali (Prof. Albano, Dott.ssa Corrado, Dr. Comi) interne al dipartimento
- Journal Club interni 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

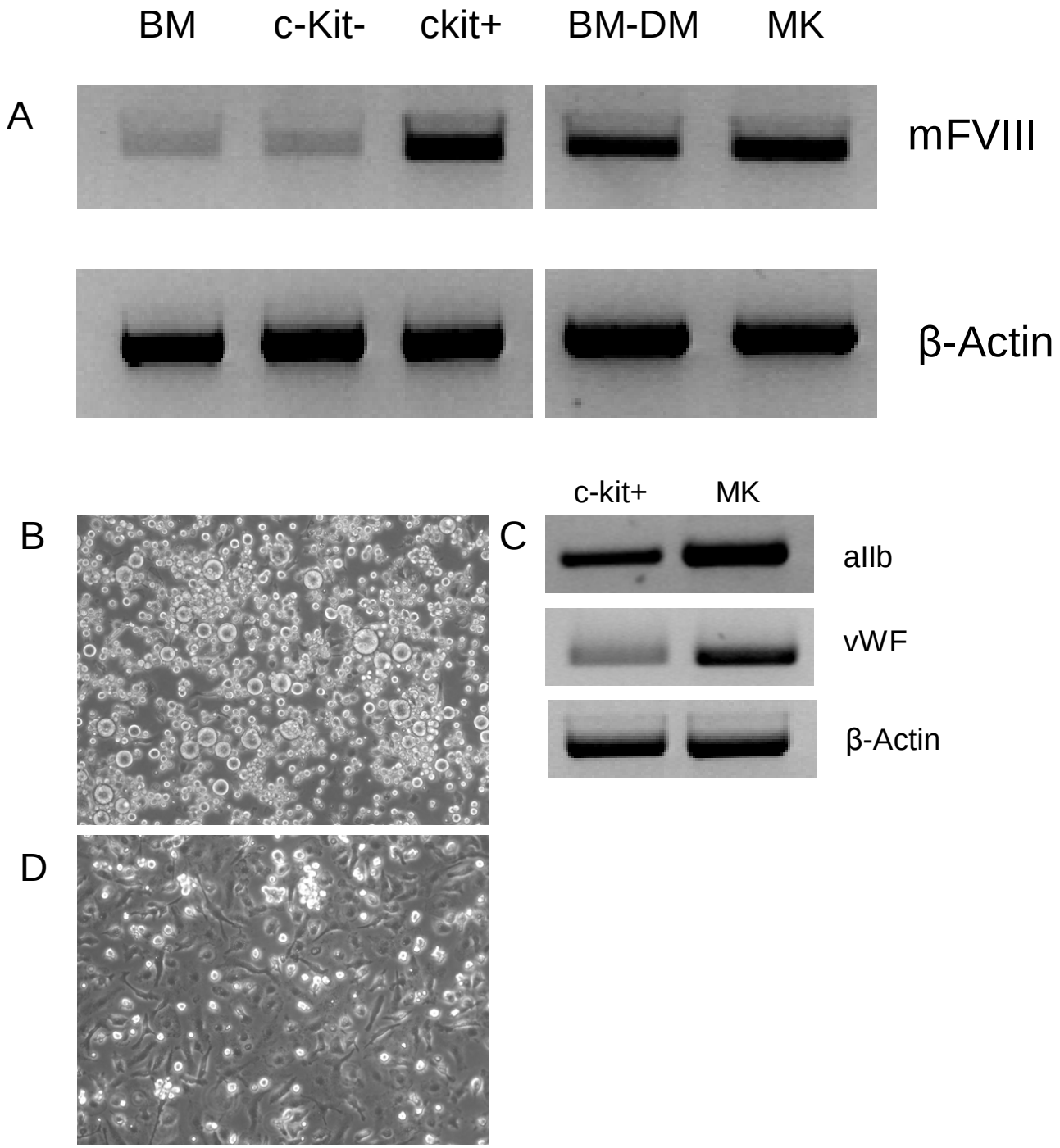


**Figure 1. Expression of FVIII mRNA in BM cells.** Cells isolated and derived from bone marrow of healthy C57BL/6 were analyzed for FVIII expression with RT-PCR. Both MK and BM-DM (d) express FVIII (a). MK were characterized for morphology (b) and expression of specific markers (c).

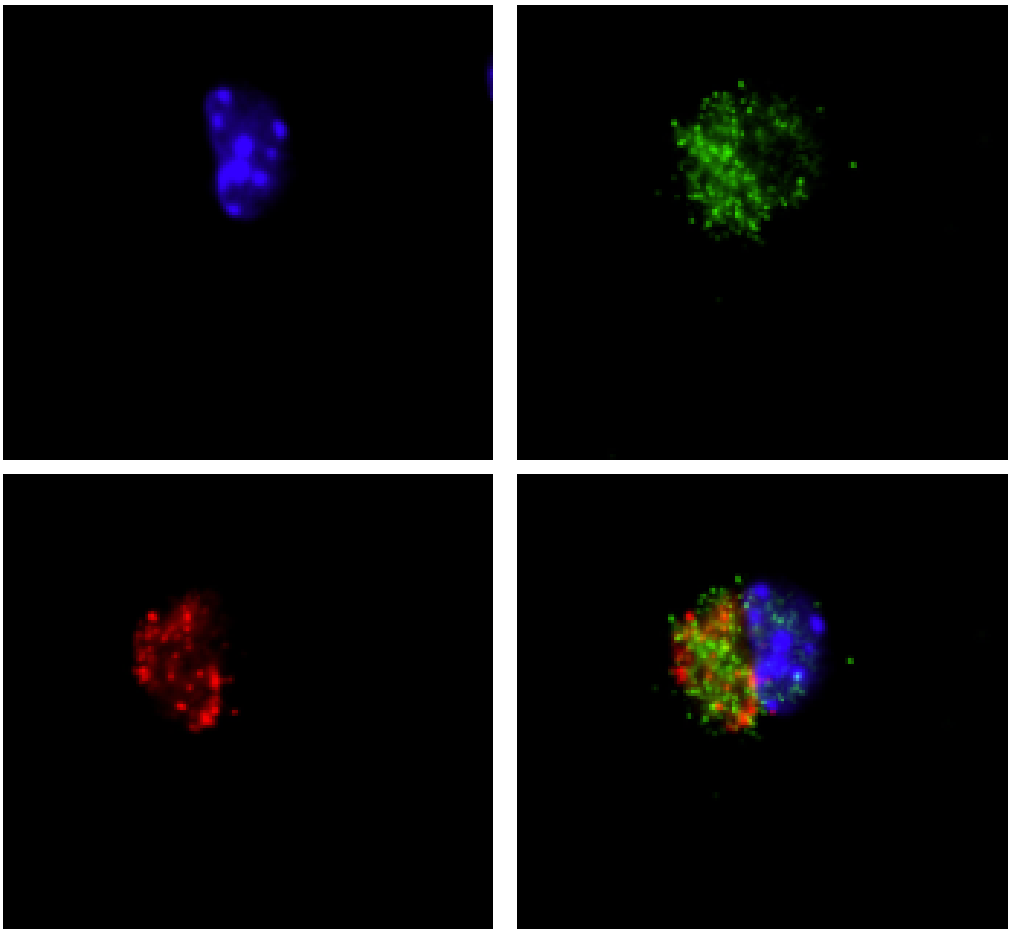

**Figure 2. Expression of FVIII in BM-DM.** Immunostaining of FVIII in BM-DM shows FVIII protein in cytoplasm (green dots) of F4/80 positive cell (red), a macrophage marker. DAPI for nuclei.

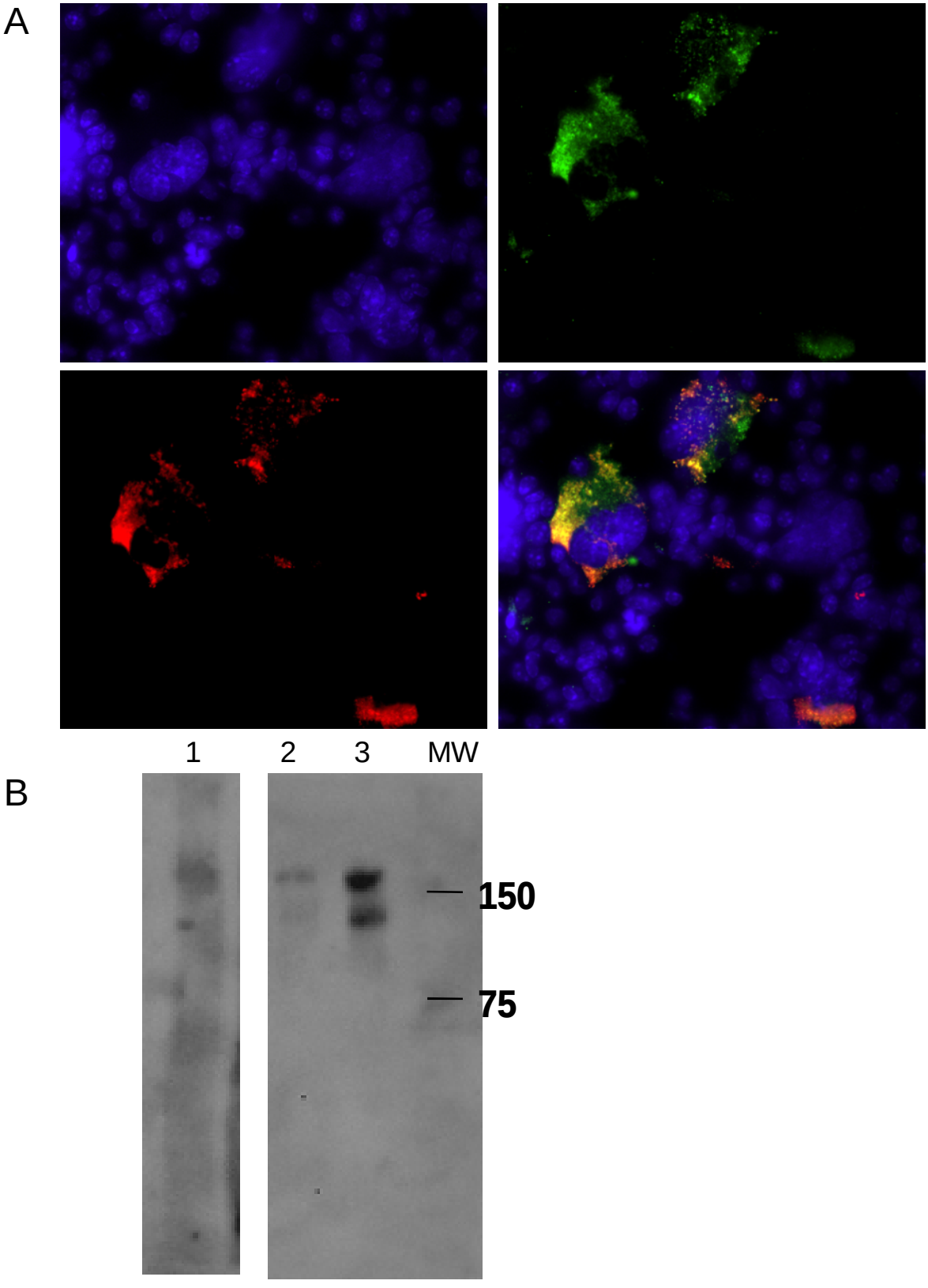


**Figure 3. Expression of FVIII in MK cells.** Immunostaining of FVIII in MKs shows that FVIII protein in cytoplasm (green dots) colocalized with vWF (red dots). DAPI for nuclei (A). Immunoblot analysis of MK (lane 1) with FVIII antibody. Purificated protein (lane 2: 10 ng,lane 3: 40 ng) was used as positive control (B).

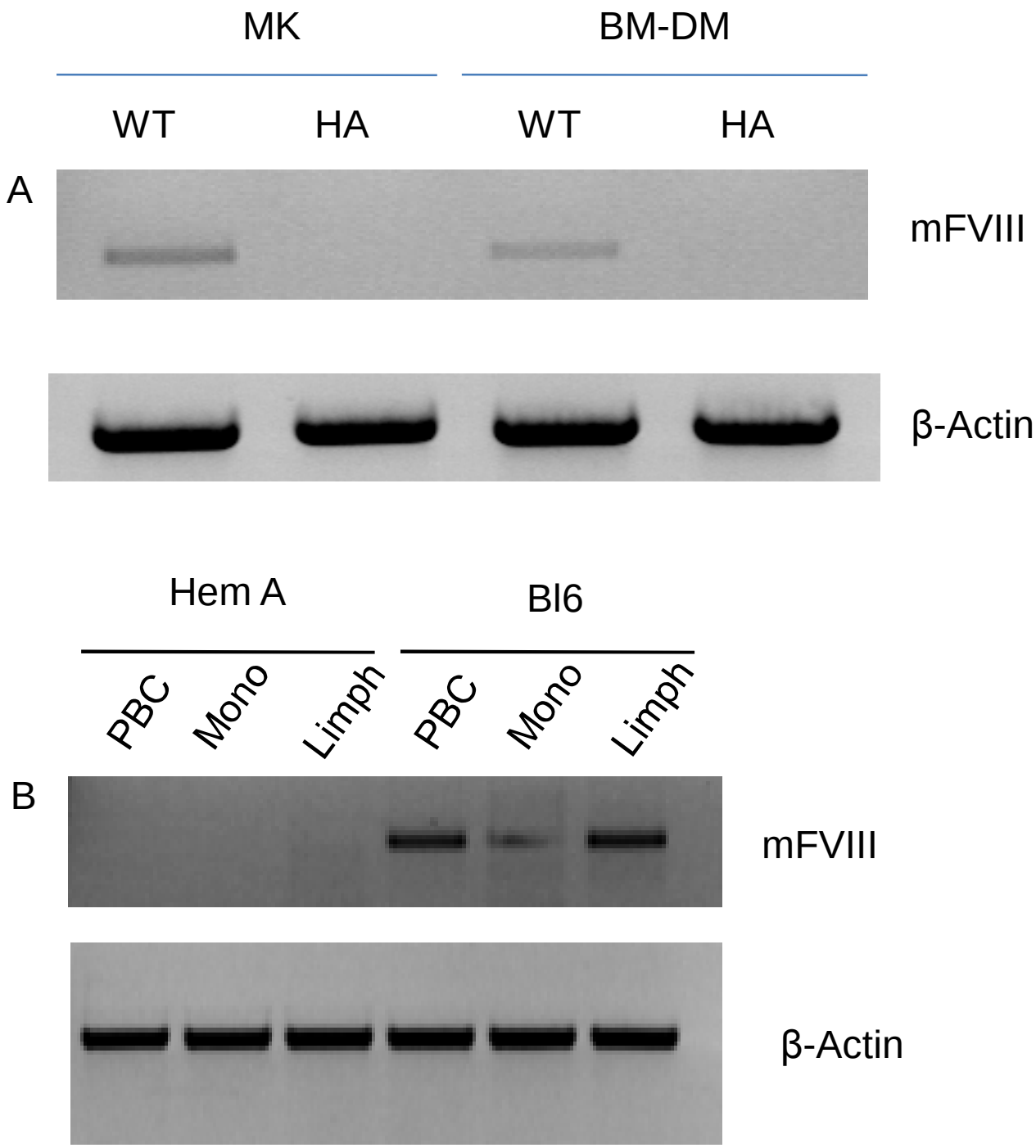


**Figure 4. Expression of FVIII mRNA in bone marrow and peripheal blood cells.** RT-PCR of cells from bone marrow-derived or isolated from peripheal blood (PBC: peripheal blood cells, Mono:monocytes,Limph:limphocytes) of hemophilia A and wt C57BL/6. Expression of β–actin shows RNA integrity.

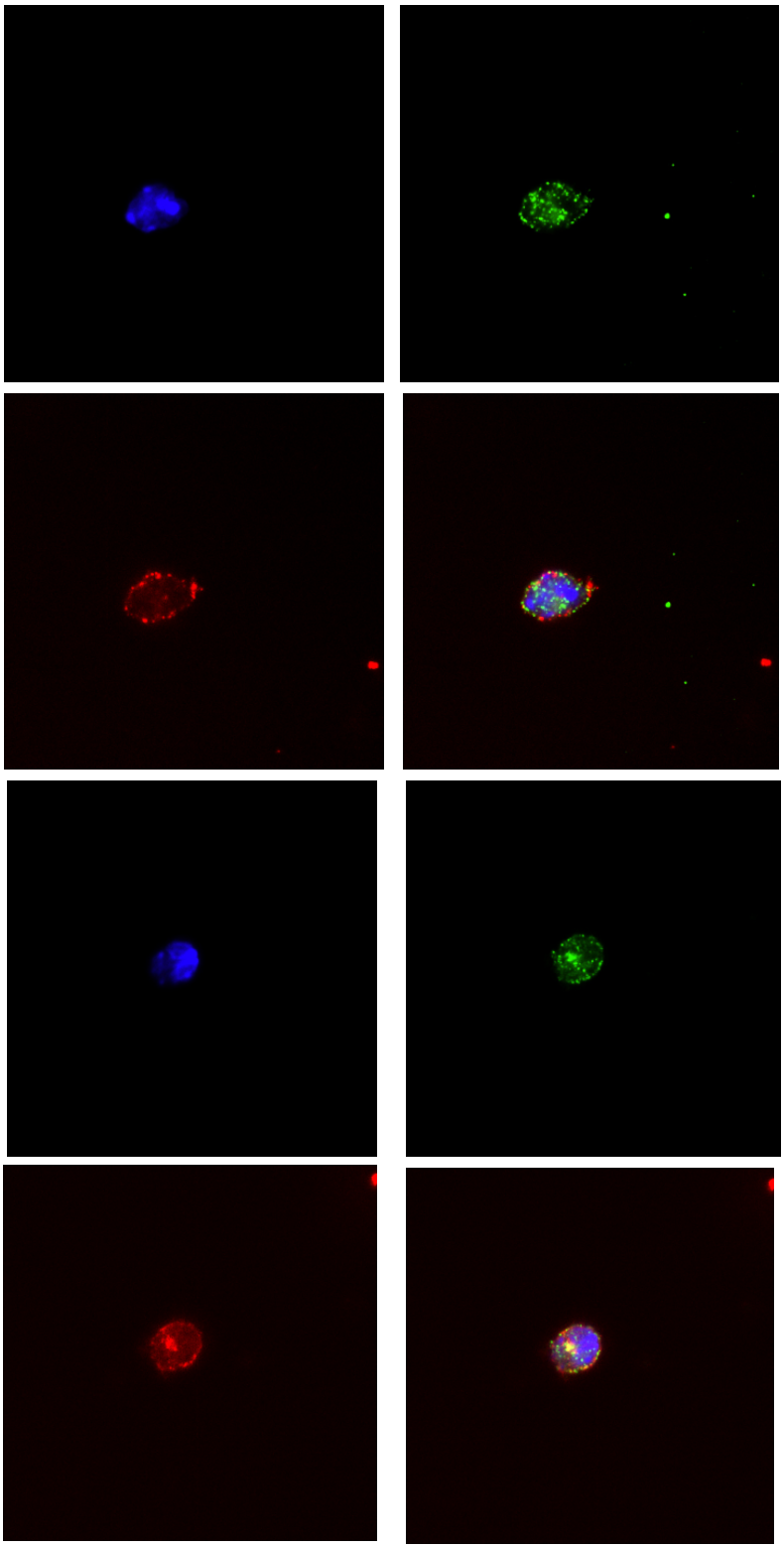

**Figure 5. Expression of FVIII in monocytes.** Expression of FVIII (green) in monocytes identified by CD14 (A,red) and CD115(B,red). DAPI staining for nuclei.

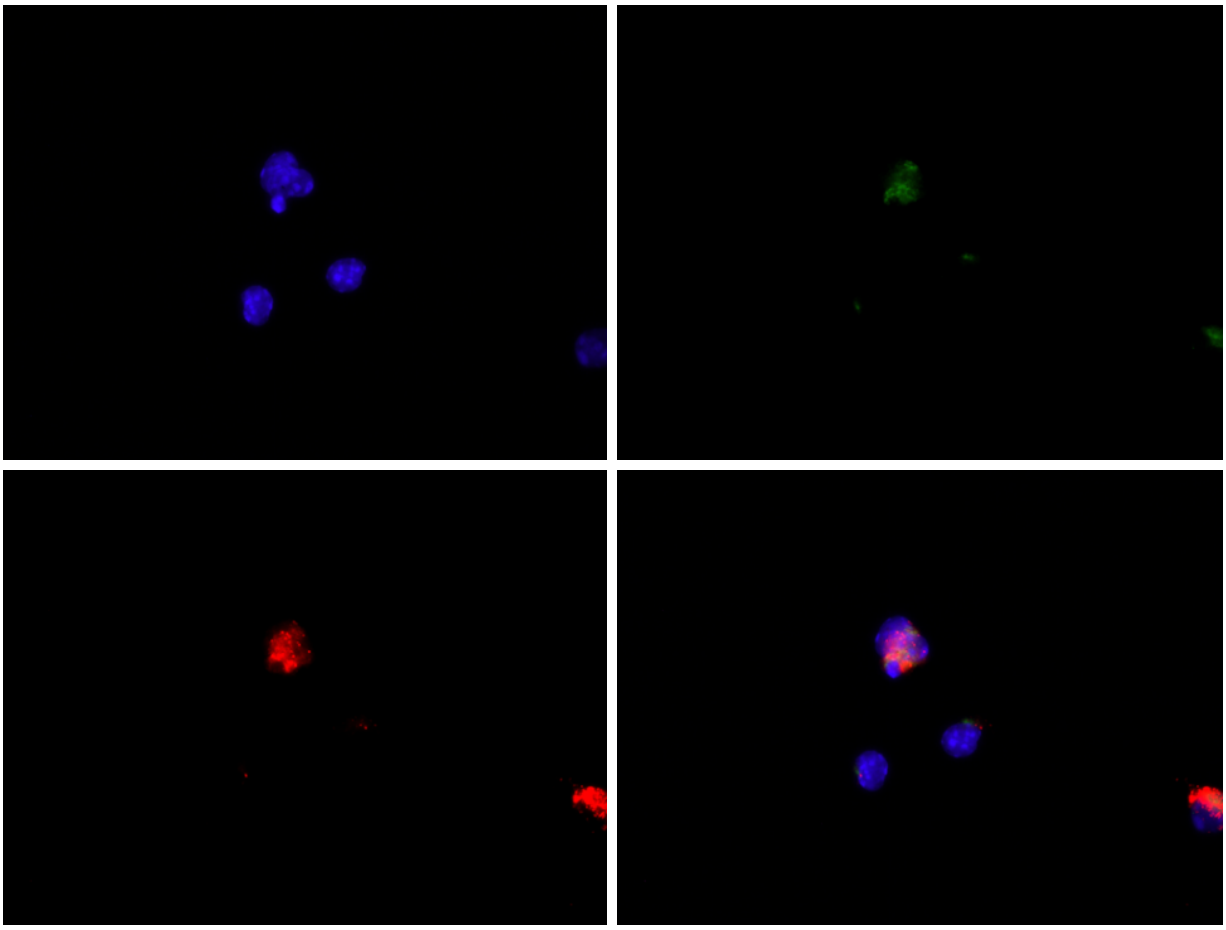

**Figure 6. Expression of FVIII in BM-DM from hemophilic mice.** Immunostaining for FVIII in hemophilic BM-DM shows that cells weren't positive for FVIII (green) in F4/80 positive cells (red).DAPI for nuclei.

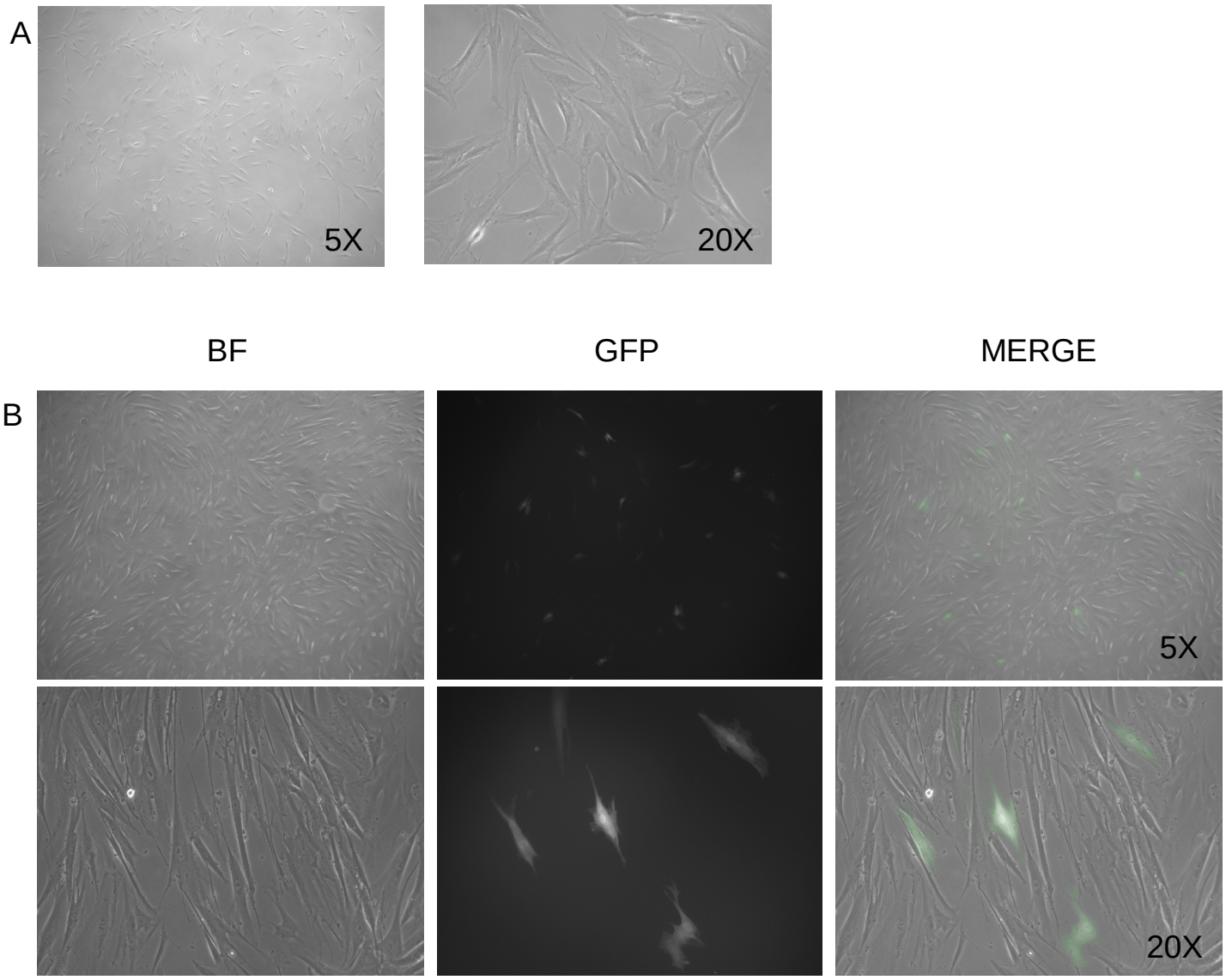


**Figure 7. Generation of fibroblast IPS cells.** Human fibroblast (A) were transducted by retroviral vectors of OCT4, SOX2,c-MYC and KLF4 . Cells transducted with GFP and OCT4, SOX2 and KLF4 were used as control of transduction (B) .

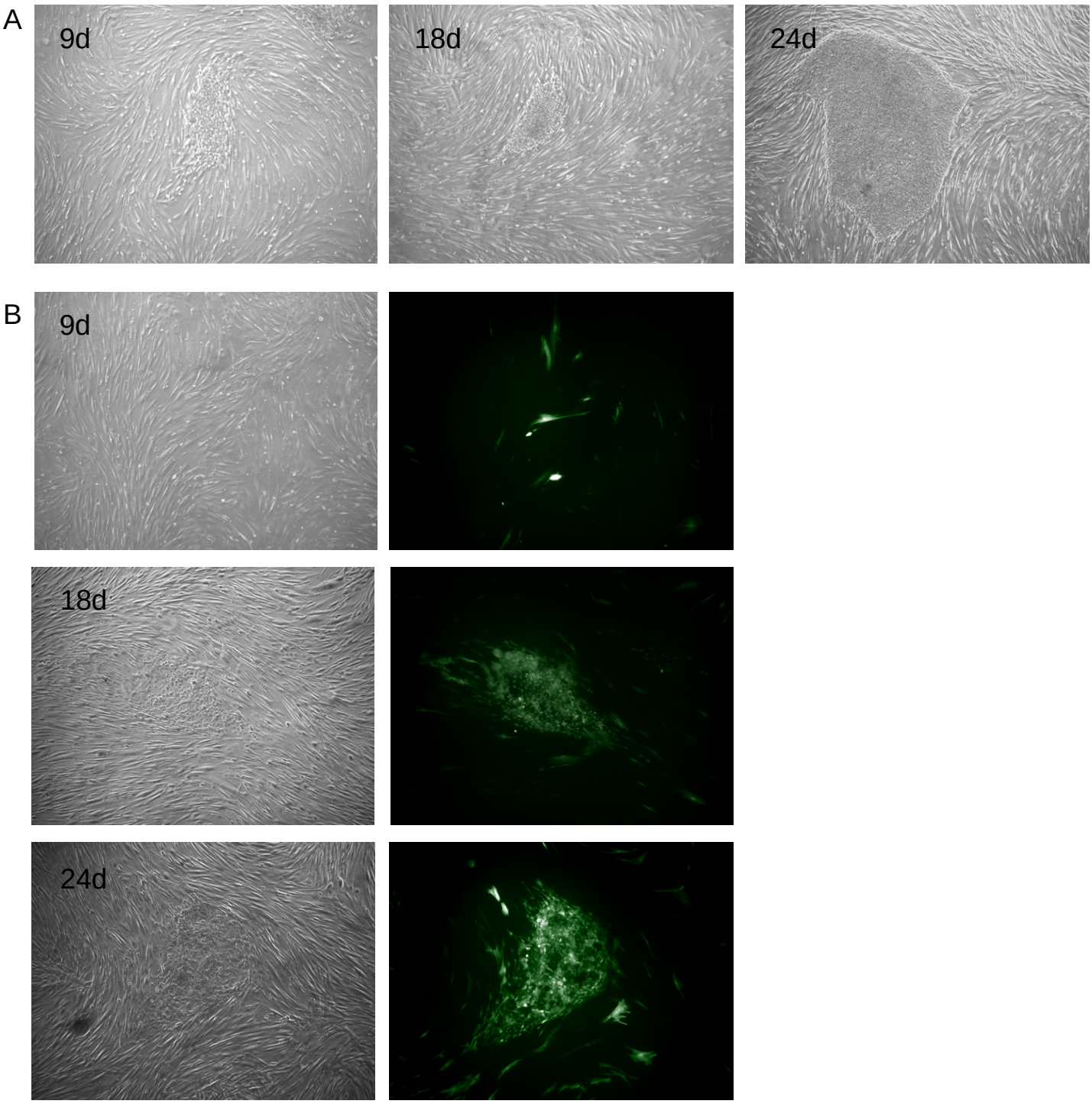


**Figure 8. Generation of fibroblast IPS cells.** Typical example of ES cell–like colony 9,18 and 24 d post-infection with four factors (A). Typical example of a ES cell–like colony 9,18 and 24 d post-infection with GFP(B).

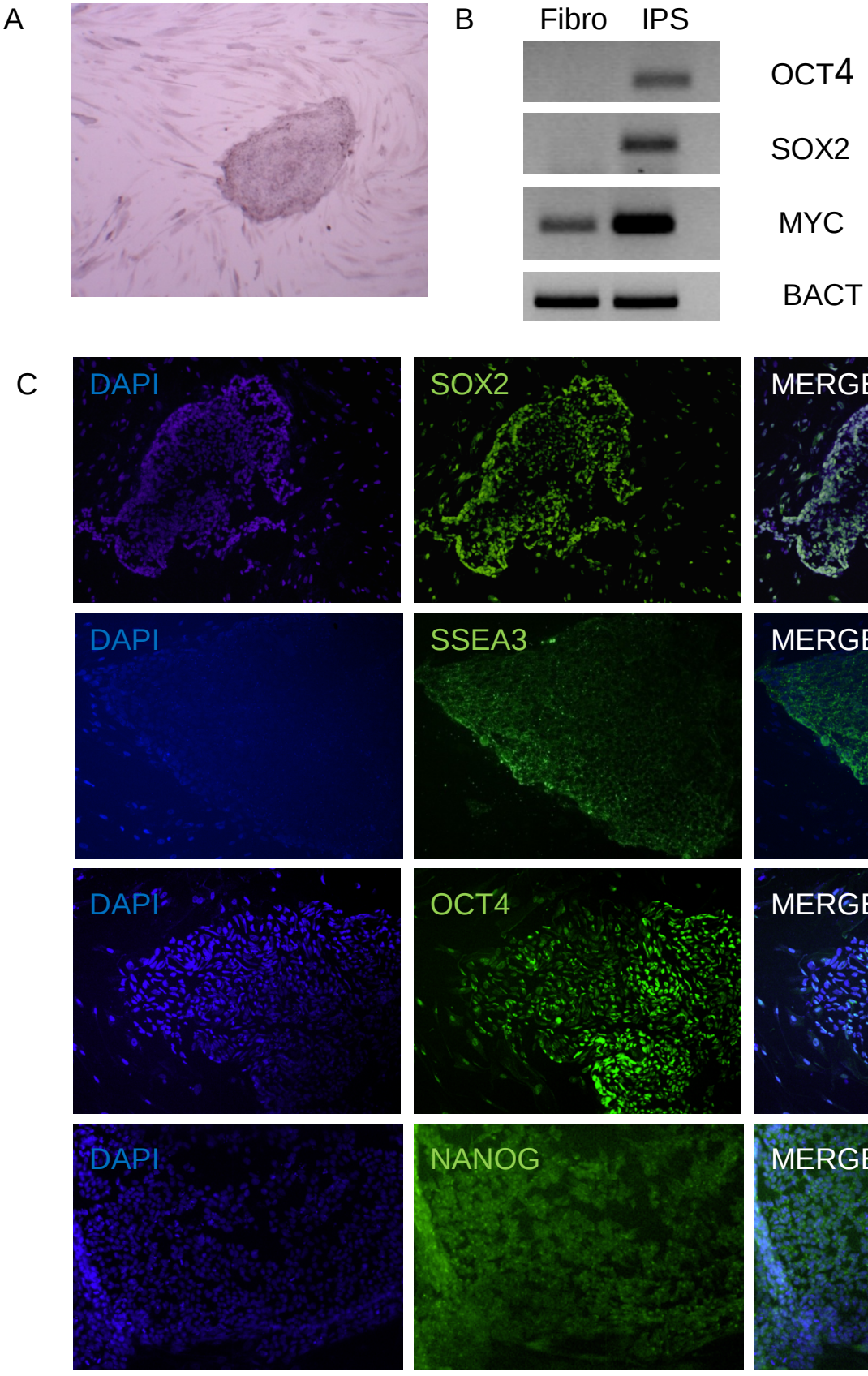


**Figura 9. Characterization of iPS cells**. A) Example of AP+ iPS colony.B) RT-PCR for reprogramming factors in fibroblasts before transduction (FIBRO) and IPS.C) Immunofluorescence of iPS colony positive for SSEA-3,SOX 2,OCT4,NANOG.DAPI for nuclei.

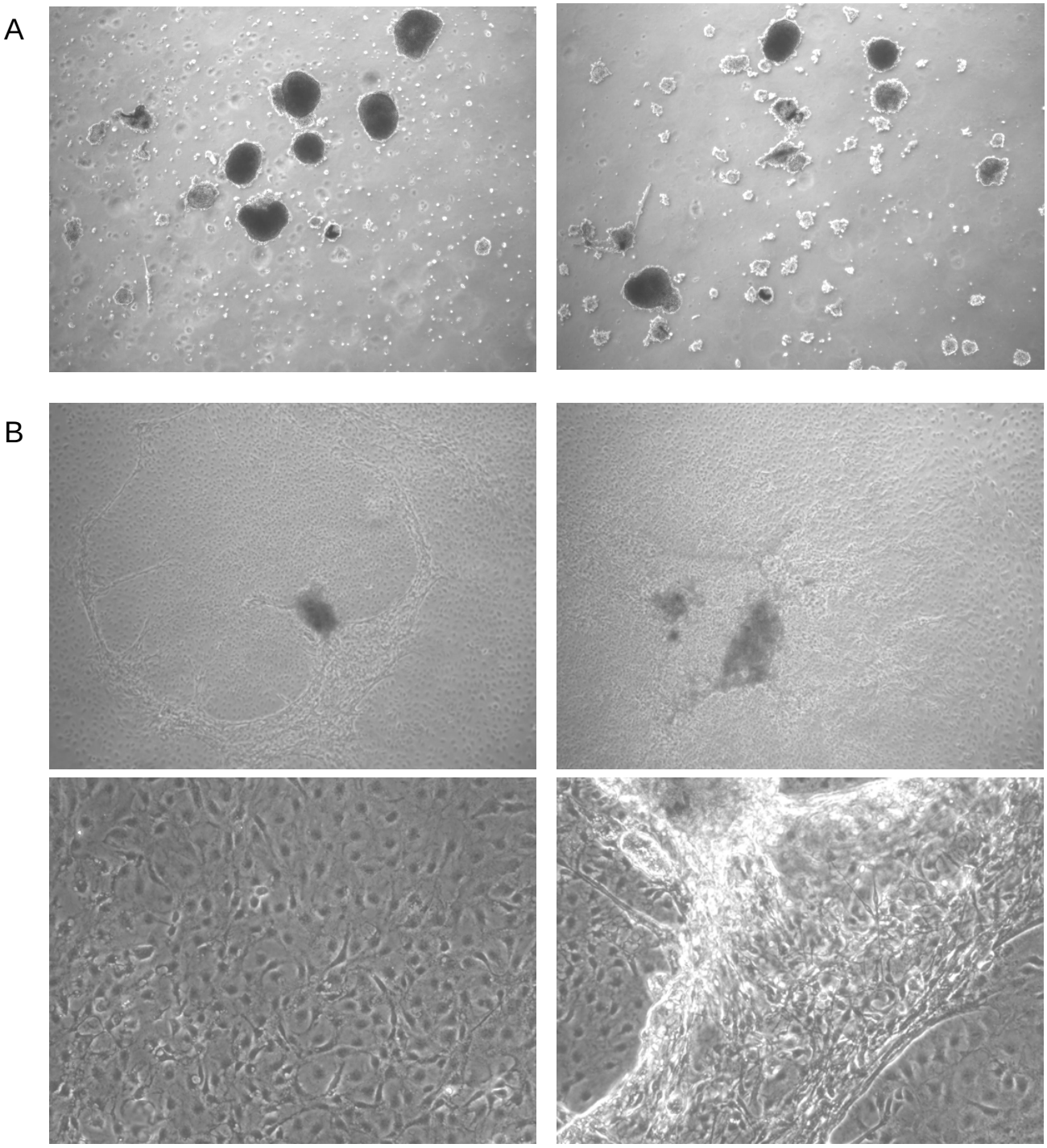


**Figura 10. Differentiation of iPS cells**. Embryo bodies from IPS colonies (A) were cultured in EB medium with Ascorbic acid for one week (B).