FACTOR IX SECRETION IN HUMAN ADIPOSE-DERIVED STEM CELLS BY NON-VIRAL GENE TRANSFER

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BACKGROUND AND OBJECTIVES

Hemophiliac patients are currently treated with intravenous infusions of either plasma derived or recombinant factors. This treatment is very efficient, but it is not a cure, and it is very costly. Cell-mediated gene therapy yielding long-term maintenance of therapeutic levels of coagulation factors, could be an optimal strategy. Due to their intrinsic properties such as self-renewal, pluripotency and almost no immunogenicity, adipose-derived stem cells (ASCs) can be considered promising for cell and gene therapy strategies *in vivo*. The aim of this study was to establish a non-viral gene therapy protocol to produce human FIX by ASCs obtained from human lipoaspirates.

METHODS

- **Cell isolation:** lipoaspirates were kindly provided by Dr. Fernández Blanco. ASCs were isolated and cultured according to a modified protocol from García-Olmo *et al* (2003).
- **Characterization:** cell cultures were analyzed by flow cytometry after staining with fluorescently-labelled monoclonal anti-CD90, 73, 29, 13, 31, 45, 34, 49e and HLA-DR.
- **Adipogenic differentiation:** to induce adipogenesis, a modified protocol from Zuk *et al.* was performed. Lipid accumulation was assessed by Oil Red O (ORO) staining.
- **Osteogenic differentiation** was performed as previously described (Zuk *et al*., 2001). After 28 days, mineralization was detected by Alizarin Red S (ARS) staining.
- **Chondrogenic differentiation** was induced for 21 days using a micromass culture technique (Dickhut *et al*., 2008). Then, micromasses were fixed and further processed for paraffin embedding. Sections were stained with a fluorescently-labelled antibody for aggrecan detection.
- **Transfection:** ASCs were transfected with a plasmid encoding an enhanced green fluorescent protein (EGFP) and human FIX linked by an IRES sequence. Nucleofection was performed in a Nucleofector II Device according to the optimized protocols for hMSC provided by the manufacturer (Lonza Cologne AG, Cologne, Germany).
- **Cytotoxicity:** cell viability was measured 24 hours after nucleofection by a modified Alamar Blue assay (Ahmed *et al*., 1994).
- **Analysis of EGFP expression:** nucleofected cells were observed by fluorescence microscopy. Transfection efficiency was also evaluated by flow cytometry.
- **RNA extraction:** total RNA was isolated from cell cultures using Trizol Reagent (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions.
- **RT-PCR:** total RNA was subjected to reverse transcription and the resulting cDNA pool was amplified by PCR using specific primers and optimized conditions of FIX (Miao *et al*., 2001). Human β-actin was used as a positive control (Cornet PB *et al*., 2002).
- **ELISA:** FIX secreted to culture medium was quantified 24 hours after transfection by an enzyme-linked inmunosorbent assay (Affinity Biologicals, ON, Canada).

RESULTS AND CONCLUSIONS

The cells obtained from lipoaspirates constitute a homogeneous population of ASCs expressing the characteristic surface antigen profile of mesenchymal stem cells (Fig. 1) and capable of differentiating towards adipogenic, osteogenic and chondrogenic lineages (Fig. 2). ASCs were then transfected with a plasmid encoding an enhanced green fluorescent protein (EGFP) and human FIX linked by an IRES sequence. Nucleofected ASCs expressed EGFP as determined by fluorescence microscopy (Fig. 3) and flow cytometry (Fig. 4), whereas RT-PCR (Fig. 5) and ELISA (Table 1) confirmed FIX expression and secretion. In conclusion, human ASCs were efficiently transfected to produce coagulation factor IX *in vitro* by nucleofection. Our results suggest that a non-viral *ex vivo* gene therapy strategy for haemophilia using ASCs as gene delivery vehicles is possible. Besides, this approach could be also applied to the treatment of other pathologic conditions where there is a protein deficiency.

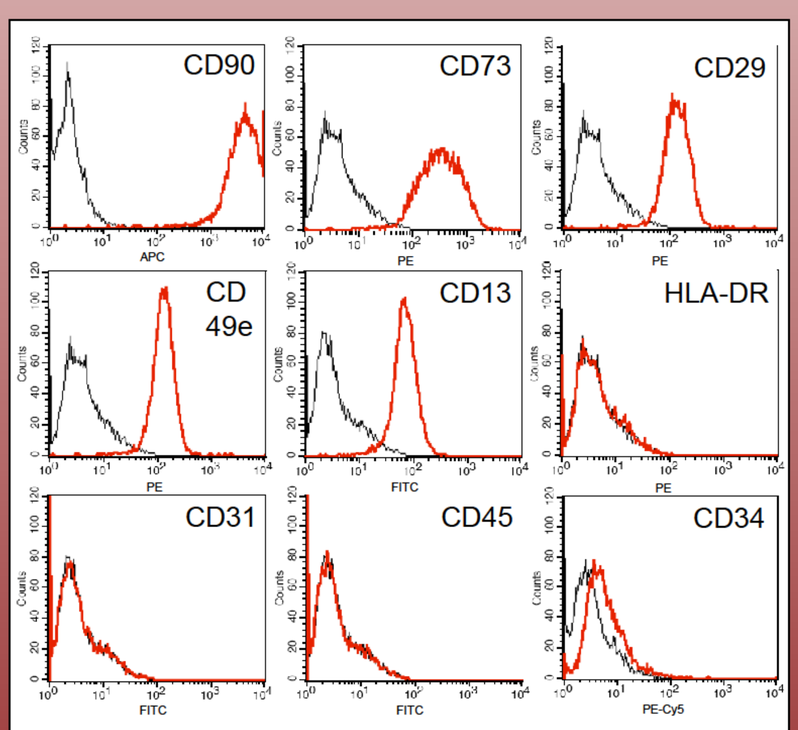

Figure 1. Characterization of ASCs by flow cytometry. *A consistent expression of CD90, CD73, CD29, CD13 and CD49e is detected, whereas cell surface markers CD31, CD45, CD34 and HLA-DR are negative. A homogeneous cell population is observed.*

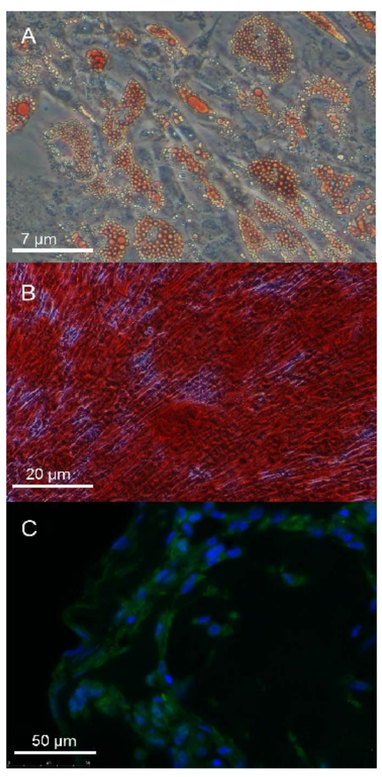

Figure 2. Differentiation assays.

(A) *ORO staining shows lipid-filled droplets accumulated by ASCs upon treatment with adipogenic medium.*

(B) *Osteogenic differentiation results in a dense ARS-stained mineralized layer.*

(C) *Immunofluorescence staining of aggrecan in the extracellular matrix surrounding ASCs in micromass culture after chondrogenic induction.*

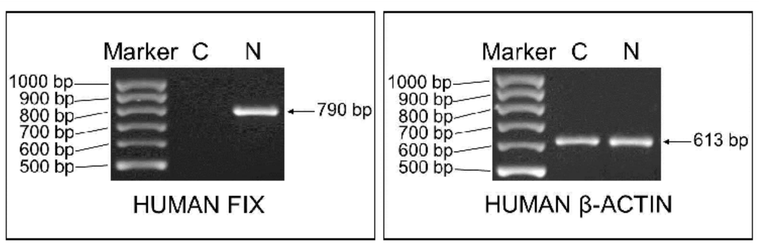

Figure 5. RT-PCR results. *Expression of human FIX* **(left)** *is only detected in nucleofected cells. Human β-actin* **(right)** *is used as a positive control, so bands are observed in both transfected and non-transfected cells.* **C**, *non-transfected cells.* **N**, *nucleofected cells.*

Table 1. Concentration of FIX within supernatants of ASCs

	Control (ng/mL)	Nucleofected (ng/mL)
Donor #3	0.02 ± 0.01	41.15 ± 3.56
Donor #9	0.02 ± 0.00	36.83 ± 6.29
Donor #11	0.00 ± 0.00	55.35 ± 7.71

Results shown are the mean (± Standard Error of Mean, SEM) of FIX concentrations from at least 3 independent experiments per donor.

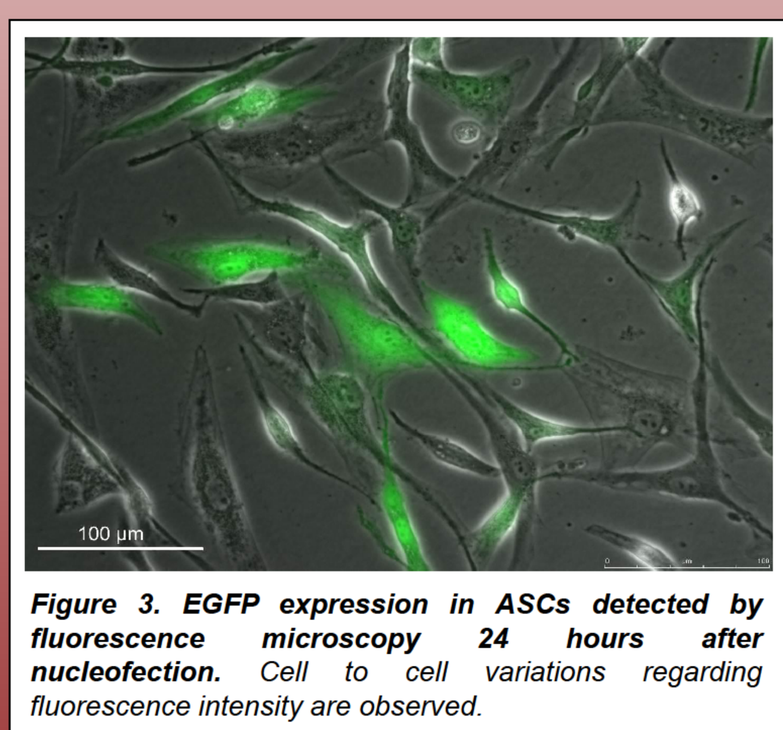

Figure 3. EGFP expression in ASCs detected by fluorescence microscopy 24 hours after nucleofection. *Cell to cell variations regarding fluorescence intensity are observed.*

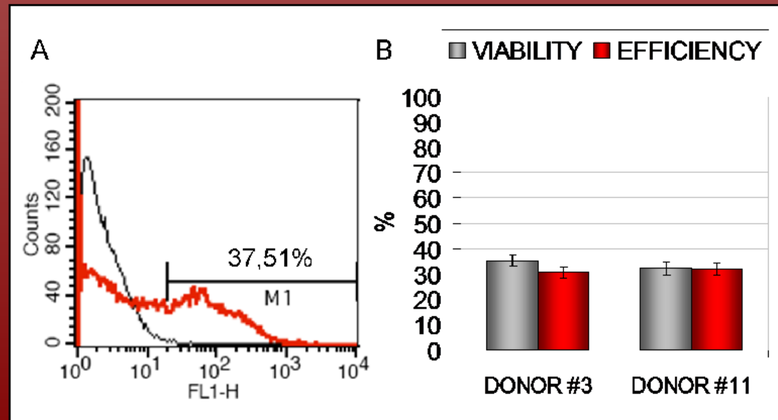

Figure 4. Nucleofection results. **(A)** *Representative flow cytometric analysis of transfection efficiency. Black and red histograms show fluorescence of non-transfected and nucleofected cells, respectively.* **(B)** *Percentage of viable and EGFP positive cells 24 hours after nucleofection. Mean ± Standard Error of Mean (SEM) from at least three independent experiments per donor are represented.*

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