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Cell therapy for factor V deficiency: An approach based on human decidua mesenchymal stem cells

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ABSTRACT

Deficiency of factor V is a congenital autosomal recessive coagulopathy associated with mutations in the *F5* gene that results in mild-to-severe bleeding episodes. Factor V is a component of the prothrombinase complex responsible for accelerating conversion of prothrombin to thrombin. At the present time there are no therapeutic factor V concentrates available. This study was designed to lay the preliminary foundations for future cell-based therapy for patients with severe factor V deficiency. The study showed that hepatospheres, which produce coagulation factors VIII, IX, and V, synthetize and store intracellular glycogen and express albumin levels up to 8 times higher than those of undifferentiated cells. Factor IX and factor V gene expression increased significantly in hepatospheres as compared to undifferentiated cells, whereas factor VIII gene expression remained constant. The factor V protein was detected in the hepatospheres' secretome. Considering the enormous potential of mesenchymal stem cells as therapeutic agents, this study proposes a highly reproducible method to induce differentiation of mesenchymal stem cells from human placenta to factor V-producing hepatospheres. This strategy constitutes a preliminary step towards a curative treatment of factor V deficiency through advanced therapies such as cell therapy.

1. Introduction

Hemostasis is the normal physiological mechanism that helps avoid significant blood loss after vascular injury. For enhanced effectiveness, this mechanism combines both cell-based and biochemical events in a coordinated manner [1,2]. In acquired and congenital coagulopathies, the mechanism may experience disruptions leading to uncontrolled bleeding and, consequently, to a significant increase in morbidity and mortality [3–5]. These coagulopathies include rare diseases like hemophilia A, characterized by a deficiency in coagulation factor VIII (FVIII) and an incidence of 1/6000 live male births; hemophilia B, caused by a deficiency in factor IX (FIX), with an incidence of 1/30,000 live male births [6]; and the so-called ultra-rare diseases such as factor V (FV) deficiency, which has an incidence of 1–9/1000,000 live births. Factor V deficiency, also known as parahaemophilia or Owrenś disease, is an autosomal recessive disorder associated to mutations in the *F5* gene [7–9].

Factor V, also called labile factor or pro-accelerin, is an indispensable coagulation factor that plays a key role in the coagulation cascade given its dual pro-coagulant and anticoagulant function [10]. Eighty percent of circulating factor V is produced by the liver, while the remaining 20% resides in platelet granules [10,11]. This circulating factor is a glycosylated 330 kDa polypeptide that loses its B-domain when activated, the protein being spliced into a 105 kDa heavy chain that contains the A1 and A2 domains, and a 71–74 kDa light chain that contains the A3, C1 and C2 domains. The heavy chain interacts with activated factor X (FIX) and prothrombin, while the light chain interacts with membrane phospholipids.

Within the coagulation cascade, factor V is a component of the prothrombinase complex, which accelerates the conversion of prothrombin to thrombin. This enzymatic complex is composed of activated factor V, calcium, phospholipids and activated factor X. Factor V

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increases the concentration of activated factor X on the surface of the cell membrane and, as a co-receptor of this factor, allosterically modifies its active site, optimizing its ability to cleave prothrombin [10]. In humans, the F5 gene has an approximate size of 80 kb, is located in chromosome 1q24.2, and consists of 25 exons and 24 introns. Factor V cDNA is 6914 bp in length [12]. Hepatocytes, found in the liver, are the main source of factor V in humans. Factor V circulates in free form in plasma, around 20% of it being stored inside megakariocytes [13,14].

Clinically, factor V deficiency is characterized by the occurrence of mild-to-severe bleeding episodes. Such episodes usually start before the age of six and are associated with a heterogeneous spectrum of hemorrhagic manifestations, ranging from mucosal or soft tissue bleeding (i.e. epistaxis or hemarthrosis) to potentially lethal hemorrhages. Abundant nasal and menstrual bleeding are distinctive features of this deficiency; profuse bleeding is also common during minor and major surgeries, as well as during dental procedures. Hemorrhagic arthropathy, hematomas, and cranial and gastrointestinal bleeding, are less frequent [7].

As opposed to hemophilia A or B, where there is a strong correlation between plasma factor levels and hemorrhagic phenotype, factor V deficiency is associated with a heterogenous clinical profile. Patients with lower levels of factor V generally face a higher bleeding risk than those with higher levels, although there are some patients with severe factor V deficiency who do not exhibit a particularly severe bleeding phenotype [15–17].

No plasma-derived or recombinant factor V concentrates are currently available to treat factor V deficiency. Available treatments consist of the administration of (virally inactivated) fresh frozen plasma or the use of Octaplas®, an alternative solvent/detergent-treated pharmaceutical product. The latter is associated with a high safety profile against emerging pathogens and prions [18], and contains an optimally controlled combination of the different coagulation factors, including factor V.

In the last few years, several research teams, including ours, have attempted to design advanced (gene or cell) therapies to find a curative treatment for congenital coagulopathies such as factor V deficiency.

This study assesses the applicability of human decidua mesenchymal stem cells (DMSC) as a treatment option for factor V deficiency. These cells, derived from the maternal portion of the placenta, possess certain characteristics that may offer the prospect of cure. In fact, DMSC have a high proliferation rate, can differentiate to the three embryonic layers, exhibit low immunogenicity, and have immunomodulatory properties. Moreover, DMSC can be obtained in large amounts without risk to the donor, are associated with a low risk of viral infection and can be cryopreserved [19–22]. The aim of this study is to analyse the differentiation of DMSC to functional hepatocytes (hepatospheres) that synthetize factor V.

2. Material and methods

2.1. Isolation and culture of DMSC

Human placentas from healthy mothers were obtained from the Department of Obstetrics and Gynaecology of the 12 de Octubre Hospital following written informed consent by the subjects and approval by the Hospital's Ethics Committee. After isolation of DMSC from placental membranes (Fig. 1A), the tissue was digested with 0.05% trypsin-EDTA (Gibco; Thermo Fisher. Scientific, Inc., Waltham, MA, USA) and the cells were seeded at 1.16×10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM glutamine, 0.1 mM sodium pyruvate, 55 μM beta-mercaptoethanol, 1% non-essential amino acids, 1% penicillin/streptomycin, 10% fetal bovine serum (FBS) and 10 ng/mL epidermal growth factor (EGF). Non-adherent cells were discarded while adherent cells were grown to confluence and re-seeded to a density of 4–5 $\times$ 10^4 cells/cm^2. In a previous study, we characterized these adherent cells as decidual cells with a mesenchymal-like phenotype and named them decidua mesenchymal stem cells (DMSC) (20).

2.2. Immunophenotypic characterization of DMSC

The phenotype of DMSC was characterized by flow cytometry with the following antibodies: CD44-FITC, CD117-PE, CD90-FITC CD13-PE, CD73-PE, CD29-PE, CD45-PerCP (BD Pharmingen, San Diego, CA); CD105-FITC (Serotec, Oxford, UK), BCRP1-FITC (Millipore, Bedford, MA); CD34-FITC, and CD133/1-PE (Miltenyi, Auburn, CA). FITC-, PE-, and PerCP-control isotypes were used as negative controls. Cell fluorescence was evaluated by a FACScan cytometer (Becton Dickinson, Lincoln Park, NJ) and data were analyzed with the CellQuest Pro 9.0 software (Becton Dickinson).

2.3. Colony forming unit (CFU) assay

Expanded DMSC were detached using trypsin-EDTA and counted in a TC-10 Automatic Cell Counter (Bio-Rad, Madrid, Spain). DMSC were plated at 100 or 500 cells per 100-mm tissue culture dish in complete culture medium and incubated for 12 days in usual culture conditions. The cells were subsequently washed with PBS, stained with 0.5% Crystal Violet in methanol for 5–10 min at room temperature and washed with PBS twice again. Visible colonies were then counted using an inverted Leica DMIL microscope (Leica Microsystems SLU, Barcelona, Spain).

2.4. Cell differentiation

Differentiation of DMSC (Fig. 1B) was carried out in the presence of oncostatin M (a cytokine that increases the efficacy of hepatocyte formation and maturation). The procedure was carried out as follows: DMSC were seeded on collagen-coated plates and cultured in the expansion medium for 6 days. To start the differentiation, the cells were cultured in a hepatocyte culture medium (HCM, Lonza, Spain), supplemented with 40 ng/mL hepatocyte growth factor (HGF, STEMCELL Technologies, Canada); 20 ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich, Spain); 40 ng/mL stem cell factor (SCF, Sigma-Aldrich, Spain); and 20 ng/mL fibroblast growth factor-4 (FGF-4, STEMCELL Technologies, Canada). Eleven days later, the cells were detached with trypsin-EDTA, centrifuged, and resuspended in the differentiation medium, to which 10 ng/mL oncostatin M (OSM, Sigma-Aldrich, Spain) was added. The cells were subsequently seeded, and the differentiation medium replaced every 3–4 days. Cell detachment at 11 days and addition of oncostatin M were the most critical experimental procedures in the differentiation process.

2.5. Assessment of hepatic differentiation

To confirm hepatic differentiation of DMSC, an analysis was performed of the hepatic biological features present in the hepatospheres. Intracellular glycogen was examined by periodic acid Schiff (PAS) staining (Sigma-Aldrich, Spain) and the stained hepatospheres were evaluated using an inverted Leica DMIL microscope (Leica Microsystems SLU, Barcelona, Spain) [19]. Other hepatospheres were stained with 1 mg/mL indocyanine green (ICG) (ICG-PULSION; PULSION Medical System, Feldkirchen, Germany) for 15 min at 37ºC. ICG uptake by hepatospheres was observed by microscopy and ICG excretion by the hepatospheres was evaluated eight hours later.

2.6. Gene expression analysis

The expression of genes encoding for FV, FVIII, FIX, albumin, and the TATA-binding protein (TBP) was analyzed by either reverse transcription polymerase chain reaction (RT-PCR) or quantitative PCR. Extraction of total RNA was performed with an NZYTech Total RNA Isolation kit (NZYTech Ltd.). Reverse transcription was carried out using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

RT-PCR was performed with Supreme NZYTaq 2x Green Master Mix

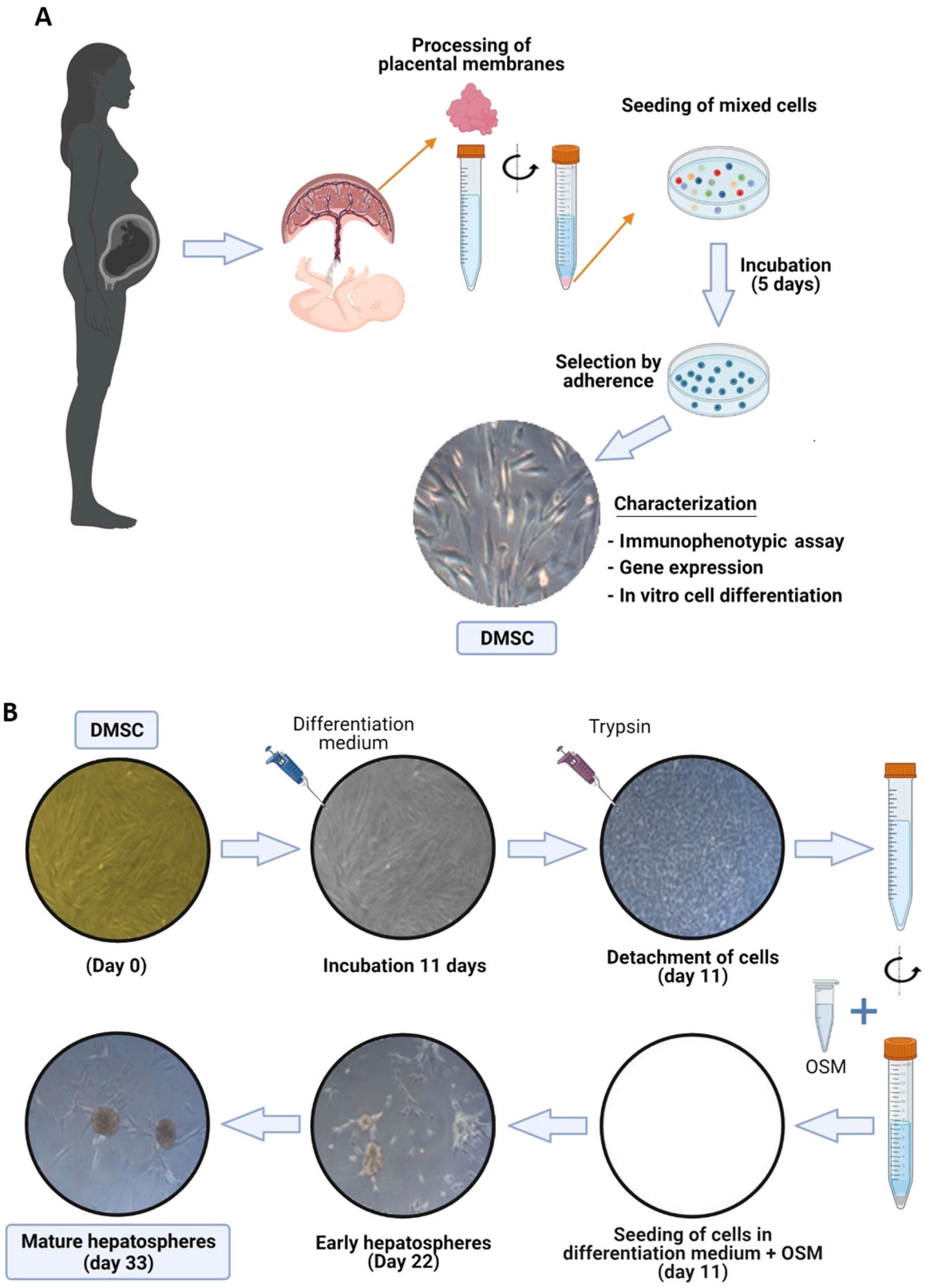

Fig. 1. Isolation, characterization and differentiation of human decidua mesenchymal stem cells. (A) Design of isolation and characterization protocol of DMSC. Cells were harvested from human placental membranes and selected by adherence to plastic prior to characterizing their phenotype. (B) DMSC differentiation method. After eleven days in the differentiation medium, the cells were detached and reseeded in differentiation medium in the presence of OSM. Early and mature hepatospheres were obtained on day 22 and 33, respectively. (Created in Biorender.com).

(NZYTech Ltd.). PCR conditions involved an initial denaturation stage at 95 ºC for 2 s followed by 30 cycles at 95 ºC, for 45 s, 59 ºC, for 45 s and 72 ºC, for 45 s, and a final extension at 72 ºC for 7 min. The specific primers for human albumin, *F5, F8, F9* and TATA-binding protein, are shown in Table 1. Products were separated in 2% agarose gel and protein bands were visualized by staining with GreenSafe Premium (NZYTech Ltd.).

Quantitative PCR was performed using GoTaq® qPCR Master Mix (Promega Biotech Ibérica, Spain) and a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). PCR reactions included the following two stages: a holding stage (50 ºC for 2 min and 95 ºC for 10 min) and a cycling stage (95 ºC for 15 s and 60 ºC for 1 min, for up to 40 cycles). *F5* gene expression with respect to the TBP housekeeping gene was calculated by applying the $2^{-\Delta Ct}$ method. For *F8*, *F9* and albumin, a comparison was made between undifferentiated and differentiated cells and data was expressed using the $2^{-\Delta\Delta Ct}$ method. Adult liver cDNA was used as positive control for all genes. cDNA from undifferentiated DMSC was used as a negative control.

2.7. Analysis of factor V expression

The human Coagulation Factor V ELISA kit (Blue Gene Biotech, China) was used to quantify the amount of factor V protein synthetized by the hepatospheres, following the manufacturer's instructions. This assay measures factor V in the 50–1000 pg/mL range. The secretome (pooled from 15 independent differentiations and tested 3 times) was obtained from the hepatospheres by collecting the culture medium at the end of the differentiation process and centrifuging it at 3000 rpm for 15 min to remove cellular debris. Protease inhibitors (Pierce Protease Inhibitor Mini Tablets, Thermofisher, Illinois, USA) were added to the secretome and samples were frozen at −80 ºC. A cell-free differentiation medium was used as control.

2.8. Data analysis

Data were analyzed using the GraphPad Prism® Software (version 9.0.0). Statistical analyses were performed by means of Student's *t*-test. Statistical significance was set at $p<0.05$. Data was expressed as means ± SD.

3. Results

DMSC cultured in an expansion medium are characterized by a fibroblastic morphology. The phenotype of DMSC was determined by flow cytometry. The cells were positive for MSC markers CD44, CD90, CD117, CD73, CD29, CD13, and CD105 and negative for hematopoietic markers CD34, CD45, CD133, and BCRP1 (Fig. 2A). In addition, DMSC were able to generate colonies when plated at low density (Fig. 2B).

Table 1
Design[a] of primers for qPCR and endpoint PCR.

Gene	Sequences	Amplified size (bp)	Gene Bank
Albumin	5-GATGCACACAAGAGTGAGGT- 3′ 5-TGGACACTGCTGAAGATACTGA- 3′	105	NM_000477.6
Factor V	5-GACCTGAGCCCACAAACTCAA- 3′ 5-AGTAGGCCCAAGAAGTCCTGAA- 3′	131	NM_000130.4
Factor VIII	5-TCAGGACAATATGGACAGTGGG-3′ 5-CTGGGTCTTGATGCCGTGAA-3′	144	NM_000132.3
Factor IX	5- GAAAGAACAACTGAATTTTGGAAGC- 3′ 5-TGCCGCCATTTAAACATGGATT-3′	76	NM_000133.3
TBP	5-TGCACAGGAGCCAAGAG-3′ 5-CACATCACAGCTCCCCAC-3′	132	NM_003194.4

[a] Primers designed with "Primers Blast," from the National Center for Biotechnology Information (NCIB).

After 11 days of differentiation, the cells acquired a polygonal morphology, typical of hepatocytes, and formed a monolayer. When detached and re-seeded, they initiated an aggregation process, which gave rise to the formation of small, rounded clusters bound to the plate through fibrous elongated cells (Fig. 1). These three-dimensional structures, known as hepatospheres, were formed around 11 days following detachment (22 days following induction of differentiation) (Fig. 3). The protocol was replicated several times with cells from different placentas. The result was in all cases the formation of hepatospheres. Cell detachment at day 11 and the addition of oncostatin M were two important milestones in the formation and maturation of hepatospheres. Relative albumin expression increased 7-fold when DMSCs were detached at day 11 as compared with undetached cells (data not shown). In addition, relative gene expression of FV and FIX increased 8-fold and 5-fold, respectively, in the presence of OSM (data not shown).

Hepatocytes and hepatospheres derived from differentiation of DMSC possess hepatic functions and are capable of synthetizing and storing intracellular glycogen (Fig. 4A-B). PAS staining resulted in pink positive cells, whereas undifferentiated cells were PAS negative. Likewise, hepatocytes and hepatospheres were capable of taking up ICG and secreting it a few hours later. However, ICG uptake was not observed in undifferentiated cells (Fig. 4C-G).

DMSC exhibited a baseline expression of albumin, FVIII and FIX genes (Fig. 5). A comparison of the gene expression of hepatospheres with that of undifferentiated DMSC showed that albumin gene expression was up to 8 times higher. FVIII levels did not change at the end of the differentiation, while FIX expression was 4 times higher in the mature 3D hepatospheres (Fig. 5). However, FV gene expression was only observed in these hepatospheres (Fig. 6). Such expression was confirmed by qPCR (Fig. 6A) and conventional PCR analysis (Fig. 6B). FV gene expression was undetectable until day 21, which is when the hepatospheres were formed, and increased until the end of the hepatosphere differentiation and maturation process (data not shown).

To analyse whether hepatospheres were able to synthetize and secrete FV, the secretome of hepatospheres was analyzed by ELISA (Fig. 7). The FV in the secretome was analyzed at the end of the differentiation process, which was when the expression of the FV gene was at its highest. After subtracting the amount of nonspecific binding protein in the cell-free medium (baseline cell-free differentiation medium) the estimated amount of FV produced by the hepatospheres was 66 ± 8 pg/mL (approximately 2.5 times higher). This amount of secreted FV was in line with the levels secreted in hepatocyte-like organoids [23].

4. Discussion

Nowadays the treatment of certain congenital coagulopathies consists mainly in the exogenous administration of highly purified coagulation factors. Some of these coagulopathies, such as factor V deficiency, however, cannot benefit from such a palliative treatment.

In the future, advanced therapies such as gene and cell therapy could constitute a curative treatment for some of these coagulopathies. Gene therapy could correct the underlying genetic mutation whereas cell therapy could generate functional healthy cells that could take over the function of defective cells.

This study proposes a preliminary approach to the use of functional hepatocytes derived from human decidua mesenchymal stem cells for the treatment of coagulopathies. DMSC are characterized by multipotency and low immunogenicity as they do not express major histocompatibility complex class II molecules or T-lymphocyte costimulatory molecules. Given their ease of harvest and high selection efficiency, DMSC have been used for a wide range of potential clinical applications for the treatment of several diseases such as liver conditions [19–22]. DMSC have been shown to differentiate into functional hepatocytes forming three-dimensional structures called hepatospheres,

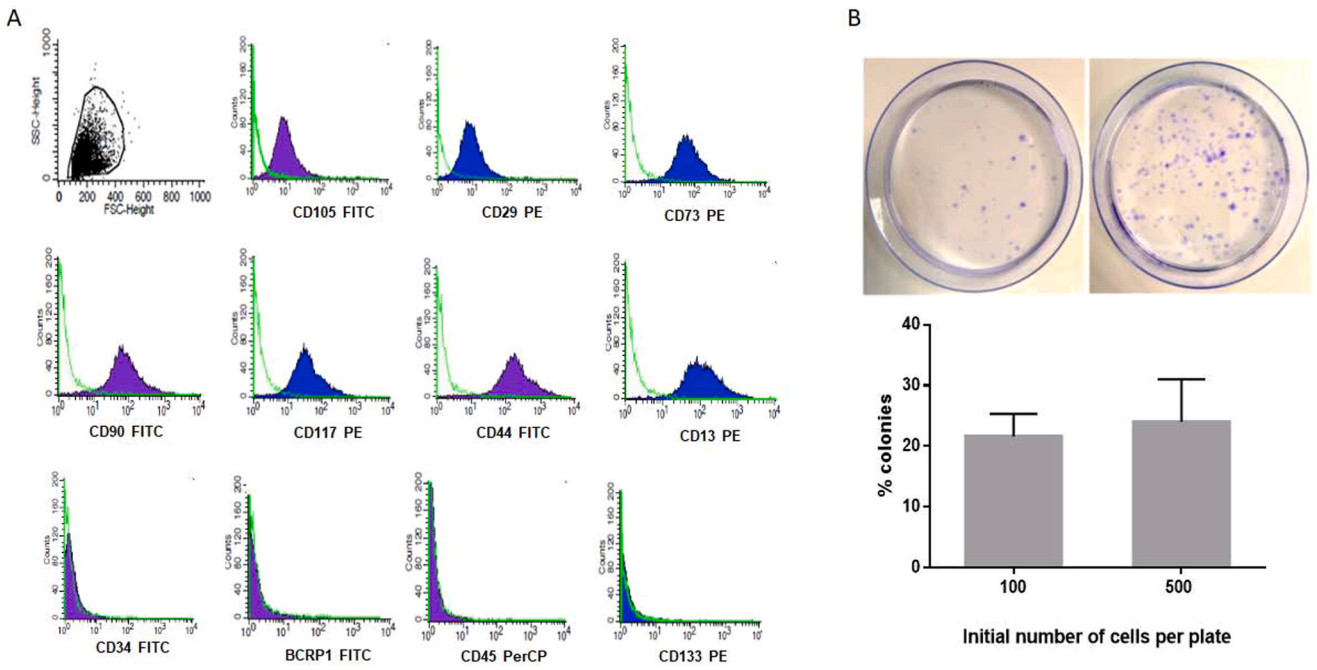

Fig. 2. Characterization of decidua mesenchymal stem cells. (A) Plots show the specific antibody staining profile (purple or blue area) versus the isotype Ig control staining (green line). (B) Representative images of crystal violet stained plates of CFU-assays performed at two different cell densities (upper panel) and CFU quantification of DMSC initially plated at 100 (n = 7) or 500 (n = 3) cells/100-mm plate.

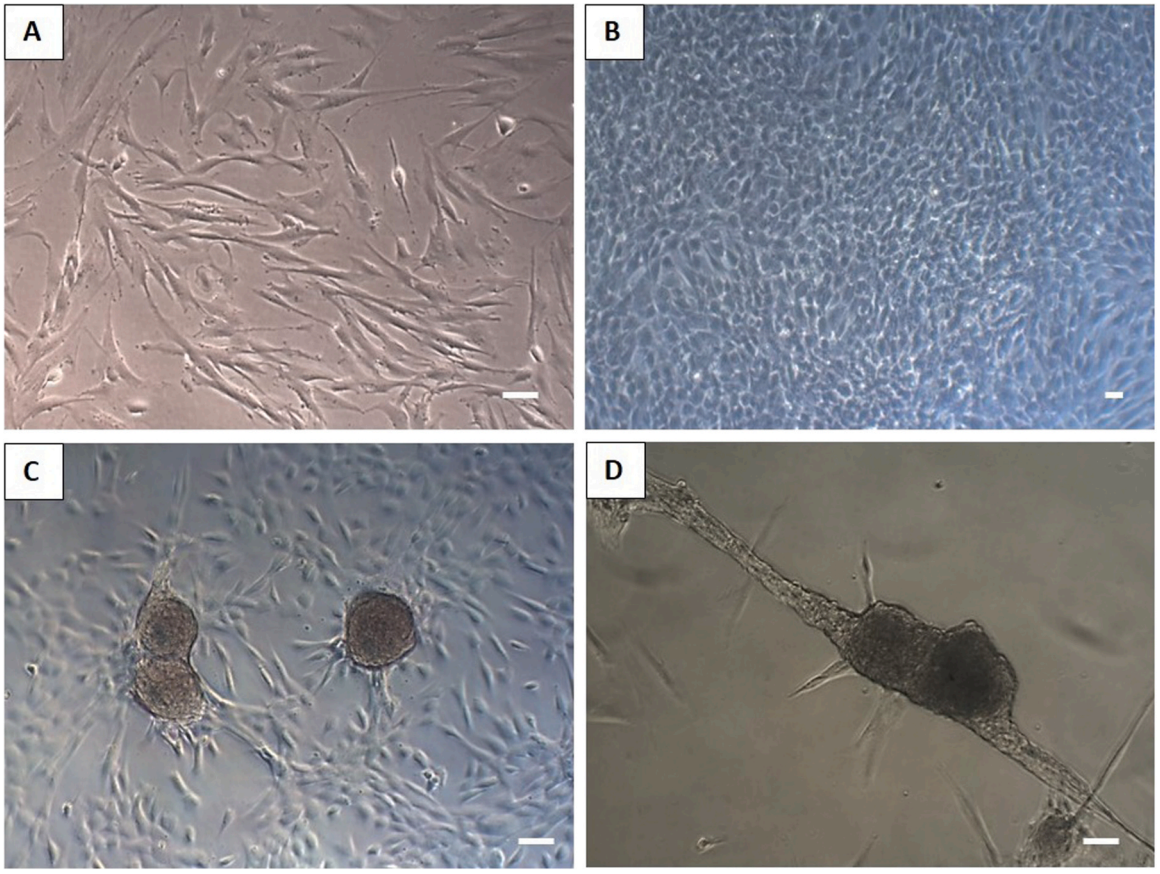

Fig. 3. Differentiation of decidua mesenchymal stem cells. (A) Undifferentiated DMSC. (B) DMSC at 11 days from differentiation. (C) DMSC at 22 days from differentiation, and (D) DMSC at 33 days from differentiation (scale bar, 100 μm).

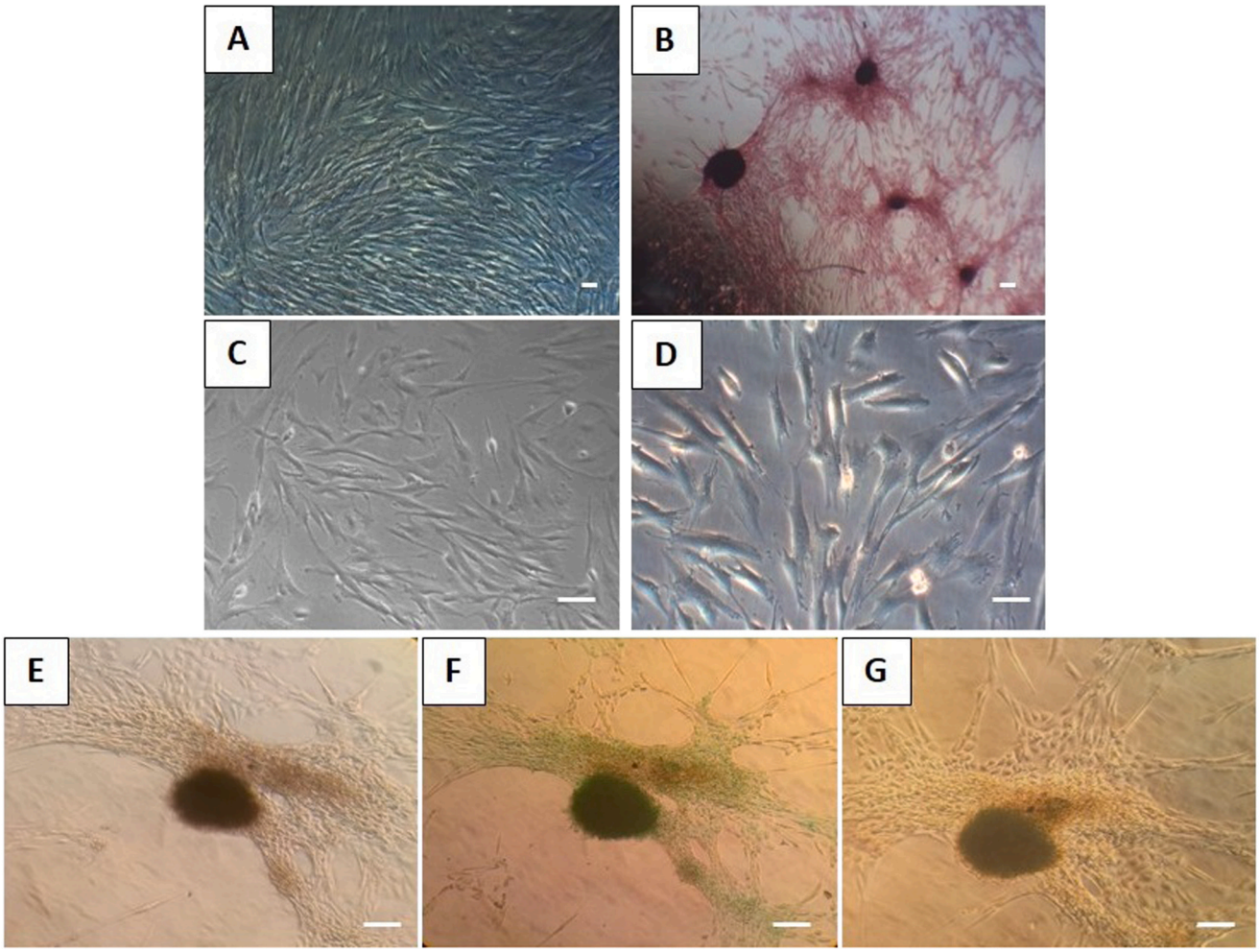

Fig. 4. PAS staining of DMSC and uptake of ICG. (A) PAS staining of undifferentiated DMSC and (B) DMSC-derived hepatospheres at 22 days from induction of differentiation. (C) and (D) Undifferentiated DMSC before and after incubation with ICG, respectively. (E) and (F) Hepatospheres formed at day 22 days from differentiation, before and after incubation with ICG, respectively. (G) Secretion of ICG after 8 h. Representative images from three different experiments (scale bar, 100 μm).

which mimic a hepatocellular environment analogous to that in the liver. Interestingly hepatospheres, which have liver-specific morphological features, also have the ability to express coagulation factors, such as *F5* and *F9*.

Some hepatocyte-specific functions occurring in hepatospheres are glycogen storage and albumin gene expression. In addition, hepatospheres can take up and subsequently secrete indocyanine green, an organic anion used to evaluate hepatocyte function [24]. Periodic acid–Schiff is a staining method used to detect polysaccharides such as glycogen, which accumulates as energy storage [25]. Indocyanine green is an organic anion used in medical diagnostics (for determining cardiac output, hepatic function, and liver and gastric blood flow, and in ophthalmic angiography. It is specifically cleared by the liver. At cellular level, ICG is taken up by hepatocytes via organic anion transport protein (OATP)1B1 (OATP-C in humans), which is exclusively expressed in the basolateral membrane of hepatocytes [26].

In this study certain experimental conditions have been established in order to favor hepatosphere maturation and therefore the expression of coagulation factors. One of these was addition of oncostatin M [27]. As regards the expression of factor IX, some reports have shown that it is expressed only by hepatocytes (in the liver) [28]. However, in the course of this study baseline levels of factor IX expression were detected in undifferentiated DMSC. On the other hand, it must be said that a 4-fold increase was observed in factor IX gene expression under hepatic differentiation. To our knowledge, this is the first report showing DMSC expression of factor IX. By contrast, factor VIII gene expression is not specific to the liver and can be observed in both undifferentiated DMSC and in cells differentiated to hepatospheres, as has been reported by other authors [29,30].

Factor V expression was not observed in undifferentiated cells, yet it was detected at the end of the differentiation process giving rise to the hepatospheres, which suggests that hepatocytes require a 3D microenvironment to express the *F5* gene. The fact that factor V appeared within hepatospheres (three-dimensional structures), and not in isolated hepatocytes (data not shown), is in line with previous reports showing that isolated hepatocytes require a three-dimensional microenvironment to preserve their functions in vitro [31]. The architecture of hepatospheres resembles that of native tissue, where cell-to-cell and cell-to-matrix interactions could contribute to preserving hepatocyte function, both in vitro and in vivo [31,32]. Importantly, hepatospheres secreted factor V protein to the culture medium.

This is the first report showing that cells differentiated to liver structures can produce and secrete factor V, indicating that the factor has undergone the required post-translational modifications in mammalian cells to become a functional protein [33].

Expression of factor VIII and factor IX does not require these complex structures. The protocols used in the present study are highly reproducible, which is essential for the development of a therapeutic

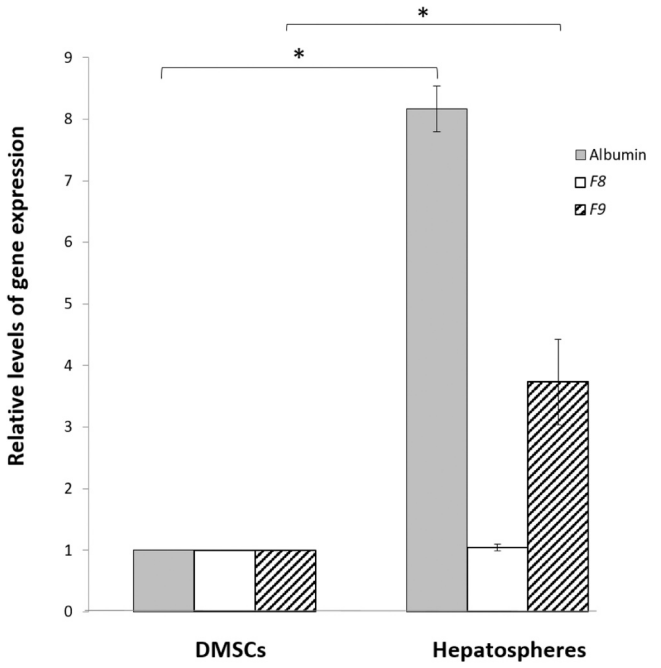

Fig. 5. Gene expression in DMSC-derived hepatospheres. Relative albumin, factor VIII and factor IX gene expression with respect to undifferentiated DMSC cells. Data was normalized to TBP expression as a housekeeping gene and presented as mean ± SD (n = 3) (*p<0.05).

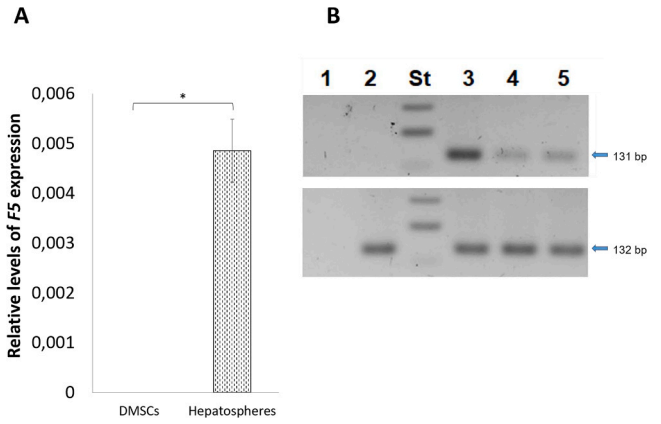

Fig. 6. Analysis of factor V gene expression using qPCR and RT-PCR. (A) Factor V gene expression in DMSC-derived hepatospheres analyzed by qPCR with respect to the (TBP) housekeeping gene (n = 4; *p<0.05). (B) RT-PCR of the factor V gene (upper electrophoresis gel) and TBP (bottom electrophoresis gel). Lane 1: Negative control. Lane 2: Undifferentiated DMSC. Lane 3: Adult human liver (positive control). Lane 4 and 5: DMSC-derived hepatospheres from two independent experiments. St: NZYTech's Leader V molecular marker.

approach for any given condition. Safety and reproducibility are two critical aspects for the implementation of any effective advanced therapy, whether gene- or cell-based.

An important short-term goal should be to increase the amount of factor V obtained from hepatospheres by further optimizing the cell differentiation protocol. A longer-term goal would be to use hepatospheres as functional liver grafts that could ensure the maintenance of adequate therapeutic levels of factor V over time [34]. The results obtained suggest that DMSC could be an excellent alternative for the development of cell therapies aimed at managing coagulation factor deficiencies. Several research teams have already started working on these therapeutic strategies in an attempt to understand the

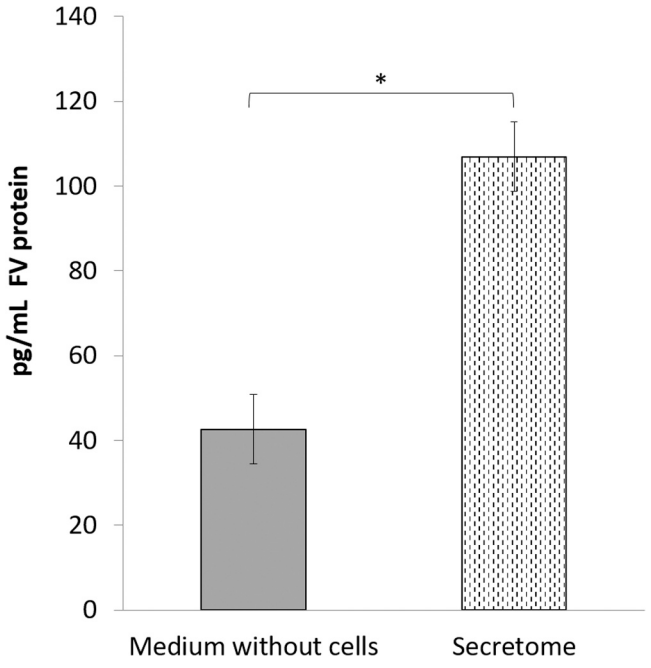

Fig. 7. Secretion of factor V by the hepatospheres. Expression of the factor V was determined by ELISA in the hepatospheres secretome. A cell-free differentiation medium was used to control for non-specific binding of the anti-factor V antibody. Data expressed as mean ± SD (n = 3) (*p<0.05).

etiopathogenic mechanisms behind such deficiencies [35–37].

Cell and gene-based approaches are particularly encouraging in the case of rare and ultra-rare diseases, which do not always benefit from the same kind of support as better-known conditions. Given that there is not yet an effective treatment available, factor V deficiency could benefit from these therapies. A highly stable recombinant form of human factor V, known as superFVa, has been recently obtained and tested in preclinical studies as a prohemostatic agent in the context of severe bleeding episodes, such as those typical of factor V deficiency and hemophilia A with inhibitors [38–40].

The present study puts forward a preliminary cell therapy approach that could help translate current research efforts from bench to bedside.

Ethics approval and informed consent

The study protocol was approved by the Ethics Committee of the 12 de Octubre, Hospital, Madrid, Spain. The study was conducted according to the principles of the declaration of Helsinki. Written informed consent was obtained from all study participants.

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CRediT authorship contribution statement

The authors' contributions were as follows: Luis J. Serrano was responsible for the collection and/or gathering of data, as well as for

data analysis and interpretation. Paz de la Torre was in charge of analyzing and interpreting the data and performed a critical review of the manuscript. Antonio Liras was in charge of the conception and design of the study, as well as for obtaining financial support and drafting the manuscript. Ana I. Flores was tasked with the design of the study, worked on financial support, and critically reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors reported no conflict of interest.

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