Article

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Platelet Rich Plasma Promotes Proliferation of Adipose Derived Mesenchymal Stem Cells via Activation of AKT and Smad2 Signaling Pathways

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Abstract

Recently, autologous platelet-rich plasma (PRP) has been proposed as a substitute for xenogenic or allogenic culture media used for in vitro cell expansion. Although PRP has been demonstrated to promote adipose-derived mesenchymal stem cell (ASC) expansion, its mechanism of action has not yet been investigated. In this study, we aimed to assess the growth factors and molecular pathways implicated in enhancement of ASC proliferation by PRP.

Cell proliferation was analyzed in ASCs cultured for 10 days with 20% autologous PRP and compared to those supplemented with 10% fetal bovine serum (FBS). The secretion of PDGF-AB, FGF, TGFβ, VEGF, and MIF in the culture media was investigated. In addition, AKT, ERK, and Smad2 signalling pathway activation involved in ASC proliferation was assessed using western blot analysis.

The expansion rate of cultured ASCs was 14 times greater with 20% PRP than with 10% FBS. Proliferation rate of ASCs was higher in 20% PRP-supplemented medium than in 10% FBS. PDGF-AB, FGF, TGFβ, and VEGF were present in the medium supplemented with 20% PRP up to 10 days. Macrophage migration inhibitory factor (MIF) secretion was confirmed in both media, and a higher level was seen in 20% PRP. The AKT, ERK and Smad2 signalling pathways were more activated in ASCs cultured with PRP compared to FBS.

In summary, our results indicate that PRP regulates ASC proliferation through secreted proteins (PDGF-AB, FGF, TGFβ, VEGF, and MIF). Growth factor/receptor complexes activate mainly AKT and Smad2 and to a lesser extent, ERK signalling pathways.

Keywords: Adipose-derived mesenchymal stem cells; Platelet-rich plasma; Macrophage migration inhibitory factor (MIF); Growth factor; Proliferation; AKT; Smad2; ERK1/2

Introduction

Adipose-derived mesenchymal stem cells (ASCs) are multipotent cells that have the ability to self-renew. They are promising candidates for gene and cell therapy because they have great differentiating potential to mesodermal lineage such as adipocyte, chondrocyte, and osteocyte [1-3], and even into endoderm and ectoderm cells [4,5].

Clinical applications of ASCs often require extensive *in vitro* cell expansion, and the culture media should be safe, biocompatible, and efficient without modifying cell phenotype or differentiation capacity. Thus far, ASCs have been studied mostly using animal-derived products such as fetal bovine serum (FBS) or allogenic additives (e.g., human serum or human platelet derivatives) as the media supplement [6-9]. However, these non-autologous supplements present some disadvantages such as infection or immunological reaction risks, limited efficiency, and high cost that limit their clinical use [10]. Therefore, the use of autologous blood-derived supplements platelet-rich plasma (PRP) has gained recent interest [11,12]. We have previously demonstrated that autologous PRP significantly enhances ASC proliferation without influencing cell characteristics [12]. PRP contains live platelets able to continually secrete various mitogenic proteins (e.g., growth factors and cytokines) for more than 10 days in cell culture [12]. Among platelet mitogenic proteins, platelet derived growth factor (PDGF-AB), transforming growth factor (TGFβ), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and macrophage migration inhibitory factor (MIF) are thought to be the most involved during *in vitro* cell expansion [13].

Macrophage migration inhibitory factor (MIF) is a 12 kDa pro-inflammatory cytokine known as a regulator of inflammation and immune response [14] and is released mainly by white blood cells when stimulated by bacterial antigens [15]. It is also ubiquitously expressed in various cells and tissues such as pancreatic beta-cells, liver and adipocytes [16,17]. It is known that ASCs [18] and platelets [19] also express MIF upon inflammatory and stress stimulation [20]. ASCs express MIF intracellularly when stimulated. MIF is known to regulate cell proliferation [21-24], migration [17], and differentiation [25]. Addition of recombinant (exogenous) MIF increases proliferation of many cell types such as stromal or progenitor neural cells, fibroblasts, smooth muscle cells, epithelial cells, and even MSCs [21-24]. It is known that both endogenous and exogenous MIF activate AKT signalling, delay cellular senescence, and promote cellular multipotency associated markers in MSCs [26].

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Binding of PDGF-AB and VEGF to PDGF receptors (PDGFR) [27-29], TGFβ to TGFβ receptors I and II (TGFβR I, II), EGF to EGF receptors (EGFR), FGF to FGF receptors (FGFR) [30], and MIF to MIF receptors (CD74, CXCR2, CXCR4) [31] triggers several downstream cascades. Mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase [7], the phosphatidylinositol 3-kinase PI3K/AKT and Smad2/3 are the main signalling pathways involved in ASC proliferation [29,32,33].

However, the link between growth factors present in PRP and proliferation of ASCs has not yet been investigated. Understanding their mechanisms of action and their pathways of controlling ASC destiny would be valuable for conducting tissue-engineering approaches both *in vitro* and *in vivo*. In this study, we focused on the primary signaling cascades that could potentially be involved in enhancing ASC proliferation. We assessed cell proliferation rate by measuring proliferating cell nuclear antigen (PCNA), which is essential for DNA synthesis [34]. We further investigated the link between proliferation and alterations in the G1/S checkpoint of the cell cycle by assessing expression level of cyclin D1, a protein, which is expressed during cell proliferation. We aimed to investigate the growth factors secreted by live platelets presented in culture media supplemented with PRP as well as their primary signalling pathways involved in ASC proliferation.

Materials and Methods

Fat tissue harvesting and ASC isolation

Adipose tissue was collected and purified from the subcutaneous abdomen layer of three consenting patients as described by Coleman [35]. Briefly, 30 mL fat tissue was manually harvested from patients who underwent abdominoplasty with a 3 mm cannula (Mentor, Santa Barbara, CA) connected to 10 mL Luer-Lok syringes (BD Biosciences, Franklin Lakes, NJ). The isolated fat tissue was purified from blood, oil, and liquid after 3 min centrifugation at 1200 g. As previously described [12], to isolate ASCs, the adipose tissue was then digested with 0.01% collagenase type I (Sigma-Aldrich, St. Louis, MO) for 30 to 45 min at 37°C with gentle agitation. After 10 min centrifugation at 500 g, the remaining pellet, known as the stromal vascular fraction (SVF), was suspended in erythrocyte lysis buffer for 5 min (Qiagen, Hilden, Germany) and then washed with the basal medium, Dulbecco's modified Eagle's medium (DMEM; Life technologies), and supplemented with penicillin and streptomycin (10,000 mg/mL; Bioconcept, Salem, NH) and 2 units/mL heparin (Liquemin 5000; Roche, Basel, Switzerland). After centrifugation at 226 g for 5 min, the SVF was then resuspended in DMEM with supplements and filtered through a 100-mm nylon cell strainer (BD Biosciences). The mean cell density in the isolated SVF was 30×10^4 cells/ml.

PRP preparation

PRP was prepared using specific tubes containing 1 mL sodium citrate as an anticoagulant and a specific gel for separating platelets and plasma from other blood components (e.g., red and white blood cells) from three patients as described previously [12]. Briefly, 8 mL of peripheral blood was collected from each patient into a Regen-BCT tube (Regen Kit; RegenLab, Le Mont-sur-Lausanne, Switzerland). To keep the technique completely autologous, for each experiment the blood donor was the same as the fat donor. The collected blood was centrifuged in a standard laboratory centrifuge for 5 min at 1500 g. Hence, the plasma and platelets were accumulated above the gel layer whereas the white and red blood cells were collected in the bottom of the tube under the separator gel. Plasma containing a high yield of platelets was homogenized by turning up and down the tube five times to obtain 4 mL PRP.

ASC culture

Cells from the SVF were plated at 2500 cell/cm^2 in a 48-well plate (BD Biosciences) and cultured with 1 mL of either 10% FBS (Gibco, Carlsbad, CA), used as a control, or 20% PRP as described previously [12]. In both cases, DMEM (Life technologies) was added and supplemented with penicillin and streptomycin (10,000 µg/mL; Bioconcept, NH, USA) and 2 units/mL heparin (Liquemin 5000; Roche, Basel, Switzerland). After 24 to 48 h, a plastic-adherent fraction was obtained, and was determined to be ASCs. Cells were cultivated at 37°C for 10 days in a standard incubator with 5% CO_2 without changing the culture media. ASC were cultured with PRP of the same patient.

Cell proliferation assessment

To assess ASC growth, 5000 cell/cm^2 were plated in 12-well plates (BD Biosciences) and cultured in DMEM (Life Technologies) with penicillin and streptomycin 10,000 µg/mL (Bioconcept, NH, USA) and 2 units/mL heparin (Liquemin 5000; Roche, Basel, Switzerland). Cultures were supplemented with 20% PRP; controls were supplemented with 10% FBS. The total number of cells under each condition was counted using an image-based cytometer (Tali; Invitrogen). The proliferation rate of ASCs was measured through the expression of PCNA using immunocytochemistry analyses according to standard protocols. Briefly, P1 ASCs seeded at 2500 cell/cm^2 with 10% FBS or 20% PRP for 8 days were fixed with 4% paraformaldehyde in Dulbecco's PBS for 30 min at room temperature. After two washes with PBS, fixed cells were blocked in PBS with 1% FBS for an additional 30 min. Cells were then incubated using rabbit anti-human PCNA antibody (Abcam, Cambridge, UK) and held overnight at 4°C. After washing with PBS, cells were incubated with an Alexa 555-conjugated anti-rabbit antibody for 2 h at room temperature. After washing with PBS, cells were incubated for 10 to 15 min with DAPI for cell nuclei detection and mounted in Fluorosave (Calbiochem). Pictures were taken with a fluorescent plate reader paradigm (6021G). Quantification of DAPI (blue) positive nuclei and PCNA positive cells (red) was performed using metamorph software. Results were expressed as the percent red-positive cells to total DAPI-positive cells per unit of area. The calculated area was the complete picture size at 20x magnification.

Growth Factors and MIF measurement

To assess the concentration of growth factors, first passage (P0) ASCs were trypsinized and cultured in 20% PRP for 24 h. The medium was then replaced by 2% PRP to stop cell signaling. After an additional 24 h, the medium was again replaced with 20% PRP and kept for 10 days during which time P1 ASCs were collected at 20 min, 5 days, 8 days, and 10 days. After centrifugation for 3 min at 1500*g*, samples were stored at -20°C until use. The amount of TGFβ, PDGF AB, VEGF, and FGF were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine®, R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions.

To specifically assess the extracellular MIF concentration in the medium, after 10 days' culture, cells were trypsinized and cultured in 10% FBS or 20% PRP for 24 h. The medium was then replaced by 2% FBS or 2% PRP to stop cell signaling. After an additional 24 h, the medium was again replaced with 10% FBS or 20% PRP and kept for 8 days during which time P1 ASCs were collected at 0, 1, 3, 5, and 8 days. Cells were washed with PBS supplemented with 10 mmol/L sodium orthovanadate on ice and then lysed prior to protein analysis

by western blot analysis in Laemmli sample buffer (62.5 mmol/L Tris–HCl, pH 7.4, 1 mmol/L EDTA, 2% (w/v) SDS, 5% (v/v) glycerol, and 1% (v/v) 2-mercaptoethanol). The Duo set ELISA kit (R&D Systems, Minneapolis, MN, USA) was used according to manufacturer's instructions for measuring MIF secretion levels of ASCs from three patients in day 0 after 20 min, day 3, day 5 and day 8 of culture in media supplemented with 10% FBS or 20% PRP.

PDGF receptor (PDGFR), TGFβ receptor (TGFβR)

The expression of PDGFR and TGFβR was determined using immunocytochemistry analyses according to standard protocols as was described in proliferation assessment by PCNA. Rabbit anti-human PDGFR antibody (Abcam, Cambridge, UK) and goat anti-human TGFβRII antibody (Santa Cruz, Texas, USA) were used as a primary antibody. Alexa 555-conjugated anti-rabbit antibody and a donkey 555-conjugated anti-goat antibody were used as secondary antibodies. After two washes with PBS, cells were incubated for 10 to 15 min with DAPI for cell nuclei detection and were mounted in Fluorosave (Calbiochem).

AKT, p-AKT, Erk1/2, p-ERK p/Smad2, GAPDH, cyclinD1, and intracellular MIF analysis

Adipose-derived mesenchymal stem cells cultured 10 days under various conditions were trypsinized and cultured again in 10% FBS or 20% PRP for 24 h at 2500 cell/cm^2. The medium was then replaced by 2% FBS or 2% PRP to stop cell signaling prior to investigating the impact of supplement addition on the signaling of ASCs. After 24 h the medium was again replaced with 10% FBS or 20% PRP and kept for 8 days. At 0, 1, 3, 5, and 8 days, ASCs were lysed and homogenized in Laemmli sample buffer, supplemented with a protease inhibitor cocktail (Roche Diagnostics) and 10 mmol/L phosphatase inhibitors sodium orthovanadate (Sigma) and sodium pyrophosphate (Sigma). Protein concentrations of all samples were determined using the amido black method as described previously (36). Then, 10 µg total proteins were loaded on 8-16% SDS-PAGE gel. Electrophoresed samples were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore) in the presence of 0.01% (w/v) SDS and 20% (v/v) methanol using a constant current of 450 mA for 1 h. The membranes were saturated for 1 h at room temperature in a 10 mmol/L Tris–HCl buffer (pH 7.4) containing 150 mmol/L NaCl, 0.1% (v/v) Tween-20 and 5% BSA, then incubated overnight at 4°C with the following antibodies: AKT, phospho-AKT (p-AKT), Erk1/2, phosphor-Erk1/2 (p-ERK), Smad2 and phospho-Smad2 (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Sigma -Aldrich, St. Louis, MO), cyclin D1 (Santa Cruz Biotech, Texas, U.S.A), and anti-endogenous MIF (a gift of Thierry Roger (University of Lausanne). Detection was performed using HRP-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Amersham Biosciences). Quantifications were then performed using the ChemiDoc XRS from Bio-Rad Laboratories and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Differences between pairs were examined for statistical significance using the *t* test, and values of p less than 0.05 were considered significant. Data are presented as mean ± SEM.

Results

ASC proliferation cultured with 20% PRP

We previously showed that 20% autologous PRP increase ASC proliferation more than other concentrations of PRP [12]. Here we showed that a media supplemented with 20% autologous PRP increased ASC expansion after 10 days of culture. The number of ASCs in 20% PRP versus 10% FBS was 14 times greater (n=14, p<0.001). The proliferation rate of ASCs cultured in media supplemented with 10% FBS or 20% PRP was assessed over 8 days by analyzing PCNA expression. The expression of PCNA was significantly higher in 20% PRP compared to 10% FBS (87.68% vs. 37.07%, respectively) after 3 days' culture (Figure 1A). In 10% FBS, it increased continuously, while in 20% PRP it showed a bell-shaped increase, maximizing on day 3. While PCNA expression of ASCs cultured in 10% FBS increased progressively from day 0 to day 8 (from 28.45% to 83.48%), in 20% PRP it increased faster and significantly from day 0 to day 3 (from 6.38% to 87.68%) and then decreased significantly from day 5 to day 8 (from 68.03% to 9.64%). Consequently, PCNA expression of ASCs cultured in 20% PRP was lower on day 8 compared to those cultured in 10% FBS.

To determine whether the difference in proliferation rate could be a consequence of cyclin D1 expression, its level was analyzed using western blot analysis (Figure 1B). Cyclin D1 expression had a tendency to increase on days 3 and 5 in PRP-treated ASCs.

Proliferation of ASCs cultured in medium supplemented with 10% FBS or 20% PRP.

Adipose-derived mesenchymal stem cells (ASCs) were cultured for 10 days in medium supplemented with 10% fetal bovine serum (gray bars) or 20% platelet-rich plasma (black bars). (**A**) After 20 min, 1 day, 3 days, 5 days, and 8 days, PCNA expression by ASCs was analyzed using immunocytochemistry and quantified using metamorph software. Results were expressed as the percent red-positive cells to total DAPI-positive cells per unit of area. The calculated area is the complete picture size. Magnification is 20x. (B) Cyclin D1 protein was analyzed using western blot analysis. Protein levels were quantified using Quantity-One software and were normalized to GAPDH n=3.

Growth factors implicated in ASCs proliferation cultured with 20% PRP

To determine the level of several growth factors in the cultured media at days 0, 5, 8, and 10 with 20% PRP, TGFβ, PDGF-AB, FGF, and VEGF levels were measured using ELISA. The PDGF-AB and FGF concentrations in the medium had bell-shaped curves, maximizing on day 5 (Figure 2A and 2B). The levels of TGFβ and VEGF continuously increased from day 0 to day 10 and reached their highest secretion levels on day 10 (Figure 2C and 2D).

Assessment of growth factor concentration in medium supplemented with 20% PRP

The secretion of PDGF–AB (**A**), FGF (**B**), TGFβ (**C**), and VEGF (**D**) was assessed using ELISA on days 0, 5, 8, and 10. The medium was not changed during the 10 days, n=3.

MIF secretion and expression in 20% PRP or 10% FBS

To investigate the role of MIF on ASC proliferation, we measured the secretion and the expression levels of MIF in 20% PRP and 10% FBS. Assessment of MIF concentration levels using ELISA showed a significantly higher MIF concentration in 20% PRP compared to 10% FBS on days 0, 3, and 5. Its secretion level in PRP-supplemented medium increased from day 0 to day 3 (0.80 ng/mL to 1.72 ng/mL respectively), maximizing on day 3 (Figure 3A). In contrast, endogenous MIF expression levels were similar during the culture period and showed

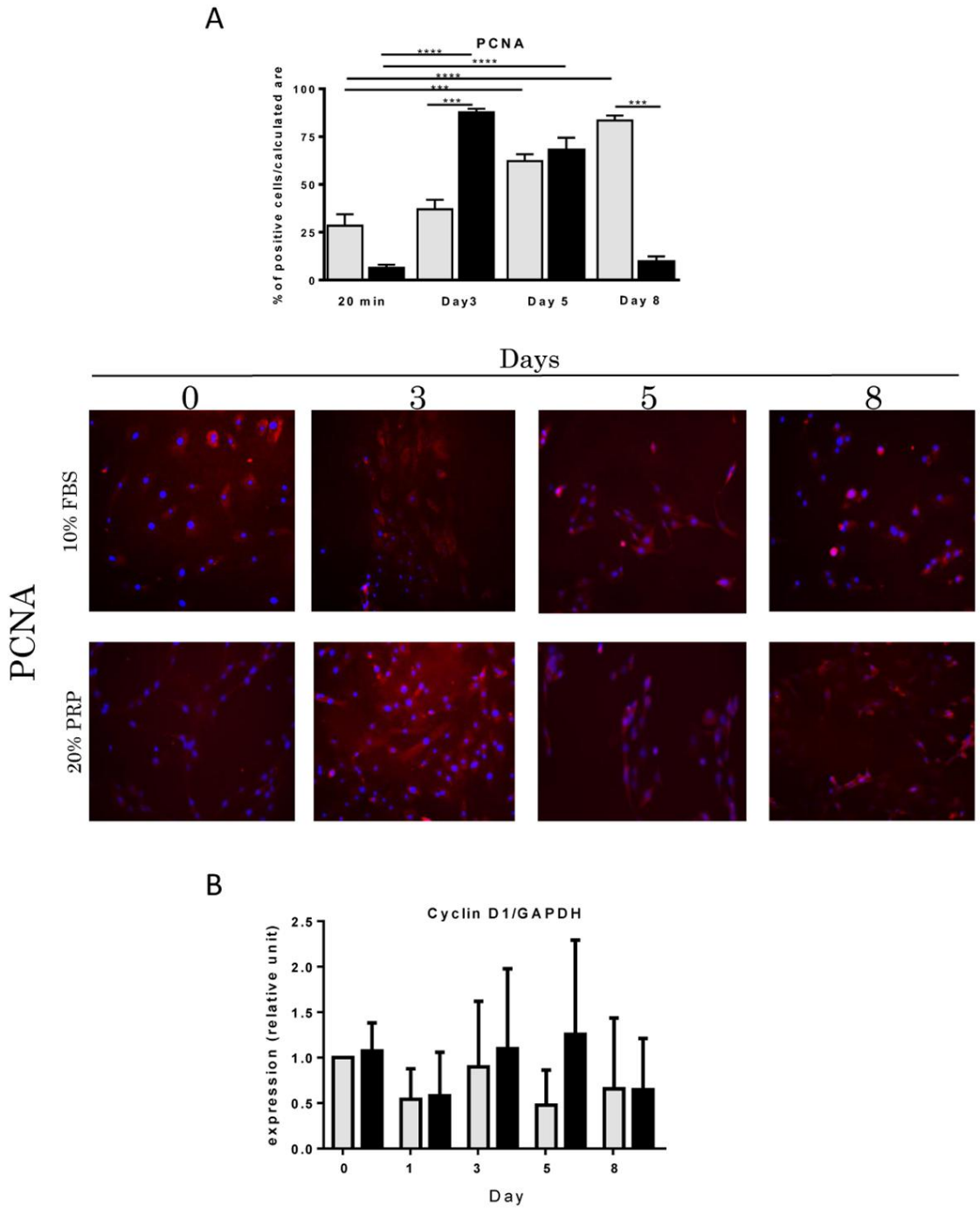

Figure 1: ASC proliferation cultured with 20% PRP.

no significant difference whether cultured with 20% PRP or 10% FBS (Figure 3B).

Assessment of MIF secretion and expression

The secretion of MIF was assessed using ELISA (A) on days 0, 3, 5, and 8 for adipose-derived mesenchymal stem cells cultured with 10% fetal bovine serum (gray bars) and 20% platelet-rich plasma (black bars). The medium was not changed. The endogenous concentration of MIF was assessed using western blot analysis (B) on days 0, 1, 3, 5, and 8. The results were then normalized to GAPDH. At each sample point, results were normalized to day 0, 10% FBS. n=3, *p<0.05 and **p<0.01.

Assessment of the growth factor receptor expression

In order to evaluate the impact of PRP and FBS on primary growth factor receptors, immunocytochemical staining was performed. The expression of the PDGF-AB receptor was similar at

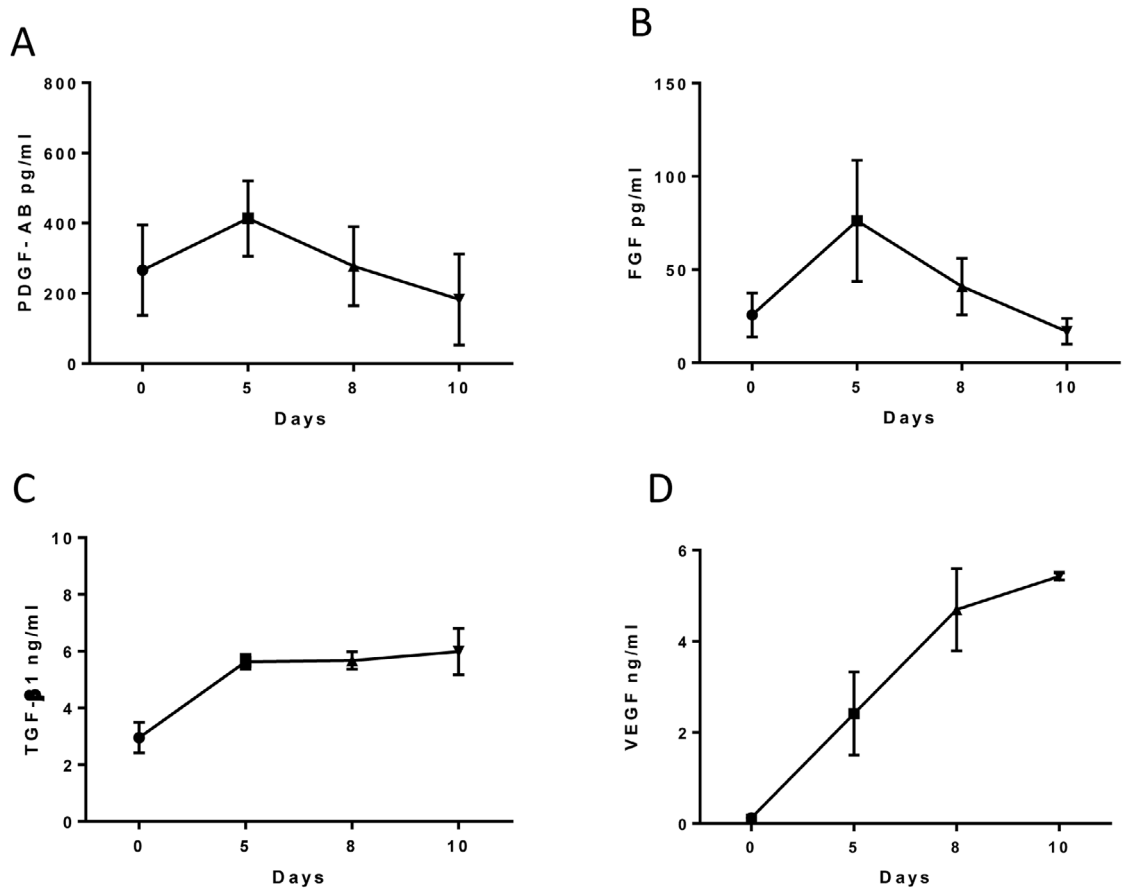

Figure 2: Growth factors implicated in ASCs proliferation cultured with 20% PRP.

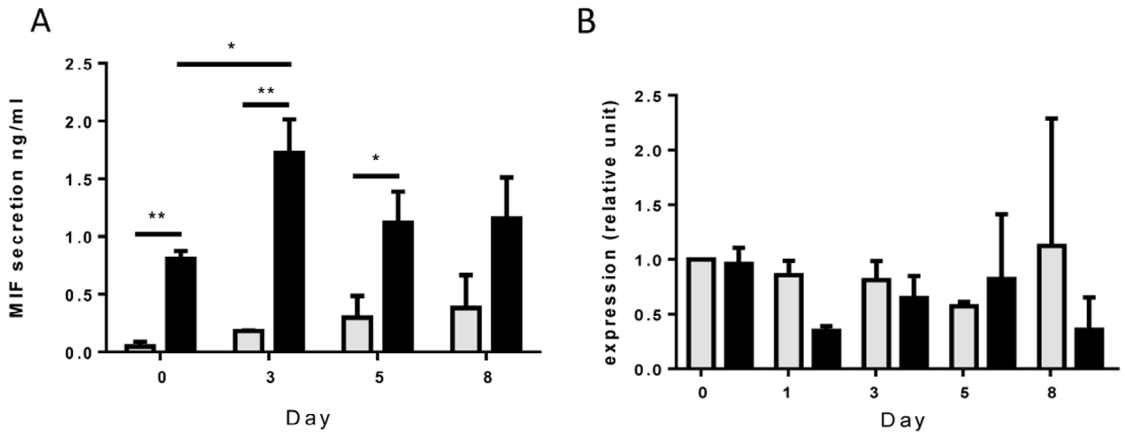

Figure 3: MIF secretion and expression in 20% PRP or 10% FBS.

day 0 (20min) in 20% PRP and 10% FBS (Figure 4A and 4C) and decreased significantly under both conditions on day 8. However, in 20% PRP, PDGF-AB receptor expression was significantly lower than in 10% FBS on day 3, day 5 and day 8 (Figure 4A and 4C). Under both conditions, TGFβ receptor expression increased from day 0 to day 5 and was stable from day 5 to day 8. However, in 20% PRP, its expression was significantly lower than in 10% FBS on day 0, day 3 and day 5 (Figure 4B and 4D).

PDGFR and TGFβR expression in ASCs

The expression of PDGFR and TGFβR were analyzed in adipose-derived mesenchymal stem cells cultured in medium supplemented

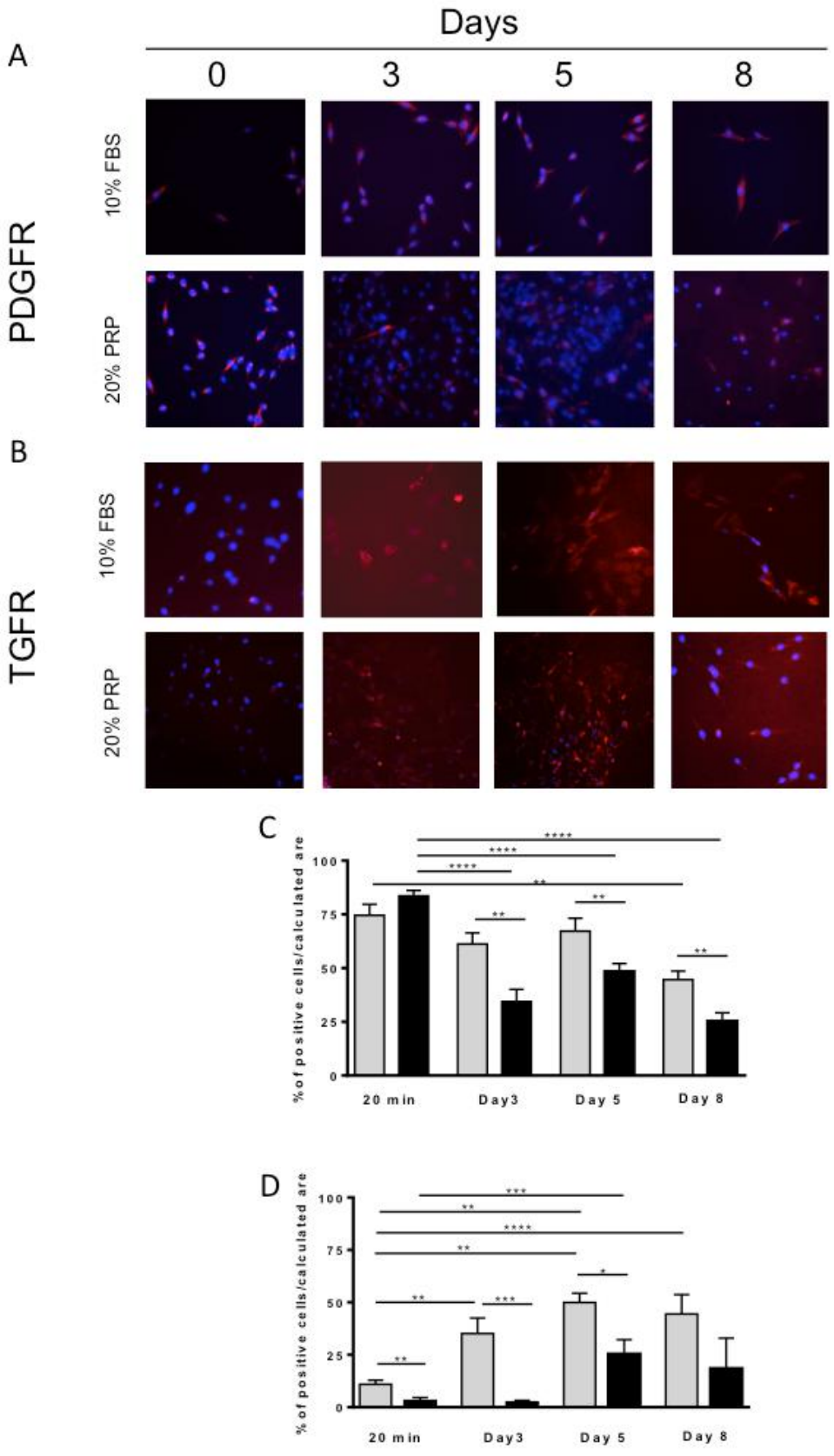

Figure 4: Assessment of the growth factor receptor expression.

with 10% fetal bovine serum (gray bars) or 20% platelet-rich plasma (black bars) after 20 min, 3 days, 5 days, and 8 days. They were fixed, permeabilized, and subjected to immunofluorescence staining for the PDGF-AB receptor (**A**) and the TGFβ receptor (**B**). Quantification of DAPI (blue) positive nuclei and PDGF-ABR (**C**) or TGFβR (**D**) positive cells (red) was performed by metamorph software. Results were expressed as the percent of red-positive cells to total DAPI-positive cells per unit of area. The calculated area is the complete picture size. Magnification is 20x. The medium was not changed through the experiments.

n=3, *p<0.05; **p<0.01, and ***p<0.001.

Activated signaling pathways in ASCs cultured in 20% PRP and 10% FBS

The expressions of molecules implicated in the main signaling cascades that are known to be involved in the proliferation of ASCs were analyzed by western blot analysis. Globally activated pAKT expression had a trend to be higher in 20% PRP than in 10% FBS at all sample points (Figure 5A). The pERK expression levels in 20% PRP tended to be higher than in 10% FBS on days 3, 5, and 8. In 10% FBS, it had a tendency to decrease progressively over time, while in 20% PRP, it increased up to day 3 and then decreased from day 5 to day 8 (Figure 5B). Finally, pSmad2 expression had a tendency to be higher in 20% PRP than in 10% FBS at all sample points (Figure 5C). Expression of pSmad2 did not change from day 0 to day 8 in 10% FBS, but in 20% PRP, it decreased from day 0 to day 3 and then increased from day 3 to day 8. Thus, pSmad2 expression level was higher at days 0 and 3 in 20% PRP than in 10% FBS and tended to also be higher at days 5 and 8.

Activated signaling pathways in cultured ASCs

The expression levels of of pAKT (**A**), pERK (**B**), and pSmad2 (**C**) were assessed for adipose-derived mesenchymal stem cells cultured with 10% fetal bovine serum (FBS, gray bars) or 20% platelet-rich plasma (black bars) at 20 min, 3 days, 5 days, and 8 days using western blot analysis. The results were normalized with total AKT, ERK, and Smad2 respectively. The results at all sample points were then normalized to 20 min FBS. n=3, *p<0.05.

Discussion

Since the 1970s, PRP has been used in the clinic for its regenerative and healing properties [12,36-38]. It has been shown to enhance cell proliferation and differentiation primarily through diverse growth factors secreted by platelets, specifically PDGF-AB, TFGβ, and FGF [39,40]. In our previous study we demonstrated that 20% PRP stimulated MSC growth and decreased their population doubling time when compared to 10%FBS, the traditional medium supplement that is widely used now-a-days for cell culture [12]. Though the stimulatory effect of PRP on ASC survival, proliferation, and *in vitro* expansion has been reported [12,41], the signaling cascades have been poorly investigated.

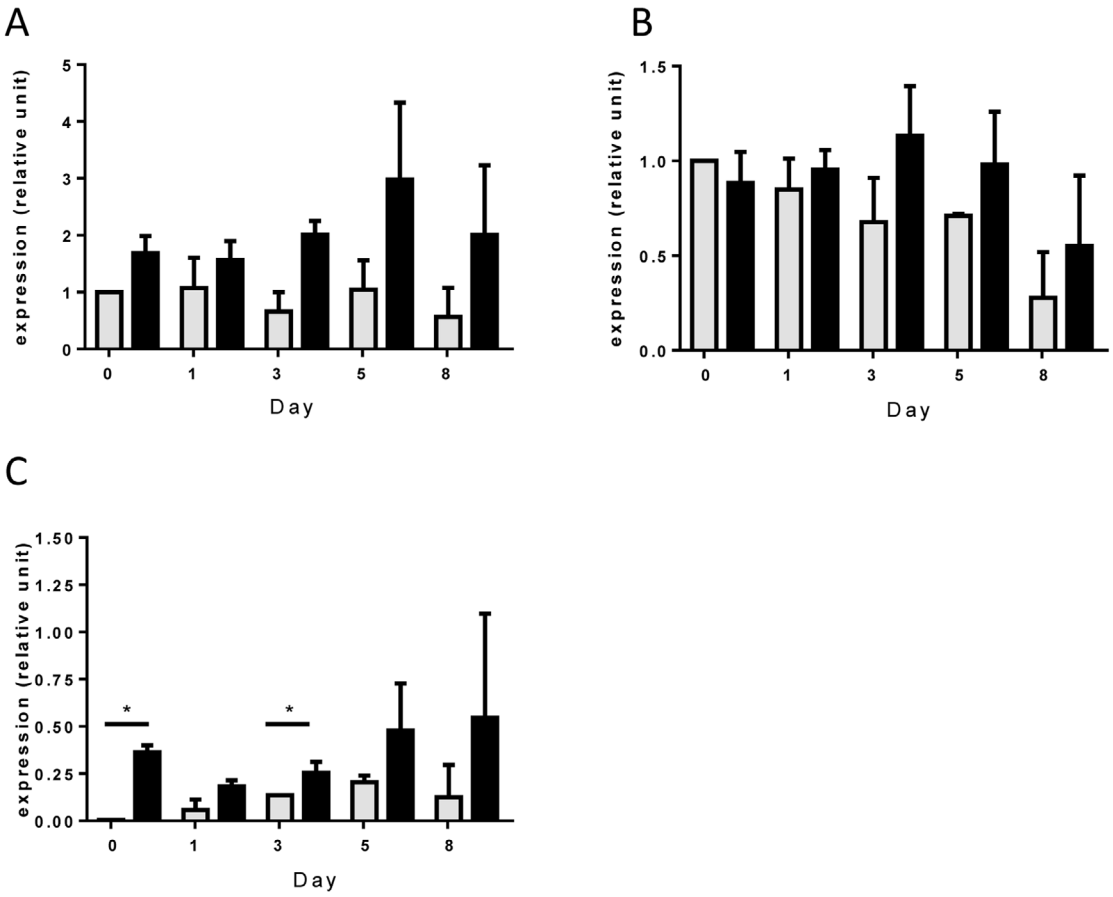

Figure 5: Activated signaling pathways in ASCs cultured in 20% PRP and 10% FBS.

Through this study, we showed that PRP, compared to FBS, increased ASC proliferation signaling pathways (Figure 6).

Primary signaling pathways regulating ASC proliferation

Binding of TGFβ to TGFβR initiates Smad2 signaling pathway; Smad2 forms a heterodimer with Smad3, which then triggers the proliferation of adipose-derived mesenchymal stem cells in the nucleus. Binding of TGFβ and FGF to their cognate receptors triggers the ERK pathway. Phosphorylated ERK then enters the nucleus and activates transcription of cellular proliferation genes. Binding of PDGF, VEGF, FGF, EGF, and MIF to their cognate receptors could initiate the AKT signaling pathway. Phosphorylated AKT prevents the expression of inhibitory proteins of ASC proliferation and activates downstream cell cycle proteins such as cyclin D1.

We observed that the levels of all growth factors studied in the 20% PRP were highest at day 3 to day 5 after culture initiation, when ASC proliferation was most enhanced. Additionally we observed that MIF concentration in the medium supplemented with PRP was significantly higher than in that supplemented with FBS. The primary signals activated in 20% PRP were AKT and Smad2.

As seen in our previous studies showing that the cells proliferated more in 20% PRP, PCNA was expressed significantly more on day 3 in PRP-cultured cells than in FBS-cultured cells. On day 8, the expression of PCNA was significantly lower in PRP-cultured cells. This could be explained by the fact that the culture was already confluent at this time and the cells were not able to proliferate any further. Cyclin D1, a cell cycle progression protein, expressed more in PRP-cultured cells compared to FBS-cultured cells at all sample points, but because of variation between patients, the difference was not statistically significant.

Our results showed that the growth factors are present in the cell culture medium over 8 days.

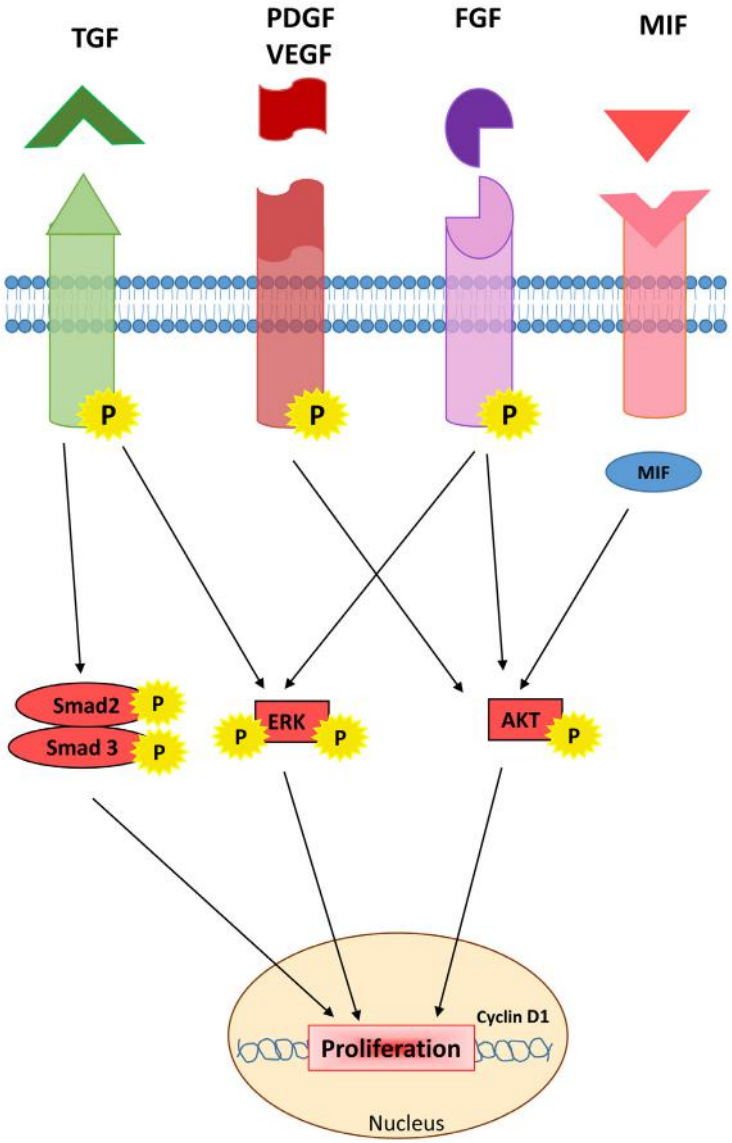

Figure 6: Primary signaling pathways regulating ASC proliferation.

It is documented that MIF secreted by ASCs decreases senescence of ASCs by inducing AKT activation/phosphorylation [26]. Moreover, MIF regulates ASC migration [17] and differentiation [25] through autocrine/paracrine effect. However, to our knowledge, its effect on ASC proliferation has not yet been investigated. We observed that supplementation with PRP, compared to FBS did not change the expression of endogenous MIF by ASCs. However, MIF concentration in 20% PRP medium was higher than in 10% FBS medium before adding them to the cell culture (data not shown). This indicates, for the first time to our knowledge, that PRP produces higher concentrations of MIF compared to FBS. Current studies reveal that the proliferation regulatory role of MIF on neural stem/progenitor cells occurs through activation of AKT signalling pathways [21,22]. We demonstrated that AKT signaling is increased in 20% PRP, and we also assumed that exogenous MIF produced by PRP could play a positive role in proliferation of ASCs.

The effects of PDGF-AB, FGF, TGFβ, and VEGF on ASC proliferation may be controlled through binding to their respective receptors. But recently it has been documented that there is no VEGFR expression in ASCs [12]. Peng et al. reported that VEGF can directly signal through PDGFR [41]. Interestingly, we found that TGFβR and PDGFR showed lower expression in PRP than in FBS. It has been reported that the proliferation of fibroblasts after exposure to mitogen is controlled through the down-regulation of growth factor receptors [42-44]. According to this concept, we can suppose that high concentrations of PDGF-AB, VEGF, and TGFβ in PRP-supplemented media could also provoke a negative feedback loop on ASC receptors.

The binding of various growth factors to their cell surface receptors activates several signaling cascades leading to an intracellular response. Currently, most studies that elucidated signalling pathways of ASC proliferation pointed to the AKT pathway [29,41]. Through this study, the expression of AKT and Smad2 showed a tendency to increase and ERK to decrease at several sample points under both culture conditions. The data could be statistically insignificant because of variations in the growth factor secretion levels in PRP between patients and because of variations in protein expression between patients. Compared to FBS, we observed a higher activation of the AKT pathway in 20% PRP, particularly at day 3, the period when the ASC proliferation rate was the highest. We also observed a higher activation of the Smad2 pathway in 20% PRP compared to FBS. We observed similar expression patterns between PDGF-AB, FGF, and pAKT, and between TGFβ and pSmad2. These results could suggest that binding of PDGF-AB, VEGF, and FGF to their specific receptors stimulates the AKT signaling pathway, while binding of TGFβ to TGFβ receptor stimulates the pSmad2 pathway, as proposed by Rodrigues et al. [33]. Activation of the AKT/Smad2 pathways resulted in enhancing proliferation of ASCs through increased expression of cell cycle progression proteins such as cyclin D1. As reported by Dalton et al. [30], we think that PDGF-AB, FGF, and TGFβ are all required to activate the AKT signaling pathway and therefore ASC proliferation. It is noteworthy to mention that in agreement with Peng et al. [41], who showed that AKT is the main signaling cascade in ASC proliferation; our results indicate that PRP induces proliferation of ASCs mainly through PDGFR/AKT and also through TGFβR/Smad2 signaling pathway. Activation of the EKR1/2 signaling pathway was not significantly different between 20% PRP and 10% FBS. As found by Gharibi et al. [29], our observations indicate that ERK could have a negligible effect on ASC proliferation. In early studies, ERK was shown to be the dominant factor responsible for ASC proliferation in the presence of PDGF-AB [45]. However, recently Ding et al. [46], demonstrated that PDGFR inhibitors do not change the phosphorylation/activation level of ERK.

In summary, based on our results, we postulate that substituting FBS with PRP would not only be safe and biocompatible, but it would also activate many signalling pathways by secreting its cocktail of growth factors. Modulation of these pathways could be a promising approach to safely and efficiently boost the proliferation of ASCs *in vitro* and in the clinic. Further studies would be necessary to characterize the exact effect of MIF on ASC proliferation and migration using MIF inhibitors and recombinant MIF.

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Conflict of Interest

The Regen BCT tubes were generously provided by Regen Lab.

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