Platelet Rich Plasma and Culture Configuration Affect the Matrix Forming Phenotype of Bone Marrow Stromal Cells

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Abstract We aim to examine the influence of platelet rich plasma (PRP) and spatial cues in cartilage/bone matrix forming proteins, and to evaluate the mitotic and chemotactic potential of PRP on human mesenchymal stem cells (hMSCs). Directed cell migration towards PRP gradients was assessed in chemotactic chambers, and recorded by time-lapse microscopy. hMSCs cultured in three-dimensional (3D) scaffolds were visualized by scanning electron microscopy; Hoechst dye was used to confirm cell confluence in 3D-constructs and monolayers before experimental treatment. MSCs were treated with 10% PRP lysate or 10% PRP lysate supplemented with TGF- β -based differentiation medium. The expression of cartilage (COL2A1, Sox9, ACAN, COMP), and bone (COL1A1, VEGF, COL10A1, Runx2) fundamental genes was assessed by real time PCR in monolayers and 3D-constructs. PRP had mitotic ($p < 0.001$), and chemotactic effect on hMSCs, Ralyleigh test $p = 1.02E - 10$. Two and three-week exposure of MSCs to PRP secretome in 3D-constructs or monolayers decreased Sox9 expression ($p < 0.001$ and $p = 0.050$) and COL2A1, ($p = 0.011$ and $p = 0.019$). MSCs in monolayers exposed to PRP showed increased ACAN ($p = 0.050$) and COMP ($p < 0.001$). Adding TGF- β -based differentiation medium to PRP increased COMP, and COL2A1 expression at gene and protein level, but merely in 3D-constructs, $p < 0.001$. TGF- β addition to monolayers reduced Sox9 ($p < 0.001$), aggrecan ($p = 0.004$), and VEGF ($p = 0.004$). Cells exposed to PRP showed no changes in hypertrophy associated genes in either monolayers or 3D-constructs. Our study suggests hMSCs have high-degree of plasticity having the potential to change their matrix-forming phenotype when exposed to PRP and according to spatial configuration.

Keywords Joint repair · Human mesenchymal stem cells (hMSCs) · Platelet rich plasma (PRP) · 3D-cultures

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Abbreviations

2D	2-dimensional
3D	3-dimensional
ACAN	Aggrecan
BM-MSC	Bone marrow-derived stem cells
COL10A1	Type X collagen
COL1A1	Type 1 collagen
COL2A1	Type 2 collagen
COMP	Cartilage oligomeric matrix protein
ECM	Extracellular matrix
GAPDH	Glyceraldehyde-3-phosphate deshydrogenase
IGF	Insulin growth factor
ITS	Insulin transferrin selenium
OD	Optical density

PDGF	Platelet derived growth factor
PLA	Poly(L-lactic acid)
PRP	Platelet rich plasma
SDF-1	Stromal-cell derived factor 1
SEM	Standard error mean
TGF- β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
XTT	(2-methoxy-4-nitro-5-sulfophenyl)-5- [(phenylamino) carbonyl]-2H-tetrazolium hydroxide

1 Introduction

A comprehensive assessment of the state of health in the world (the Global Burden of the Disease launched by the World Bank and the World Health Organization) revealed that osteoarthritis is among the 25 leading causes of healthy life lost, accounting for more than 17 million years lived with disability in 2010 [1]. The latter results in economic substantial costs for affected individuals and societies. Knee is the most commonly affected joint.

Knee osteoarthritis (OA) is no longer a condition of the elderly, and nearly 1.5 million of those with primary total knee replacement are in their 50–60 s [2, 3], and therefore prone to costly revision surgery and other long-term complications. Prevention of OA is challenging because cartilage has poor intrinsic resources for repair, so lesions evolve to progressive joint deterioration. The lack of treatments other than palliative and the need to reduce the impact of joint degeneration has centered the attention on biological therapies.

The first widely accepted regenerative therapy for cartilage repair was autologous chondrocyte implantation (ACI) [4]. However, the needs to harvest healthy cartilage and perform two-step surgery, with a three-week delay, are few among many drawbacks. Moreover, the fact that bone marrow derived mesenchymal stem cells (BM-MSCs) produced similar results than ACI [5] has focused joint repair strategies on the MSCs alternative.

Bone marrow can be harvested by needle-aspiration from the iliac crest, and BM-MSCs are efficiently isolated based on their plastic adhesive properties, and characterized by a distinctive surface profile and trilineage differentiation potential. Despite the rare occurrence of these cells (0.01–0.001%) in the stromal compartment [6], several millions of cells can be obtained after expansion in culture. BM-MSCs have at least tri-lineage differentiation capabilities (bone, fat and cartilage), thus it was presumed that they could engraft, and form new cartilage when implanted either by surgical procedures or simply injected within the joint. To date, adult mesenchymal stromal/stem cell have been implanted to treat focal chondral and osteochondral defects in more than ten studies, and also

they have been injected percutaneously within the joint as a potential remedy for osteoarthritis [7].

Platelet rich plasma (PRP) is often used as an adjuvant for MSC injection or implantation [8–11] because it provides a complex mixture of autologous growth factors, and other cytokines involved in healing mechanisms [12]. In addition, a recent meta-analysis revealed that intraarticular PRP injections provide pain relief (moderate evidence), and functional improvement (limited to moderate evidence [13]. But how PRP plus MSCs can improve joint conditions is intriguing.

Actually, opportunities for developing an effective treatment for the joint will only arise after unveiling enigmas of combination products (i.e. PRP + MSCs). We investigated whether changes induced by PRP can provide a suitable BM-MSC product for cartilage therapy, to be used in implantation or injection procedures. We first examined the influence of PRP in BM-MSC proliferation and chemotaxis. We cultured BM-MSCs in 3D-scaffolds and 2D-conditions, mimicking cell implantation and cell injection therapeutic approaches, with and without PRP and then we examined differences in the expression of ECM molecules that build cartilage, meniscus or bone.

2 Materials and methods

2.1 Bone marrow derived mesenchymal stem cells (BM-MSCs)

Human BM-MSCs, from three adult donors, were purchased from Lonza, (Basel Switzerland). For cell expansion, MSCs were cultured in DMEM GlutaMAX^{TM} plus 1% antibiotics (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St-Louis, Mo, USA) at 37 °C in 5% CO₂. Experiments were performed at passage 4–5.

2.2 Platelet rich plasma preparation

The study protocol was approved by our institutional review board (CEIC Galdakao-Usansolo Ospitalea #13-27). Blood donors provided written-informed consent. PRP was prepared using one-step centrifugation [14]. Briefly, peripheral blood, withdrawn in citrated tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria), was centrifuged at 570 g for 7 min, and the plasma layer was collected avoiding aspiration of the buffy coat. PRP had a moderate enrichment in platelets (2x) and no leukocytes. PRP lysates were prepared by three freeze–thaw cycles performed at –20 °C/37 °C, and subsequent sterile filtration through 0.22 μ m filters (Merck Millipore, Billerica, USA). PRP lysates from twenty donors were pooled to avoid inter-donor variations;

aliquots were made for short-term storage at -20°C . Before PRP use in cell cultures, heparin (Hospira, Lake Forest, IL, USA) was added at a concentration of 2 U/ml.

2.3 Three-dimensional (3D) construct preparation and monolayer cultures (2D)

3D-constructs consisted on MSCs grown at high cell density in poly-lactic acid (PLA) scaffolds. PLA scaffolds were placed in Ultra-Low Attachment Corning® 12 well plates (Corning, NY, USA), and treated overnight with DMEM GlutaMAX™, to augment their hydrophilic capacities and enhance cell-seeding efficacy. For the seeding procedure, 2×10^5 cells were re-suspended in 50 μl , and placed on the scaffold and kept for 2 h at 37°C in 5% CO_2 to allow cell attachment. Once cells had adhered, wells were filled with complete growth medium.

Cell adhesion and scaffold/cell interactions were examined using scanning electron microscopy (SEM). For SEM observation, scaffolds were washed twice with PBS, fixed with 2% glutaraldehyde in cacodylate buffer (0.1 M pH 7.4) 2 h at room temperature and post-fixed in 1% OsO_4 for 1 h. Then, they were washed and subjected to graded ethanol dehydration, and dried through the CO_2 critical point. Finally, samples were sputter-coated with a thin layer of gold and observed in a Hitachi SEM (Hitachi S-3400 N). The voltage used was 15.0 kV.

Hoechst staining was achieved by adding 1 $\mu\text{g/ml}$ to the wells containing the scaffolds, incubated 4 h at 37°C and 5% CO_2 and cells were observed using a Nikon Eclipse T-2000 (Nikon, Tokyo, Japan).

For 2D cultures, cells were harvested by trypsinization (TrypLE™ Select, Gibco, Life Technologies, Carlsbad, CA, USA), counted in a Bürker Chamber (ThermoFisher Scientific, Waltham, MA, USA) seeded in 12 well plates (Corning, NY, USA) at a density of 2×10^5 cells/ cm^2 , and were incubated until a monolayer was observed in an optic phase contrast microscope (Nikon, Tokyo, Japan).

2.4 Cell treatments

Three-dimensional constructs and monolayers were exposed to PRP, or to PRP supplemented with 10 ng/ml TGF- β 1 (plus 10^{-7} M dexamethasone and 50 $\mu\text{g/ml}$ ascorbic acid) for 14 and 21 days. Controls were cultured with DMEM and ITS + 1 (Insulin–transferrin–sodium selenite, linoleic-BSA) supplement (Sigma Aldrich, St-Louis, Mo, USA). Media and supplements were changed every 3 days.

2.5 Chemotaxis assay with PRP

Chemotaxis experiments were performed in μ -Slide Chemotaxis 3D chamber (Ibidi GmbH, Martinsried, Germany).

Cells were counted using an automatic counter (TC10 Automated Cell Counter, BioRad, Hercules, California, USA) using Trypan blue (Sigma, St Louis, Mo, USA) exclusion, and 12,000 viable cells suspended in 6 μl of DMEM GlutaMAX™ supplemented with 2% FBS were introduced in the chambers. The latter were incubated for 2 h at 37°C , in a 5% CO_2 atmosphere to allow cell attachment. Afterward, one reservoir was filled with PRP or ITS and the opposite with DMEM. Additionally, we set positive (ITS/ITS or PRP/PRP) and negative controls (DMEM/DMEM). Time-lapse microscopy (Nikon Eclipse TE2000-E, Tokyo, Japan) was performed over a period of 24 h with a 10 min interval between images (ORCA ER camera, Hamamatsu, Japan). Data were imported into ImageJ software (National Institutes of Health, Bethesda, USA). Minimum 30 cells were tracked in each experiment using the “Manual Tracking” plugin (Fabrice P. Cordelières, Institut Curie, Orsay, France). The ImageJ “Chemotaxis and Migration Tool” plugin (Ibidi GmbH, Martinsried, Germany) was used to calculate the center of mass (COM), forward migration indices (FMI) in directions parallel and perpendicular to the gradient, velocity ($\mu\text{m/h}$), directness (μm), and significance using Rayleigh test. In addition to trajectory and velocity, these parameters describe the tendency of cells to travel towards the chemotactic gradient.

2.6 XTT assays

The viability and proliferation was examined using XTT ((2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) colorimetric assay (Cell Proliferation Assay Kit, Roche, Basel, Switzerland). Cells were seeded in 96 micro-well plates (Corning, NY, USA) at a density of 4000 cells/ cm^2 , and the number of metabolically active cells was assessed at 0, 24, 48, 72 and 96 h, following manufacturer’s instructions. Color developed in micro-wells was determined using a plate reader (PolarStar Omega, BMG Labtech, Offenburg, Germany) at 470 nm.

2.7 RNA extraction and RT-PCR

To extract RNA from the constructs, they were frozen at -80°C in 50 ml Falcon tubes and next 500 μl of High Pure RNA Isolation Kit Lysis Buffer was added. The constructs were incubated for 15–20 min with continuous vortexing, and the supernatant was collected. In parallel, trypsin was added to 2D cultures and pellets were obtained centrifuging cells at 1500 rpm for 5 min. Total RNA from 3D-constructs and monolayers were extracted using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) following manufacturer instructions. Total RNA

concentration and purity was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). 1 µg from each RNA sample was reverse transcribed using the SuperScript® III First-Strand Synthesis System (Invitrogen). Real time PCR was performed in triplicates using SYBR Green PCR Master mix (Applied Biosystems), on 7900HT Fast Real-Time PCR System (Applied Biosystems). For normalization, GAPDH gene expression was used. Primer sequences are available upon request.

2.8 In-cell western

In-Cell Western assay was performed to evaluate type 1- and 2- collagen expression in MSCs exposed to PRP plus TGF-β for 21 days in 3D conditions. Paraformaldehyde (4%) was used for fixation during 20 min at room temperature. Cells were then permeabilized (PBS + Triton 0.1%) and blocked in Odyssey Blocking buffer during 1 h at room temperature. Mouse monoclonal primary antibodies diluted in Odyssey Blocking buffer were added to scaffolds to detect either Type 1 Collagen (abcam, ab6308) or Type 2 Collagen (abcam, ab3092) expression, and incubated at 4 °C overnight. After washing the scaffolds (PBS + Tween 20 0.1%) an infrared secondary goat anti-mouse antibody (IRDye800CW) diluted in Odyssey blocking buffer + Tween 20 0.2% was added, and incubated for 1 h at room temperature. CellTag 700 was used to normalize cell number on scaffolds. Scaffolds were then washed and immediately scanned. Results were analyzed using the In-Cell Western Odyssey software (LI-COR, Lincoln, Nebraska).

2.9 Statistical analyses

Chemotaxis and XTT data represent mean and mean standard error. One-way ANOVA was used for comparisons. All RT-PCR data is presented as median and 25–75 percentiles of BM-MSCs from three different donors, and repeated experiments. For analysis of gene expression profiles, fold changes were analyzed by the Mann-Whitney U test. $p \leq 0.050$ was considered statistically significant. Data were analyzed using SPSS for windows, version 18.0, SPSS (SPSS Inc, Chicago, IL USA).

3 Results

After cell seeding MSCs established interactions with PLA scaffold in all planes (shown in Fig. 1A). Before starting the cell treatments, confluence in monolayers and 3D-constructs was confirmed by Hoechst dye. The experiment was carried out as depicted in Fig. 1B.

Fig. 1 **A** Representative view of the PLA scaffold seeded with MSCs at scanning electron microscopy; note that cells are scattered inside the scaffold in all planes and that cells are in close contact with the scaffold. **B** Schematic diagram illustrating the design of our experiment after confluence in three-dimensional constructs and monolayers was achieved

3.1 PRP has chemotactic and proliferative effects on BM-MSCS

As shown by the Rayleigh test that discriminates arbitrary movement from directed migration, PRP but not ITS is chemotactic for MSCs, $p = 1.02E - 10$ and $p = 0.803$, respectively. Cell velocity and directness was similar in PRP, and ITS gradients but higher than the negative control (DMEM/DMEM), $p < 0.001$ for both. Table 1 shows the main parameters describing cell movement in each experimental condition. Figure 2A shows representative trajectory plots of BM-MSCs migrating towards a gradient of PRP.

As expected, MSCs proliferate significantly with PRP compared to controls (ITS supplement), $p < 0.001$. Addition of 10 ng/ml TGF-β1 did not modify the proliferative potential of the molecular pool present in PRP lysates.

Table 1 Main parameters describing cell movement in the time period from 0 to 24 h: Euclidean distance, distance (μm) between two points, cell in time zero and cell after 24 h; accumulated distance represents the sum of all incremental movements

Parameters	PRP versus DMEM	PRP versus PRP (+/+)	ITS versus DMEM	DMEM versus DMEM (–/–)
Mean velocity (SD) $\mu\text{m}/\text{min}$	0.26 (± 0.08)	0.27 (± 0.07)	0.28 (± 0.08)	0.19 (± 0.06)
Mean accumulated distance (μm)	312 (± 98)	329 (± 86)	211 (± 58)	231.2 (± 70)
Mean Euclidean distance (μm)	196 (± 107)	250 (± 89)	120 (± 57)	81 (± 48)
Center of mass (μm)	185	29	16	21
Rayleigh test	$p = 1.02\text{E} - 10$	$p = 0.901$	$p = 0.803$	$p = 0.412$
FMI II	0.57	0.05	0.05	0.04
FMI I	–0.01	–0.01	–0.07	–0.05

Accordingly, TGF- β 1 individually has no effect on proliferation (Fig. 2B).

3.2 PRP affects the matrix-forming phenotype of BM-MSCs in 3D-constructs

In 3D high-density cell cultures, PRP lysate down regulated the expression of Sox9 by five fold at 14 days and 21 days ($p = 0.004$ and $p < 0.001$, respectively), and markedly decreased the expression of COL2A1 by 11-fold and 20-fold at 14 days and 21 days ($p = 0.031$ and $p = 0.011$). ACAN and COMP did not change significantly in these experimental conditions (Fig. 3A).

PRP lysate did not induce changes in cell hypertrophy-related genes, including COL1A1, VEGF, COL10A1 and Runx2 (Fig. 3B).

3.2.1 PRP + TGF- β based differentiation medium

Priming BM-MSCs with 10 ng/ml TGF- β 1 (plus 10^{-7} M dexamethasone and 50 $\mu\text{g}/\text{ml}$ ascorbic acid) in the molecular context of PRP increased COL2A1 at 21 days, $p < 0.001$, and COMP at 14 days, $p < 0.001$ (Fig. 4A). Instead, TGF- β 1 (plus 10^{-7} M dexamethasone and 50 $\mu\text{g}/\text{ml}$ ascorbic acid) did not modify hypertrophy-related genes (Fig. 4B). The expression of type 1- and type 2-collagen under these conditions was also confirmed at the protein level (Fig. 4C).

3.3 PRP affects the matrix-forming phenotype of BM-MSCs in high-density 2D-cultures

COL2A1 expression is reduced by fourfold in PRP treated MSCs at 21 days, $p < 0.019$; Sox9 decreased by two fold at both time points, $p = 0.024$ and $p = 0.050$. On the other hand, ACAN increased by three fold ($p = 0.050$), and COMP increased by three fold at 14 days, $p < 0.001$ and by six fold at 21 days, $p < 0.001$. The expression of molecules related to cell hypertrophy including COL1A1,

VEGF, COL10A1 and Runx2 were not modified by PRP lysates in monolayers (Fig. 5).

3.4 PRP + TGF- β 1 based differentiation medium in monolayers

Addition of 10 ng/ml TGF- β 1 (plus 10^{-7} M dexamethasone and 50 $\mu\text{g}/\text{ml}$ ascorbic acid) in monolayer cultures decreased Sox9 at 14 days and 21 days, $p < 0.001$ and $p = 0.001$ Respectively (Fig. 6A). ACAN and VEGF decreased by two fold at 14 days, $p = 0.004$ (Fig. 6B).

3.5 Culture configuration (2D versus 3D) affects the matrix-forming phenotype of BM-MSCs

Cells cultured for 14 days in monolayers with PRP showed higher expression of COL2A1 ($p = 0.014$) and Sox9 ($p = 0.008$) than in 3D-constructs. However, we could not confirm this difference after 21 days. Concurrently, the expression of COMP and COL1A1 were increased in monolayers by seven and six fold, at 14 and 21 days, $p < 0.001$, and by three fold, $p = 0.003$ and $p < 0.001$, respectively (Fig. 7).

4 Discussion

Percutaneous injection or surgical implantation of cultured MSCs has become a focus of research in joint repair strategies. Moreover, PRP is frequently used as supplement during MSC expansion, *in vitro*, before implantation [15]. In addition, PRP is used as a vehicle in direct MSCs injections, and also to encourage cell implantation, and new tissue formation [7]. However, unpredictable tissue type and quality is a major challenge after MSC injection or implantation with or without PRP.

We addressed this issue by examining the matrix forming phenotype of human MSCs, and showed that the expression of COL2A1 and Sox9 (crucial transcription

Fig. 2 Chemotaxis and proliferation. **A** Chemotaxis: Representative plot of MSCs migrating towards PRP gradient (*upper left*, PRP/DMEM); positive control for PRP showing cells migrating towards PRP gradients (*upper right*, PRP/PRP); ITS supplement did not show chemotactic properties (*down left*, ITS/DMEM); negative control showing that cells migrate in arbitrary directions (DMEM/DMEM). **B** Cell proliferation was assessed at 4000 cell/cm² starting cell density using the XTT assay. Results show mean $\pm$ SEM from three repeated experiments, ^{a,b} $p < 0.001$ compared to **A** ITS or **B** ITS + TGF- β 1. MSCs, mesenchymal stem cells; PRP platelet rich plasma, ITS insulin transferrin selenium, DMEM culture media, TGF- β 1 transforming growth factor beta 1, SEM standard error of the mean

factor controlling chondrogenesis) were decreased significantly by PRP. These results were found in MSCs cultured at high-density in both, monolayers and 3D-constructs.

Cell–cell contact and 3D-configuration might be necessary, but are not sufficient to induce chondrogenesis in

our experimental conditions. Moreover, addition of PRP did not recreate the composition of hyaline cartilage in terms of COL2A1 expression. Besides, the expression of Sox9 was reduced by PRP in both culture systems. Reduced COL2A1 and Sox9 expression is not unusual in

Fig. 3 Matrix-forming phenotype of MSCs cultured with PRP in 3D-scaffolds was displayed relative to MSCs cultured with media supplemented with ITS in 3D-scaffolds. **A** PRP modulates expression of cartilage matrix forming genes, including COL2A1, and Sox9. Aggrecan (ACAN) and COMP remained unchanged. **B** PRP does not modify the expression of bone matrix forming genes, including COL1A1 ($p = 0.057$), VEGF, COL10A1 and Runx2. Boxplots shows median and 25–75 percentiles, $*p \leq 0.05$; $**p < 0.01$; $***p < 0.001$

cell culture models that do not involve micro-mass pellets [16]. The latter enhance chondrogenesis by mimicking multicellular structures analogous to cartilaginous condensations found in embryonic cartilage development [17]. Actually, rabbit MSCs encapsulated in PRP hydrogels showed enhanced chondrogenesis [18]. Accordingly, canine MSCs encapsulated in alginate showed enhanced chondrogenesis with adjuvancy of PRP [19]. But as shown here, high-density MSCs in monolayers or 3D-constructs do not seem to be committed to chondrogenesis or osteogenesis in our experimental conditions.

These two culture models (2D and 3D) can be relevant to clinical practice because ordinarily, MSCs are injected intraarticularly after 2D expansion for two to three weeks. Alternatively these expanded cells can be implanted following specific surgical procedures, and 3D-configuration *per se* could enhance the expression of cartilaginous matrix molecules [20].

Typically, optimized differentiation media for chondroinduction are supplemented with TGF- β based cocktails, which favor chondrogenesis through Smad3 and Wnt-associated b-catenin signaling [21]. Interestingly, adding exogenous TGF- β 1 (plus dexamethasone and ascorbic acid) to PRP cultures enhanced COL2A1 expression, although merely in 3D cultures. PRP contains significant concentrations of TGF- β 1 (typically about 20–30 ng/ml in our pure PRP) [22]. Supplementing cell cultures with 10% PRP, which involves 2–3 ng/ml, may be insufficient to induce chondrogenesis. According to the present results, concentrations above 10 ng/ml can induce COL2A1 expression in the molecular context of PRP. TGF- β 1 is a pleiotropic cytokine, and its biological effects are concentration and context dependent. Remarkably, supplementing ITS cultures with 10 ng/ml TGF- β 1 in 3D-constructs did not enhance chondrogenesis, instead decreased COL2A1 (data not shown).

Whether MSCs can help in forming hyaline cartilage is uncertain. The best *in vitro* procedure to prime MSCs to hyaline cartilage commitment has been investigated in the past decades, but controlling details are not completely understood [23]. Not only controlling spatial cues, but also stimulating cells with dynamic molecular microenvironments is paramount [24]. Other authors [25] suggest that the presence of chondrocytes in MSC cultures can help enhance chondroinduction through upregulation of Sox9, COL2A1 and ACAN. In parallel, genes involved in hypertrophy (Runx2 and COL10A1) were down regulated in these experiments.

The joint is an organ composed of different tissues, including cartilage, synovium meniscal fibrocartilage, ligaments and subchondral bone. Whether injected MSC target preferentially some tissues over others is unknown. Actually, straightforward intraarticular injections have been used with relative successful outcomes to target meniscus [26], or cartilage [27, 28]. Therefore undifferentiated MSCs may be desired to fulfill individual joint requirements. In fact, Vaugnness et al. [26] in a randomized clinical trial, reported increased meniscal volume in some of the patients treated with allogeneic MSCs injections following subtotal meniscectomy.

Osteoarthritic joints are prone to form osteophytes, a matter of concern when injecting MSCs with trilineage differentiation capabilities. In this regard, Gelse et al. [29] have shown molecular differences between osteophyte cartilage and articular cartilage, using microarray technologies. Here we show that the pattern of gene expression of MSC phenotype when exposed to PRP secretome is clearly different to the osteophyte phenotype. In fact, osteophyte cells showed enhanced expression of Runx2 (transcription factor controlling osteogenesis) and COL1A1, and neither gene is overexpressed with PRP.

Fig. 4 Matrix-forming phenotype of MSCs cultured with PRP + TGF- β (plus dexamethasone and ascorbic) was displayed relative to MSCs cultured with PRP in 3D-constructs, 2 $^{-\Delta\Delta Ct}$. **A** Addition of TGF- β based differentiation medium enhanced COL2A1 (21 days), and COMP (14 days) expression. **B** Cell hypertrophy related genes (COL1A1, VEGF, COL10A1 and Runx2) remained unchanged. **C** In-Cell Western assay was performed to assess qualitatively type 1 and 2 collagens. Fluorescence intensity showed that both proteins, type 1 and 2 collagens, were present within the cells after 21 days of PRP plus TGF- β treatment. *Boxplots* shows median and 25–75 percentiles, *** $p < 0.001$

MSCs showed relevant expression of COMP, and PRP further enhanced COMP expression in monolayer cultures at 14 and 21 days. COMP is a pentameric protein of the thrombospondin family present in mechanically loaded tissues including tendon, cartilage, and meniscus. COMP catalyzes fibrillogenesis, stabilizes and maintains the fibrillar structures. Elevated levels of COMP are found in the

synovial fluid of patients with osteoarticular pathology. Fragmentation of COMP is found during inflammatory processes and disease stage specific differential cleavages have been reported in osteoarthritis [30].

PRP intraarticular injections are the simplest regenerative medicine intervention for joint conditions. Positive effects rely not only on the anti-inflammatory effects.

Fig. 5 Matrix-forming phenotype of MSCs cultured in monolayers with PRP is displayed relative to MSCs cultured in monolayers with serum-free media (supplemented with ITS) ($2^{-\Delta\Delta Ct}$). **A** PRP modified the expression of COL2A1, Sox9, ACAN, and COMP in MSCs cultured in 2D-monolayers. **B** PRP does not alter the expression of bone matrix forming genes (COL1A1, VEGF, COL10A1 and Runx2) in these experimental conditions. *Boxplots* shows median and 25–75 percentiles * $p \leq 0.05$; *** $p < 0.001$

Importantly, PRP supports MSCs chemotaxis [31]. Synergic effects of cytokines, including SDF-1 α and PDGF, are responsible for PRP chemotactism. This feature is especially relevant when cartilage conditions are treated by microfracture or drilling procedures aiming to stimulate joint regeneration supported by the mobilization of endogenous subchondral progenitor cells. In addition to suchondral bone marrow, several other MSC niches have been identified in the joint organ including Hoffa fat, synovium, and pericyte cells [32]. Chemotactic properties of PRP can help in mobilizing MSCs from the endogenous niches to colonize injured tissue. This effect is synergic with the mitotic and anabolic potentials of PRP in enhancing tissue repair embodied by growth factors such as IGF-1, and TGF- β 1.

With the present study we have addressed the commitment to the musculoskeletal lineages of the hMSCs in 2D and 3D culture situation in an attempt to emulate the

Fig. 6 Matrix-forming phenotype of MSCs cultured with PRP + TGF- β (plus dexamethasone and ascorbic) in monolayers is displayed relative to MSCs cultured in monolayers with PRP, $2^{-\Delta\Delta Ct}$. **A–B** TGF- β 1 (plus dexamethasone and ascorbic) decreased Sox9 at 14 and 21 days in the PRP context. Furthermore, ACAN and VEGF were down regulated at 14 days. *Boxplots* shows median and 25–75 percentiles. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$

percutaneous (intraarticular) injection (2D) and cell implantation (3D) approaches that are being used as advanced therapies for the osteoarticular defects. The obtained results have shown how the spatial configuration and the addition of PRP and/or TGF- β has an impact in the expression of some fundamental transcription factors, and matrix forming elements of the osteoarticular tissues.

4.1 Limitations

One limitation of the present study is the choice of the rigid synthetic PLA scaffold. Although PLA is a biocompatible material, initially used in the manufacturing of resorbable sutures, other malleable, collagen-based natural scaffolds can be more appropriate to approach surgical techniques of cell implantation. However, 3D-PLA constructs could better mimic *in vitro* the behavior of MSC in a hard osteoarticular surface.

Fig. 7 Matrix-forming phenotype of MSCs in 2D monolayers is displayed relative to 3D-constructs, both supplemented with PRP **A** At 14 days COL2A1, and SOX9, are up-regulated in monolayers relative to 3D-constructs. **B** COMP and COL1A1 are increased in 2D at both time points. The relative gene expression values, $2^{-\Delta\Delta C_t}$ were plotted in a boxplot showing the median and 25–75 percentiles. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$

Furthermore, we have performed all cultures in normoxia, but we are aware that to better mimic the *in vivo* joint environment, hypoxia along with mechanical stimulation has to be included in the *in vitro* models [33].

Because MSCs can function by paracrine mechanisms, modulating inflammation and angiogenesis *in vivo*, another limitation of this study is that we have not assessed the angiogenic and inflammatory status of MSCs. The latter could provide further insights into MSC biology in these experimental conditions.

Our results are only valid for pure PRP formulations [34]. The presence of high concentration of leukocytes in the PRP, i.e. L-PRP, could induce changes in MSC commitment that have not been explored in this study. Further experimental work is needed to explore dose-effects of TGF- β ideally in the context of different PRP formulations.

The 2D culture compared to our 3D-construct showed a limited commitment to musculoskeletal lineages in spite of the clear benefits observed as improved functionality, pain relief and increase in cartilage quality observed in clinical

trials based on intra-articular injections of MSCs cultured in 2D-conditions [26, 35].

The combination of TGF- β and PRP (but not TGF- β alone) in our 3D-model induces the expression of the established hyaline cartilage marker COL2 at RNA and protein level. The presence of PRP regulates expansion and migration but not the musculoskeletal commitment of MSCs. However, TGF- β 1 supplemented PRP combined with MSCs could provide greater benefits than the cells alone to treat joint conditions.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical statement This study was approved by our institutional review board (CEIC Galdakao-Usansolo Ospitalea #13-27).

References

- Murray CJ, Lopez AD. Measuring the global burden of disease. *N Engl J Med*. 2013;369:448–57.
- Losina E, Weinstein AM, Reichmann WM, Burbine SA, Solomon DH, Daigle ME, et al. Lifetime risk and age at diagnosis of symptomatic knee osteoarthritis in the US. *Arthritis Care Res (Hoboken)*. 2013;65:703–11.
- Andia I, Maffulli N. Meeting current musculoskeletal health demand through deeper insights into tissue homeostasis and regeneration. *Expert Opin Biol Ther*. 2015;15:767–71.
- Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med*. 1994;331:889–95.
- Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH. Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. *Am J Sports Med*. 2010;38:1110–6.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143–7.
- Pak J, Lee JH, Kartolo WA, Lee SH. Cartilage regeneration in human with adipose tissue derived stem cells: current status in clinical implications. *Biomed Res Int*. 2016;2016:4702674.
- Kim YS, Kwon OR, Choi YJ, Suh DS, Heo DB, Koh YG. Comparative matched-pair analysis of the injection versus implantation of mesenchymal stem cells for knee osteoarthritis. *Am J Sports Med*. 2015;43:2738–46.
- Koh YG, Jo SB, Kwon OR, Suh DS, Lee SW, Park SH, et al. Mesenchymal stem cell injections improve symptoms of knee osteoarthritis. *Arthroscopy*. 2013;29:748–55.
- Pak J, Chang JJ, Lee JH, Lee SH. Safety reporting on implantation of autologous adipose tissue-derived stem cells with platelet-rich plasma into human articular joints. *BMC Musculoskelet Disord*. 2013;14:337.
- Buda R, Vannini F, Castagnini F, Cavallo M, Ruffilli A, Ramponi L, et al. Regenerative treatment in osteochondral lesions of the talus:

- autologous chondrocyte implantation versus one-step bone marrow derived cells transplantation. *Int Orthop*. 2015;39:893–900.
12. Andia I, Maffulli N. Platelet-rich plasma for managing pain and inflammation in osteoarthritis. *Nat Rev Rheumatol*. 2013;9:721–30.
  13. Laudy AB, Bakker EW, Rekers M, Moen MH. Efficacy of platelet-rich plasma injections in osteoarthritis of the knee: a systematic review and meta-analysis. *Br J Sports Med*. 2015;49:657–72.
  14. Andia I, Rubio-Azpeitia E, Maffulli N. Platelet-rich plasma modulates the secretion of inflammatory/angiogenic proteins by inflamed tenocytes. *Clin Orthop Relat Res*. 2015;473:1624–34.
  15. Rubio-Azpeitia E, Andia I. Partnership between platelet-rich plasma and mesenchymal stem cells: in vitro experience. *Muscles Ligaments Tendons J*. 2014;4:52–62.
  16. Goude MC, McDevitt TC, Temenoff JS. Chondroitin sulfate microparticles modulate transforming growth factor- β 1-induced chondrogenesis of human mesenchymal stem cell spheroids. *Cells Tissues Organs*. 2014;199:117–30.
  17. Klumpers DD, Smit TH, Mooney DJ. The effect of growth-mimicking continuous strain on the early stages of skeletal development in micromass culture. *PLoS One*. 2015;10:e0124948.
  18. Moroz A, Bittencourt RA, Almeida RP, Felisbino SL, Deffune E. Platelet lysate 3D scaffold supports mesenchymal stem cell chondrogenesis: an improved approach in cartilage tissue engineering. *Platelets*. 2013;24:219–25.
  19. Elder S, Thomason J. Effect of platelet-rich plasma on chondrogenic differentiation in three-dimensional culture. *Open Orthop J*. 2014;8:78–84.
  20. Beitzel K, McCarthy MB, Cote MP, Durant TJ, Chowanec DM, Solovyova O, et al. Properties of biologic scaffolds and their response to mesenchymal stem cells. *Arthroscopy*. 2014;30:289–98.
  21. Jian H, Shen X, Liu I, Semenov M, He X, Wang XF. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. *Genes Dev*. 2006;20:666–74.
  22. Anitua E, Sánchez M, de la Fuente M, Azofra J, Zalduendo M, Aguirre JJ, et al. Relationship between investigative biomarkers and radiographic grading in patients with knee osteoarthritis. *Int J Rheumatol*. 2009;2009:747432.
  23. Somoza RA, Welter JF, Correa D, Caplan AI. Chondrogenic differentiation of mesenchymal stem cells: challenges and unfulfilled expectations. *Tissue Eng Part B Rev*. 2014;20:596–608.
  24. Correa D, Somoza RA, Lin P, Greenberg S, Rom E, Duesler L, et al. Sequential exposure to fibroblast growth factors (FGF) 2, 9 and 18 enhances hMSC chondrogenic differentiation. *Osteoarthritis Cartilage*. 2015;23:443–53.
  25. Cooke ME, Allon AA, Cheng T, Kuo AC, Kim HT, Vail TP, et al. Structured three-dimensional co-culture of mesenchymal stem cells with chondrocytes promotes chondrogenic differentiation without hypertrophy. *Osteoarthritis Cartilage*. 2011;19:1210–8.
  26. Vangsness CT Jr, Farr J 2nd, Boyd J, Dellaero DT, Mills CR, LeRoux-Williams M. Adult human mesenchymal stem cells delivered via intra-articular injection to the knee following partial medial meniscectomy: a randomized, double-blind, controlled study. *J Bone Joint Surg Am*. 2014;96:90–8.
  27. Orozco L, Munar A, Soler R, Alberca M, Soler F, Huguet M, et al. Treatment of knee osteoarthritis with autologous mesenchymal stem cells: two-year follow-up results. *Transplantation*. 2014;97:e66–8.
  28. Vega A, Martín-Ferrero MA, Del Canto F, Alberca M, García V, Munar A, et al. Treatment of knee osteoarthritis with allogeneic bone marrow mesenchymal stem cells: a randomized controlled trial. *Transplantation*. 2015;99:1681–90.
  29. Gelse K, Ekici AB, Cipa F, Swoboda B, Carl HD, Olk A, et al. Molecular differentiation between osteophytic and articular cartilage—clues for a transient and permanent chondrocyte phenotype. *Osteoarthritis Cartilage*. 2012;20:162–71.
  30. Bay-Jensen AC, Reker D, Kjølgaard-Petersen CF, Mobasheri A, Karsdal MA, Ladel C, et al. Osteoarthritis year in review 2015: soluble biomarkers and the BIPED criteria. *Osteoarthritis Cartilage*. 2016;24:9–20.
  31. Xu X, Zhu F, Zhang M, Zeng D, Luo D, Liu G, et al. Stromal cell-derived factor-1 enhances wound healing through recruiting bone marrow-derived mesenchymal stem cells to the wound area and promoting neovascularization. *Cells Tissues Organs*. 2013;197:103–13.
  32. Lee JK, Lee S, Han SA, Seong SC, Lee MC. The effect of platelet-rich plasma on the differentiation of synovium-derived mesenchymal stem cells. *J Orthop Res*. 2014;32:1317–25.
  33. Müller J, Benz K, Ahlers M, Gaissmaier C, Mollenhauer J. Hypoxic conditions during expansion culture prime human mesenchymal stromal precursor cells for chondrogenic differentiation in three-dimensional cultures. *Cell Transplant*. 2011;20:1589–602.
  34. Dohan Ehrenfest DM, Andia I, Zumstein MA, Zhang CQ, Pinto NR, Bielecki T. Classification of platelet concentrates (Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives. *Muscles Ligaments Tendons J*. 2014;4:3–9.
  35. Jo CH, Lee YG, Shin WH, Kim H, Chai JW, Jeong EC, et al. Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial. *Stem Cells*. 2014;32:1254–66.