Identification of factors to stimulate *in situ* cartilage regeneration through endogenous stem cell recruitment

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Summary of the thesis to obtain the Master of Science Degree in Biomedical Engineering

October 2016

Abstract: Articular cartilage (AC) reconstitution remains a challenge for tissue engineering. Endogenous mesenchymal stem cells (MSCs) has become a promising tool for AC healing, due to the location nearby the joint, the migratory potential to injured sites and their chondrogenic differentiation. This study evaluated not only the migration of MSCs derived from bone marrow (BM) and synovium (SYN) but also the chondrogenesis of the recruited cells in response to different factors. The migration of MSCs was carried out in 3D collagen gel, supplemented with medium containing thirteen different factors and analysed under a confocal microscope after 48 hours. The chondrogenesis was performed in 3D pellet culture in the presence of transforming growth factor (TGF)-β1 along with the factors which stimulated cell migration, and measured through glycosaminoglycan presence stained with thionin. The *in vitro* migration of BM- and SYN-MSCs was stimulated in the presence of platelet growth factor (PDGF)-BB, bone morphogenetic protein (BMP)-2, nerve growth factor (NGF)-β, stromal cell derived factor (SDF)-1α and regulated on activation, normal T cell expressed and secreted (RANTES). In chondrogenesis, the combinatorial effect of TGF-β1/BMP-2 improved the chondrogenesis of BM- and SYN-MSCs, TGF-β1/SDF-1α did not decrease chondrogenesis and different responses were verified for TGF-β1/PDGF-BB and TGF-β1/NGF-β; chondrogenesis of BM-MSCs was slightly inhibited in response to TGF-β1/PDGF-BB, but chodrogenesis of SYN-MSCs did not decrease, and on the other hand TGF-β1/NGF-β inhibited chodrogenesis of SYN-MSCs, but did not reduce chodrogenesis of BM-MSCs. These findings have great promise for *in vivo* cartilage treatment through endogenous regeneration of cartilage.

Keywords: Chemokines, Chondrogenesis, Growth Factors, Mesenchymal Stem Cells, Migration

Introduction

Articular cartilage (AC) has a limited self-repair capacity, likely due to the absence of blood vessels and innervation [1, 2]. Therefore, cartilage defects caused by degenerative diseases such as osteoarthritis (OA), congenital malformation or trauma of cartilage tissue are unable to repair.

In recent years, the use of mesenchymal stem cells (MSCs) has been more and more extensively applied as a therapeutic to restore the cartilage tissue structure and function, due to their ability to migrate to damage tissues and to differentiate into chondrocytes. MSCs presence in sources nearby or within the joint such as synovium (SYN), bone

marrow (BM), AC, infrapatellar fat pad and synovial fluid raises the chance of using factors that may stimulate the recruitment of endogenous MSCs instead of the need to administrate exogenous MSCs. However, strategies based on manipulating endogenous MSCs for cartilage tissue are very limited. A few recent studies have used chemokines, for instance monocyte chemoattractant protein (MCP)-1/chemokine C-C motif ligand (CCL)-2, macrophage inflammatory protein (MIP)-1α/CCL3, Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES)/CCL5, and stromal cell derived factor (SDF)-1/chemokine C-X-C motif (CXCL)-12 to induced migration of human and rabbit MSCs in transwell assays. Growth factors, such as platelet-derived growth factor (PDGF)-BB, -AB, and -AA, epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), insulin-like growth factor (IGF)-1, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), trombin, transforming growth factor (TGF)-β3 and inflammatory cytokines, including interleukine (IL)-6, IL-8, IL-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, also have played a role in MSCs recruitment [3-9]. Although many factors have gained significant interest as chemoattractants, few studies tried to combine the recruitment of MSCs and their chondrogenic differentiation potential. Mendelson et al. has shown migration and chondrogenesis of human MSCs in gelatin microspheres supplemented with TGF-β3, and TGF-β3 plus SDF-1β [10] and Lee et al. has demonstrated either the stimulation of human MSCs migration within the marrow cavity and the healing of athymic nude rat osteochondral defects after implantation of heparin-conjugated fibrin loaded with PDGF-AA and TGF-β1 [7]. Additionally, BMP-2, a member of the TGF superfamily, is a factor of great interest which has shown a positive impact in chondrogenic differentiation when in presence of TGF-(β1, β2 and β3) [11-14]. However, it remains elusive the sources of endogenous cells for cartilage regeneration and whether endogenous cell homing, without cell transplantation, can be reinvigorated by specific bioactive conditions that might improve cartilage tissue healing.

Accordingly, this thesis aims primarily at evaluating whether chemotactic factors can only stimulate the migration of MSCs derived from SYN and BM or are also able to induce chondrogenic differentiation of the migrated cells. The author hypothesises that cartilage regeneration is achieved not only by the use of recruitment factors that induce migration of MSCs to injured places but also by the presence of factors that induce chondrogenesis of the recruited cells. This hypothesis was investigated by testing the chemotactic migration capacity of human MSCs derived from four donors of BM and four donors of SYN tissue in response to five chemokines (MCP-1/CCL2, MCP-3/CCL7, MIP-1α/CCL3, SDF-α/CXCL12, and RANTES/CCL5), five growth factors (PDGF-BB, nerve growth factor (NGF)-β, BMP-2, IGF-1, and TGF-β1), and three inflammatory cytokines (IL-1α, IFN-γ, and TNF-β) in a 3D collagen gel. After having selected the factors that stimulate significant migration of the MSCs, chondrogenic differentiation was analysed by immunohistochemistry after 28-32 days of pellets culture formation, in the presence of chondrogenic medium supplemented with TGF-β1 and the selected factors. Finally, through this process, the quality of the formed cartilage was evaluated in the different conditions.

Methods

Human MSCs derived from bone marrow and synovium

BM and SYN derived MSCs were acquired by ethical approval and informed consent of eight OA patients who underwent joint replacement surgery (Orthopaedics Department, Erasmus MC, University

Medical Centre Rotterdam, The Netherlands). Bone marrow was aspirated from the iliac crest according to the local ethical committee of the Erasmus MC. Cells were platted at 30×106 nc/T175 to 30×10^6 nc/T175 in 20 mL of α-MEM (1:1, Invitrogen) supplemented with 10% screened foetal calf serum (FCS), gentamycine (1:1000, Invirtogen), fungizone (1:167, Invirtogen), bFGF (1:5000, AbD Serotec) and 0.1 mM ascorbic-acid (1:500, Sigma), and incubated at 37°C, 5% CO_2 and 90% humidity. After 24h, all nonadherent cells were washed off by changing the medium and BM-MSCs, the adherent cells, were further expanded when sub-confluent. BM-MSCs were used in passage 2 or 3 for the migration assays and chondrogenesis.

The synovium tissue was chopped into small pieces and digested for 2-3 hours on a shaker at 37°C in medium containing HANKS BSS, collagenase IV (2 mg/ml) and dispase II (0.2 mg/ml). The digested tissue was passed through a syringe of 16 G needle a few times, neutralised with serum (to a 5% final concentration) and sieved through a 100 u and 40 u sieves. After a PBS wash the cells were seeded in culture medium, and incubated at 37°C, 5% CO_2 and 90% humidity. On day 3, all nonadherent cells were removed by changing the culture medium. SYN-MSCs, when sub-confluent, were trypsinized (0.25% trypsin/EDTA, Life Technologies) and resuspended in culture medium for a late passage. SYN-MSCs were used in passage 1 and 2 for the migration assays and chondrogenesis.

Migration assay

The first step of migration assays is the formation of spheroids from BM- or SYN-MSCs in 3D Petri Dish (Sigma-Aldrich) of 400 µm in diameter and 800 µm in depth per micro-well. The mould of the 3D Petri Dish was filled with 500 µL of melted sterile agarose (1g of agarose dissolved in 50 mL of saline solution) and after its solidification, the agarose micro-wells were removed by squeezing the mould into a 12 well plate. After 3 washes in α-MEM containing 10% FCS, fungizone (Invitrogen) and gentamycin (Invitrogen), 190 µL mono-dispersed cells previously stained with CFDA were seeded per agarose mould (500 cells per micro-well) and 1.5 mL of medium was added in each well after 10 minutes of cell settlement at 37°C. During the next 24 hours cells self-assembled and formed spheroids. For collecting the spheroids, the micro-wells were inversed and placed into 1 mL of medium and then centrifuged to dislodge the spheroids out from the micro-wells to the medium which was collected for the next phase.

The migration of the spheroids was evaluated for MCP1/CCL2, MCP-3/CCL7, MIP-1α/CCL3, SDF-α/CXCL12, PDGF-BB, NGF-β, BMP-2, and IGF-1 (50 and 100 ng/mL), RANTES/CCL5 (10, 25, 35, 50 and 100 ng/mL), TGF-β1 (10, 50 and 100 ng/mL), IL-1β, IFN-γ, and TNF-α (1 and 10 ng/mL) in a 3D collagen gel. A volume of 1 mL of gel was prepared by mixing 680 µL of H_2O, 100 µL of PBS, 25 µL of NaOH and 193 of µL collagen I. 100 µL gel was solidified at 37°C, 95% humidified air and 5% CO_2 for 30 minutes in a 8 well chamber slide (Lab-Tek) of 0.7 cm^2 growth area per well. After a 10 minute wash with 200 µL of serum free medium containing ITS, fungizone and gentamycin, a volume of 200 µL containing the spheroids supplemented with each factor was added into each well and incubated at 37°C and 5% CO_2 for 48 hours. Two wells with spheroids in medium without factors were seeded as a negative control. The chemokines used were recombinant factors of human origin and purchased from PeproTech.

Imaging and data analysis

The migration was analysed under a confocal laser scanning microscope (Leica TCS SP5, imaging department of Erasmus MC, the Netherlands) 48 hours after the induction of migration. For each condition three to six spheroids were scanned alive or after fixation in 4% paraformaldehyde through

their depth in XYZ-mode with a 10× dry objective and a Argon laser source (λ=488 nm). Images of 512×512 pixels were obtained. The data extracted was then measured using ImageJ [15]. Each image with one single spheroid was decomposed into two components, such as the core and sprouting cells (the main core of cells and the cells that move from their initial position but stay attached to the core), and migrating cells (single or group of cells that detached from the main core). The macro analyses a single spheroid at the time and it gives the cell area of the core and sprouting cells, and of the migrating cells as a function of the distance to the core centre in µm. Two parameters are extracted from the migrating cells component: the migratory cell area and maximum migratory cell distance. A recruitment factor is selected when significantly stimulate these two parameters, not just one. Image processing and measurements were executed using *Microsoft Excel 2010* and *GraphPad Prism 5*.

Statistics

Data were analysed with *IBM SPSS Statistics 21*. A linear mixed model was used for migratory cell area and maximum migratory cell distance data. Multiple comparisons were analysed with Sidak post hoc test. Conditions and donors were considered as fixed and random parameters, respectively. Normal distribution of the data or the residuals of the data were confirmed by both Kolmogorov-Smirnov and Shapiro-Wilk tests. Differences were considered significant when p value was *<0.05, **<0.01 and ***<0.001.

Pellet culture

Pellets were formed by centrifugation of 0.2×10^6 BM- or SYN-MSCs at 1100 rpm for 8 minutes in 15 ml conical polypropylene tubes (VWR). MSCs were cultured in 0.5 mL of medium containing DMEM-high-glucose (1:1, Invirtogen), gentamycine (1:1000, Invirtogen), fungizone (1:167, Invirtogen), sodium pyruvate 1mM (1:100, Invirtogen), 40 µg/ml proline (1:500, Sigma), ITS (1:100, B&D systems), 0.1mM ascorbic-acid (1:500, Sigma) and 100 nM dexamethasone (1:10000, Sigma). The medium was supplemented with 10 ng/mL of TGF-β1 (positive control), 50 ng/mL of NGF-β, SDF-1α, BMP-2, and PDGF-ββ, the combination of those factors with 10 ng/mL of TGF-β1, and an addition condition of 25 ng/mL of RANTES along with 10 ng/mL of TGF-β1. The tubes were incubated at 37°C with a humidified atmosphere of 5% CO_2. The medium was carefully refreshed twice a week for 28-32 days.

Immunostaining

After 28-32 days, the cells were fixed in 4% formalin embedded in paraffin blocks. Sections of 6 µm were stained with thionin to detect glycosaminoglycan (GAGs), a component of the cartilage matrix. Images were taken under an optical microscope. To quantify the chondrogenic differentiation a qualitative scale based on thionin staining was used (Table 3).

Table 1 – Qualitative scale of chondrogenic differentiation used to quantify pellet culture.

Score	Chondrogenic differentiation	Intensity of thionin staining	Cell morphology
0	None	None	Fibroblast-like morphology
+	Low	Low	Fibroblast-like morphology
++	Moderate	Moderate	Small chondrocyte-like morphology
+++	High	High	Chondrocyte-like morphology

Results

Factors that Induce Migration of BM- and SYN-MSCs

The migration potential of BM- and SYN-MSCs was evaluated in a 3D collagen gel supplemented with different factors, after 48 hours. A panel of 13 molecules: 5 chemokines (MCP-1, MCP-3, MIP-1α, SDF-1α, and RANTES), 3 inflammatory cytokines (IL-1α, IFN-γ, and TNF-β) and 5 growth factors (PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1), were tested. Single spheroids were imaged under the confocal microscope and then decomposed into two components: core and sprouting cells, and migrating cells (Figure 1, red and green, respectively).

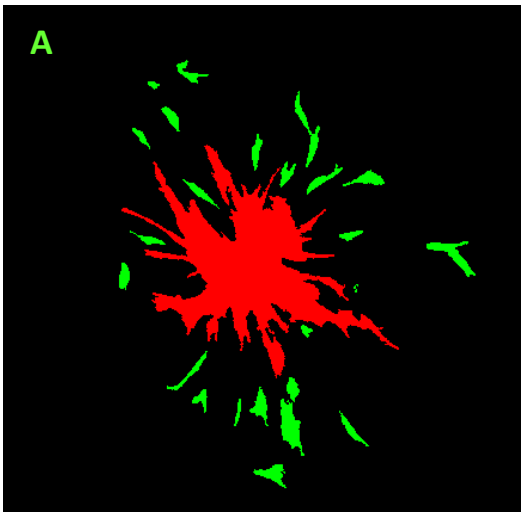

Figure 1 – Decomposition of a single spheroid after 48 hours of migration. Core and sprouting cells (red) and migrating cells (green).

Only migrating cells were used to evaluate the migratory potential of BM- and SYN-MSCs in response to different factors. Two parameters were quantified: the migratory cell area (sum of the values of the migrating cell area) and the maximum migratory distance (last distance reached by the migrating cells). It was considered a recruitment factor, those which significantly stimulate the migratory cell area and maximum migratory distance.

Based on the evaluation of the migratory cell area (Figure 2A) and maximum migratory cell distance (data not shown) of BM-MSCs in the presence of the factors, the highest and significant migration potential of BM-MSCs was observed in response to 1 and 10 ng/mL of IL-1β and 50 ng/mL of PDGF-BB, followed by 1 ng/mL of TNF-α, 50 ng/mL SDF-1α, 50 ng/mL of RANTES, 50 ng/mL of NGF-β, and 50 ng/mL of BMP-2. For SYN-MSCs, the findings of the migratory cell area (Figure 2B) and the maximum migratory cell distance (data not shown) demonstrated that 50 and 100 ng/mL SDF-1α, 10 and 25 ng/mL of RANTES, 50 ng/mL of β-NGF, 50 ng/mL of BMP-2, and 50 ng/mL of PDGF-BB significantly improve migration of SYN-MSCs. The comparison of migration of BM- and SYN-MSCs demonstrated that IL-1β and TNF-α were recruitment factors for BM-MSCs, but not for SYN-MSCs. MCP-1 showed a significant increase in SYN-MSCs migration, however this behaviour was not evident for BM-MSCs. In addition, BM-MSCs migrated more in the presence of 50 ng/mL of RANTES, but SYN-MSCs responded better for lower concentrations of RANTES (10 and 25 ng/mL). On the other hand, both cell types showed significant migration in response to 50 ng/mL of PDGF-BB, NGF-β, SDF-1α and BMP-2.

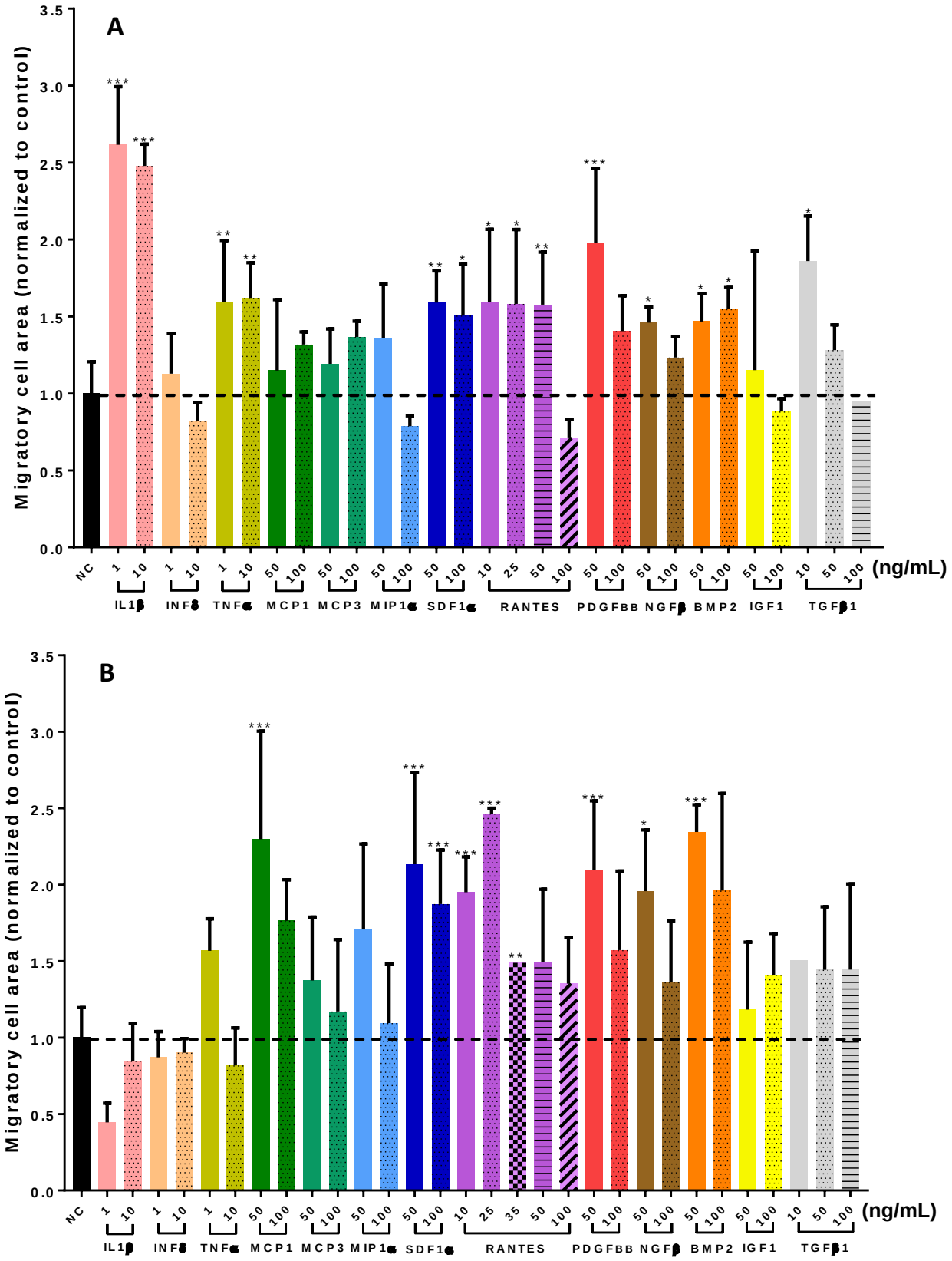

Figure 2 - Migration of MSCs derived from bone marrow (A) and synovium (B) in response to different factors. *In vitro* migration of MSCs in collagen gel supplemented with culture medium containing different factors was evaluated after 48 hours under confocal microscopy. Migratory cell area, a parameter used to quantify the migration potential of MSCs in response to IL-1α, IFN-γ, TNF-β, MCP-1, MCP-3, MIP-1α, SDF-1α, RANTES, PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1, is expressed as normalised values with respect to the negative control, NC, (n=4 independent experiments. * p<0.05; ** p<0.01; *** p<0.001 where p is evaluated with reference to the NC).

Chondrogenesis of BM- and SYN-MSCs

Cartilage regeneration not only requires the migration of MSCs to the defects but also the chondrogenesis of the recruited cells. Therefore, it is of interest to determine whether the factors which induce significant migration of MSCs evaluated in the previous assays have an effect on chondrogenesis. To achieve this, a 3D pellet culture was induced for 3 BM donors and 1 SYN donor during 28 or 32 days. For BM donor 1, conditions such as 10 ng/mL TGF-β1 (positive control), 50 ng/mL NGF-β, PDGF-BB, BMP-2, and SDF-1α, or the combination of those with TGF-β1, respectively TGF-β1/NGF-β, TGF-β1/PDGF-BB, TGF-β1/BMP-2, and TGF-β1/SDF-1, were tested. For BM donors 2 and 3, conditions such as NGF-β, BMP-2, and SDF-1α were excluded because they did not show chondrogenic differentiation in donor 1. The condition of PDGF-BB, despite having not demonstrated chondrogenesis, was still tested to check if this condition would show the same increase in the number of cells with a fibrosblast-like morphology for the next two donors.

The findings obtained were consistent within the BM donors (Figure 3). It was seen no thionin staining in the presence of PDGF-BB and an increment in the proliferation of the cells with a fibroblast-like morphology in response to PDGF-BB and TGF-β1/PDGF-BB. In addition, it was observed very similar intensity of the thionin staining and chondrocyte-like morphology in response to TGF-β1/NGF-β and TGF-β1/SDF-1α compared to TGF-β1 (PC), and the condition TGF-β1/BMP-2 showed the highest accumulation of GAGs and chondrocyte-like morphology.

Additional studies of chondrogenesis were carried out with SYN-MSCs in order to compare to the obtained outcomes of BM-MSCs. For SYN donor 1 (data not showed), PDGF-BB was excluded and the combination of TGF-β1 and 25 ng/mL of RANTES (TGF-β1/RANTES) was introduced. The data showed the highest GAGs expression in pellets treated with TGF-β1/BMP-2. In addition, certain conditions, such as TGF-β1/SDF-1α, and TGF-β1/PDGF-BB, did not differ much from the TGF-β1 (PC). Finally, TGF-β1/NGF-β, and TGF-β1/RANTES decreased the chondrogenesis compared to TGF-β1.

In summary, the treatment of TGF-β1/BMP-2 in pellet culture of BM- and SYN-MSCs showed the highest GAG expression and chondrocyte-like morphology for all the donors and therefore indicating close proximity to cartilage tissue. In addition, BM- and SYN-MSCs cultured in chondrogenic medium supplemented with PDGF-BB and TGF-β1/PDGF-BB showed larger pellet size and an increase in cell number with a fibroblastic-like morphology compared to the pellets of the remaining conditions.

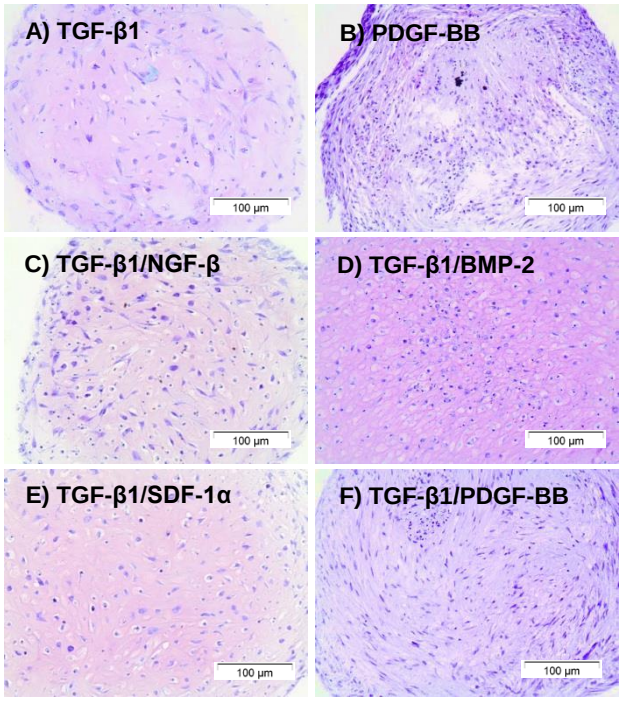

Figure 3 - GAGs analyses of BM-MSCs donor 3 after 28 days of pellet culture treated in chondrogenic medium supplemented with additional factors. The correspondent scores for each condition after application of the qualititive scale are: A) TGF-β1 (++), B) PDGF-BB (0), C) TGF-β1/NGF-β (++/+++), D) TGF-β1/BMP-2 (+++), E) TGF-β1/SDF-1α (++), and F) TGF-β1/PDGF-BB (+). Images are representative of three pellets of BM donor 3. Magnification 100×.

Discussion

Articular cartilage has limited self-repair capacity after damage, which often leads to progressive degeneration of the articulation. It is a paradox that cartilage has mesenchymal stem/progenitor cells within or nearby the joint (such as in bone marrow, articular cartilage, synovium, synovial fluid and infrapatellar fat pad), but no spontaneous healing occurs. This work hypothesizes that the presence of recruiting factors can stimulate the migration of MSCs to the injured cartilage sites, followed by chondrogenic differentiation of the migrated cells through chondrogenic factors. Accordingly, factors such as PDGF-BB, BMP-2, NGF-β, SDF-1α and RANTES significantly stimulated the migration of BM- and SYN-MSCs in a 3D collagen matrix. Interestingly, factors IL-1β, TNF-α, TGF-β1 were able to stimulate significantly the migration of BM-MSCs, whereas factor MCP-1 recruited substantially only SYN-MSCs. Beyond the recruitment of MSCs, the combination of TGF-β1/BMP-2 stimulated the chondrogenic differentiation of MSCs from the two sources. In addition, the TGF-β1 induced chondrogenesis was not inhibited when combined with NGF-β or SDF-1α in BM-MSCs, and in the presence of PDGF-BB or SDF-1α in SYN-MSCs.

One of the key findings of the present study was that BMP-2 not only stimulated the recruitment of both sources of MSCs (bone marrow and synovium), but also induced chondrogenic differentiation of those cells when combined with TGF-β1. Although BMP-2 in combination with TGF-β1 has already been shown to improve chondrogenic differentiation in BM-MSCs [16] and SYN-MSCs [59], its role as a recruitment factor in a 3D collagen matrix was verified for the first time.

The chemotactic response of factors is commonly evaluate in transwells and Boyden chambers, however these assays are 2D, complex and time consuming systems [17]. In this study, a 3D collagen matrix was used to assess the migration of BM- and SYN-MSCs in response to different factors. The motivations for the design of this system were to permit the recruitment of the cells by specific factors in a 3D matrix and to simplify the quantification of the stimulation of the migrating cells. This stimulation was evaluated through an innovative technique which quantified the migratory cell area and maximum migratory cell distance in response to the application of different factors. These two parameters were used to select the factors which efficiently stimulated the cell migration. Thirteen factors were tested; factors IL-1β and PDGF-BB proved to be the best chemoattractants for BM-MSCs. The high chemotactic potential of PDGF-BB on BM-MSCs is confirmed from reports of other studies [3, 8]; however, so far IL-1β had shown only moderate chemoattractant capacity [9]. Additional factors which produced significant migration of BM-MSCs were TNF-α, TGF-β1, BMP-2, NGF-β, SDF-1α and RANTES. Some previous studies corroborated the chemotactic potential of SDF-1α [18-20], TNF-α [9] and BMP-2 [6]. RANTES however has shown conflict within the findings, because some studies demonstrated that high concentrations of RANTES stimulated migration of MSCs [4, 5] and low concentrations of RANTES evidenced no chemotaxis [5, 8]; however, in this study the recruitment of MSCs occurred for low concentrations of RANTES, but it did not for high concentrations. Moreover, MCP-1 and MIP-1α, which were considered chemoattractant factors for BM-MSCs in previous reports [5, 7, 21], in this study did not significantly stimulate the amount of cells that migrate (i.e. the migratory cell area), but they only increased the distance reached by migrating cells (i.e. the maximum migratory cell distance). This low chemotaxis of MCP-1 and MIP-1α was however confirmed in other studies [4, 9].

An additional reservoir of MSCs is synovium. Its higher chondrogenic potential compared to BM, adipose tissue, muscle, periosteum and infrapatellar fat pad [22] makes synovium a promising cell source for cartilage regeneration; however, very few studies tried to evaluate the migration of SYN-MSCs in response to different stimuli. This study assessed the capacity of different factors to stimulate migration of SYN-MSCs in a 3D collagen gel. The findings showed for the first time significant migration of SYN-MSCs in the presence of PDGF-BB, BMP-2, NGF-β, MCP-1 and RANTES. SDF-1α showed also an increase in the recruitment of SYN-MSCs, confirming results already reported in the literature [4, 5, 10].

Although many factors have gained significant interest as chemoattractants, few studies tried to combine the recruitment and chondrogenic differentiation potential of MSCs. In this study, not only the recruitment of MSCs, but also the chondrogenesis of MSCs from two available sources adjacent to articular cartilage, such as bone marrow and synovium, was assessed in the 3D pellet culture. Both cell types responded superiorly to combinatorial TGF-β1/BMP-2 stimulation, as evidenced by the upregulation of GAG expression, a cartilage-specific matrix component. Previous studies corroborated this finding for specific cell sources, such as BM [13, 16, 23], adipose tissue [12] and articular cartilage [13]. Moreover, obtained findings demonstrated that chondrogenic differentiation of BM-MSCs appears to be decrease in response to TGF-β1/PDGF-BB; the same behaviour was reported in a previous study where MSCs derived from AT were tested in the presence of TGF-β1/PDGF-BB [12]. On the other hand, the chondrogenesis of MSCs derived from synovium under the same condition showed no inhibition in this study. An additional condition which also demonstrated different behaviours in MSCs derived from BM and SYN was TGF-β1/NGF-β; in BM-MSCs the chondrogenesis was not improved nor inhibited, but in SYN-MSCs it was inhibited. Furthermore, two other combinations were studied for the first time, such as TGF-β1/SDF-1α and TGF-β1/RANTES; TGF-β1/SDF-1α showed no inhibition of chondrogenic differentiation of both sources of MSCs and TGF-β1/RANTES, just assessed for SYN-MSCs, demonstrated inhibition of chondrogenesis. These findings indicate that distinctive cell sources respond differently to diverse stimuli.

To conclude, the findings of this work can significantly contribute to improve the recruitment of BM- and SYN-MSCs to injured cartilage sites and to induce the differentiation into chondrocytes; thereby, they are of great interest for understanding how to regenerate cartilage by recruitment of endogenous MSCs without cell transplantation. However, more prolonged experiments, first in osteochondral models and later on in animal models, are needed to validate the recruitment of MSCs into cartilage defects in response to BMP-2, PDGF-BB, NGF-β, SDF-1α and RANTES, and the effect on chondrogenic differentiation of MSCs in the presence of recruitment and chondrogenic factors such as BMP-2 and TGF-β1. Moreover, further investigation on factors controlled release and biosafety must be conducted to allow their utilization in the human medicine.

References

1. Clouet, J., et al., *From osteoarthritis treatments to future regenerative therapies for cartilage.* Drug Discov Today, 2009. **14**(19-20): p. 913-25.
2. Roelofs, A.J., J.P. Rocke, and C. De Bari, *Cell-based approaches to joint surface repair: a research perspective.* Osteoarthritis Cartilage, 2013. **21**(7): p. 892-900.
3. Ozaki, Y., et al., *Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells.* Stem Cells Dev, 2007. **16**(1): p. 119-29.

4. Ponte, A.L., et al., *The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities.* Stem Cells, 2007. **25**(7): p. 1737-45.
5. Rice, C.M. and N.J. Scolding, *Adult human mesenchymal cells proliferate and migrate in response to chemokines expressed in demyelination.* Cell Adh Migr, 2010. **4**(2): p. 235-40.
6. Haasters, F., et al., *Mesenchymal stem cells from osteoporotic patients reveal reduced migration and invasion upon stimulation with BMP-2 or BMP-7.* Biochem Biophys Res Commun, 2014. **452**(1): p. 118-23.
7. Lee, J.M., et al., *In vivo tracking of mesechymal stem cells using fluorescent nanoparticles in an osteochondral repair model.* Mol Ther, 2012. **20**(7): p. 1434-42.
8. Phipps, M.C., Y. Xu, and S.L. Bellis, *Delivery of platelet-derived growth factor as a chemotactic factor for mesenchymal stem cells by bone-mimetic electrospun scaffolds.* PLoS One, 2012. **7**(7): p. e40831.
9. Vertelov, G., et al., *High targeted migration of human mesenchymal stem cells grown in hypoxia is associated with enhanced activation of RhoA.* Stem Cell Res Ther, 2013. **4**(1): p. 5.
10. Mendelson, A., et al., *Chondrogenesis by chemotactic homing of synovium, bone marrow, and adipose stem cells in vitro.* Faseb J, 2011. **25**(10): p. 3496-504.
11. Kim, H.J. and G.I. Im, *Combination of transforming growth factor-beta2 and bone morphogenetic protein 7 enhances chondrogenesis from adipose tissue-derived mesenchymal stem cells.* Tissue Eng Part A, 2009. **15**(7): p. 1543-51.
12. Yoon, I.S., et al., *Proliferation and chondrogenic differentiation of human adipose-derived mesenchymal stem cells in porous hyaluronic acid scaffold.* J Biosci Bioeng, 2011. **112**(4): p. 402-8.
13. Murphy, M.K., et al., *TGF-beta1, GDF-5, and BMP-2 stimulation induces chondrogenesis in expanded human articular chondrocytes and marrow-derived stromal cells.* Stem Cells, 2015. **33**(3): p. 762-73.
14. Weiss, S., et al., *Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells.* J Cell Physiol, 2010. **223**(1): p. 84-93.
15. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nat Methods, 2012. **9**(7): p. 676-82.
16. Noth, U., et al., *Chondrogenic differentiation of human mesenchymal stem cells in collagen type I hydrogels.* J Biomed Mater Res A, 2007. **83**(3): p. 626-35.
17. Kramer, N., et al., *In vitro cell migration and invasion assays.* Mutat Res, 2013. **752**(1): p. 10-24.
18. Wynn, R.F., et al., *A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow.* Blood, 2004. **104**(9): p. 2643-5.
19. Son, B.R., et al., *Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases.* Stem Cells, 2006. **24**(5): p. 1254-64.
20. Croitoru-Lamoury, J., et al., *Human mesenchymal stem cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-beta, and Copaxone.* J Interferon Cytokine Res, 2007. **27**(1): p. 53-64.
21. Wang, L., et al., *MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture.* Hematology, 2002. **7**(2): p. 113-7.
22. Sakaguchi, Y., et al., *Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source.* Arthritis Rheum, 2005. **52**(8): p. 2521-9.
23. Toh, W.S., et al., *Combined effects of TGFbeta1 and BMP2 in serum-free chondrogenic differentiation of mesenchymal stem cells induced hyaline-like cartilage formation.* Growth Factors, 2005. **23**(4): p. 313-21.