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

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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 glycosaminoglycans 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

Resumo

A reconstrução da cartilagem permanece um desafio para o ramo da engenharia de tecidos. As células estaminais mesenquimais (MSCs) endógenas tornaram-se uma ferramenta promissora na reparação da cartilagem, devido à sua localização próxima da articulação, do seu potencial migratório para áreas danificadas e da sua diferenciação em condrócitos. Neste estudo não só se avaliou a migração das MSCs extraídas da medula óssea (BM) e sinóvia (SYN), mas também a condrogénese das células recrutadas em resposta a diferentes fatores. A migração das MSCs foi realizada num gel de colagénio 3D suplementado por meios contendo treze factores diferentes, e analisada sob um microscópio confocal após 48 horas. A condrogénese foi efectuada em cultura de pellet na presença de Factor de transformação de crescimento (TGF)-β1 juntamente com os factores que estimularam migração celular e medida através da presença de glicosaminoglicanos coradas com tionina. A migração *in vitro* das BM- e SYN-MSCs foi estimulada na presença de Factor de crescimento plaquetário (PDGF)-BB, Proteína morfogenética óssea (BMP)-2, Factor de crescimento neural (NGF)-β, Factor derivado do estroma da medula óssea (SDF)-1α e Regulada sob activação expressa e secretada por células T normais (RANTES). Na condrogénese, o efeito combinatório de TGF-β1/BMP-2 melhorou a condrogénese das BM- e SYN-MSCs, TGF-β1/SDF-1α não diminuiu a condrogénese e diferentes respostas foram verificadas perante TGF-β1/PDGF-BB e TGF-β1/NGF-β; a condrogénese de BM-MSCs foi ligeiramente inibida em resposta a TGF-β1/PDGF-BB, mas a condrogénese das SYN-MSCs não diminuiu, e por outro lado, TGF-β1/NGF-β não inibiu a condrogénese das BM-MSCs, mas reduziu a condrogénese das SYN-MSCs. Estes resultados são promissores para o tratamento *in vivo* da cartilagem através da regeneração endógena da cartilagem.

Palavras-chave: Células Estaminais Mesenquimais, Condrogénese, Fatores, Migração, Quimiocinas

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Glossary

α-MEM	Alpha-minimum essential medium
AC	Articular cartilage
AGCN	Aggrecan
AT	Adipose tissue
ASCs	Adipose stem cells
BM	Bone marrow
BMP	Bone morphogenetic protein
Col2A1	Collagen type II
Col10A1	Collagen type X
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular matrix
FCS	Foetal calf serum
GAGs	Glycosaminoglycans
GFP	green fluorescent protein
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukine
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MSCs	Mesenchymal stem cells
MPCS	Mesenchymal progenitor cells
NC	Negative control
NGF	Nerve growth factor
OA	Osteoarthritis
PC	Positive control
PDGF	Platelet-derived growth factor
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
SDF	Stromal cell derived factor
SM	Synovial membrane
SSCs	Synovium stem cells
SYN	Synovium
SOX9	SRY (sex determining region Y)-box 9
TGF	Transforming growth factor
TNF	Tumor necrosis factor

1. Introduction

Hyaline cartilage or articular cartilage (AC) is a connective tissue present on the articulation of the joints. AC is characterized by an extracellular matrix (ECM) which is rich in collagens type II, IX and XI, proteoglycans, and water. This composition makes cartilage a viscoelastic tissue that absorbs shocks and provides sliding areas in the joints thus allowing the bones to move. Additionally, AC is composed by chondrocytes, cells that reside within the ECM, which play a role in tissue homeostasis by synthetizing and degrading the extracellular matrix [1]. Unlike other tissues, AC has a limited self-repair capacity, likely due to the absence of blood vessels and innervation [2, 3]. Therefore, cartilage defects caused by degeneration, congenital malformation or trauma of cartilage tissue are unable to repair.

Cartilages are often affected by osteoarthritis (OA), a degenerative joint disease which can be described by a decrease in the thickness of articular cartilage, subchondral bone sclerosis, osteophytes formation and synovium inflammation [2]; it causes joint pain, cartilage stiffness and movement limitations, and consequently affects the patients daily life. Although the most predisposed articulations are knees, hips and spine, owing to their weight bearing function, OA also affects non-weigh bearing joints, such as fingers and toes. The National Health Interview Survey considered OA the main cause of disability in United States between 2008 and 2013, affecting 52.5 million (22.7%) adults (aged more than 18 years) [4]. Current clinical mid-stage OA treatments are oral nonsteroidal anti-inflammatory drugs which act on symptom reduction. In advanced stages of OA, arthroplasty (total joint replacement) is commonly used as final solution [2]. Furthermore, the repair of joint function and structure has been relied on therapeutics such as microfracture technique, mosaicplasty, and autologous chondrocyte implantation. However, these repair strategies are more suitable to regenerate non-degenerative cartilage lesions and not degenerative lesions, such as in OA. Mosaicplasty is a technique that harvest many small osteochondral plugs from non-weight bearing areas of the femur and transplant them to injured articular cartilage sites [3, 5]. In microfracture procedure, multiple millimetre size holes are produced in the subchondral bone, in order to induce migration of marrow elements to the joint injured spaces [3, 6, 7]. After the application of these techniques improvement in terms of pain relief and in function are shown in the patients for short periods of time [3]; however insufficient results have been shown so far over long-term periods [8] due to the formation of fibrous cartilage which affects the mechanical properties of the AC [9]. The third prevailing technique, the autologous chondrocyte implantation procedure, requires the removal of healthy chondrocytes from non-weight bearing areas of the joint, followed by *in vitro* expansion and further implantation of culture-expanded chondrocytes in the injured cartilage areas of the same individual. Autologous chondrocyte implantation has been developed as an alternative approach to classical surgical techniques and enhances the function of AC for longer periods of time compared with the previous surgical treatments; however the main drawback of this technique is the unstable phenotype of chondrocytes that can lead to fibrocartilage formation in place of normal cartilage development [3].

In recent years, the use of mesenchymal stem cells (MSCs) has been more and more extensively applied as a therapeutic alternative to restore the cartilage tissue structure and function. MSCs are a promising cell source for cartilage tissue engineering due to their *in vivo* and *in vitro* long term

self-renewal capacity, and their ability to migrate to damage tissues and to differentiate into chondrocytes. One of the findings to date in the use of MSCs for cartilage repair is that a lower number of cells remain in the joint or in the injured place after implantation. In addition, the regenerated tissue is fibrous and therefore it has very poor mechanical properties compared to normal cartilage. Although MSCs displayed low engraftment into the injured sites, it was evident that they establish a microenvironment auspicious for recruitment and activation of endogenous stem/progenitor cells for cartilage regeneration [3]. This raises the chance of using growth factors and chemokines 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, MIP-3α/CCL20, CCL-19, CCL-22, stromal cell derived factor (SDF)-1/chemokine C-X-C motif (CXCL)-12 and fractalkine/chemokine C-X3-C motif ligand (CX3CL)-1, 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), thrombin, transforming growth factor (TGF)-β3 and inflammatory cytokines, including interleukin (IL)-6, IL-8, IL-1β, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, also have played a role in MSCs recruitment [10-27]. However, some disadvantages are involved within chemotaxis assay: it is a 2D assay, it requires a complex set up for the transwells, it needs to determinate a migration optimal time for each cell source, and it is a time consuming technique [28]. In order to overcome the main disadvantage of the transwell assays, the 2D system, 3D cell migration systems that mimic better the real joint environment were considered. Phipps et al. used a bone-mimetic scaffold coated with PDGF-BB to recruit MSCs [25], and Ozaki et al. tested the chemotactic potential of PDGF-BB, PDGF-AA, HB-EGF, TGF-α, EGF, bFGF, IGF-1, HGF, thrombin in a wound migration assay; PDGF-BB, PDGF-AA, HB-EGF, and thrombin significant enhanced rabbit MSCs migration [17].

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 chondrogenic differentiation of human MSCs in gelatin microspheres supplemented with TGF-β3, and TGF-β3 along with SDF-1β [24] 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 [23]. 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 or -β3 [29-32]. 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 synovium and bone marrow 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 chondrogenic differentiation of the recruited cells. This hypothesis was investigated by testing the chemotactic migration capacity of human MSCs derived from four donors of bone marrow and four donors of synovium tissue in response to five chemokines (MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, MCP-3/CCL7 and SDF-α/CXCL12), five growth factors (PDGF-BB, TGF-β1, IGF-1, BMP-2 and Nerve growth factor (NGF)-β), and three inflammatory cytokines (IL-1α, TNF-β and IFN-γ) in a 3D collagen gel matrix. After having selected the factors that stimulate significant migration of the MSCs, chondrogenic differentiation was analysed by immunohistochemistry after 28-32 days of pellet 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.

2. State-of-the-art

Tissue engineering is the research field that, combining cells, engineering and materials, aims at developing a functional alternative to improve or substitute the damaged tissues. As stated in section 1, cartilage is not able to self-repair after injury due to the absence of vasculature and innervation. Therefore, techniques able to regenerate cartilage defects are of high interest in tissue engineering field. For example, transplantation of exogenous MSCs alone or within a scaffold to injured sites has been adopted as a strategy to repair AC defects. However, the results have demonstrated that lower number of cells remain in the joint or in the damaged tissue after transplantation [3]. Heterogeneity of MSCs population and the inhospitable inflammatory environment of the injured cartilage are the probable reasons for this low cell engraftment in the recipient sites [33]. Even though MSCs did not show direct repair through cell replacement after transplantation, it is suggested that MSCs create a microenvironment that stimulate the recruitment of endogenous stem/progenitor cells for tissue repair [4]. From this fact, therapeutics that rely on activation and mobilization of a large number of endogenous stem/progenitor cells to injured sites and promotes their differentiation into chondrocytes without the need to administrate exogenous cells hold great promise for cartilage tissue engineering and clinical practice [9]. Accordingly, recent research has been using chemokines and growth factors, which can stimulate the mobilization of endogenous MSCs to injured cartilage followed by *in situ* cartilage regeneration.

Hence, this chapter starts with an overview of the factors that induce the recruitment and the chondrogenic differentiation of MSCs, followed by a summary of the endogenous cell sources for regenerating cartilage.

2.1 Chemokines and Growth Factors for Recruitment and Chondrogenic Differentiation of Endogenous Stem or Progenitor Cells

Growth factors, chemokines and cytokines are biologically active proteins produced endogenously by various cell types. These proteins mediate proliferation, differentiation and survival of cells. In addition, they attract cells to the injury site in order to maintain and repair the tissues [34].

2.1.1 Recruitment factors

Many chemokines and growth factors have the correspondent receptors expressed by MSCs. Included in these receptors are CXC chemokine receptors 1 and 2 (CXCR1 and CXCR2) [35], CXCR4 [11], CC receptor 1 and 2 (CCR1 and CCR2) [15], vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR)-a and -b [23]. The correspondent ligands are IL-8, SDF-1, MIP-1α, MCP-1, placenta growth factor (PlGF) and PDGF [14, 19, 36]. Although the mechanisms regulating the circulation of MSCs to peripheral blood and their recruitment to appropriate microenvironments are still unknown, the use of some of these growth factors and chemokines has been shown to induce migration of MSCs [10-15, 17-27, 37, 38] (Table 1). Migration is a process of cell movement, that can be characterized by a directional migration in response to a gradient of soluble stimuli (chemotaxis) or by a random motility (chemokinesis) [28].

SDF-1, a chemokine protein of the CXC subfamily, also known as CXC motif chemokine 12 (CXCL12), is one of the chemokines that can stimulate cell migration. SDF-1 and its receptor CXCR4 can mediate the migration of MSCs to bone marrow [11]. Previous works [24, 37] showed that SDF-1 has also a chemotactic effect in adipose and synovium stem cells. Moreover, a dose dependent effect of SDF-1 on the mobilization of human BM-MSCs was shown in Boyden chamber and transwell assays [11, 14, 16, 19]. In addition, the chemokines of the CC subfamily that played a part in MSC recruitment are MCP-1, MIP-1α and RANTES, respectively known as CCL2, CCL3 and CCL5. MCP-1 stimulates migration and infiltration of monocytes and macrophages in order to induce normal response to inflammation and immunological survival of tissues [39]. MIP-1α is mainly expressed by immune cells, fibroblasts and epithelial cells [40] and therefore its expression is increased in injury areas. RANTES is expressed by a large number of cell types such as natural killer cells, memory T cells, eosinophils and dendritic cells [41]. Some studies have tested the response of MSCs in the presence of MCP-1, MIP-1α and RANTES in Boyden chamber or transwell assays; however, some contradictory results were found: on the one hand, *in vitro* migration of BM-MSCs was demonstrated in response to MCP-1, MIP-1α and RANTES [10, 14, 15, 18, 20, 23]; and on the other hand, no significant recruitment of BM-MSCs was observed in the presence of these chemokines [19, 21, 26, 27]. A few other chemokines, including CXCL21, CXCL1, CXCL16 and CXCL19, have been shown induction of chemotaxis of BM-MSCs [14].

Besides chemokines, cell recruitment has been presented in response to some pro-inflammatory cytokines. For instance, IL-8 or CXCL8, which belongs to the CXC chemokine subfamily, showed a chemotactic response in human BM-MSCs in *in vitro* studies [10, 19, 23, 27]. Furthermore, inflammatory chemokines, including TNF-α, IL-6, and IL-1β, chemotactically stimulated hMSCs derived from BM, as demonstrated in transwell chamber [27].

Finally, several growth factors have also been playing a role in migration of stem cells. PDGF, as a homodimer (PDGF-AA and PDGF-BB) or a heterodimer (PDGF-AB), demonstrated the greatest effect on MSCs migration in *in vitro* and *in vivo* models [17, 23, 25, 26]. Several other growth factors such as HB-EGF, EGF, HGF, IGF-1, FGF-2, and thrombin have been showing a chemotactic response in BM-MSCs demonstrated by Boyden or transwell assays [13, 17, 23, 26, 27]. Moreover, TGF-β3, a chondrogenic factor, proved to be an chemotactic factor to adipose stem cells (ASCs), BM-MSCs, and synovium stem cells (SSCs) when codelivered with SDF-1 [24]. In addition, the chemotactic response of BMP-2 demonstrated an improvement in chemotaxis chamber coated with collagen IV and in transwell invasion assays [22], but on the other hand another study described that green fluorescent protein (GFP)-expressing human MSCs in Boyden chamber did not respond to BMP-2 [25]. Moreover, the response of MSCs in the presence of NGF-β, a growth factor that accelerated wound healing [42], was tested in Boyden chamber assay, but no chemotactic response was shown [17].

Table 1 – Migration assays: relevant literature concerning the different factors and concentrations of experimental models. Bold values represent the concentrations that showed significant cell migration in response to the correspondent factor; the other concentrations showed no significant stimulation.

Reference	Assay method	Cell source	Factors and concentrations
Fiedler et. al., 2002 [12]	Boyden chamber, 4 h	hMPCs derived from BM	rhbFGF (0.001, 0.01, 0.1 and 1 ng/mL) rhTGF-β1 (0.001, 0.01, 0.1 and 1 ng/mL) rhPDGF-BB (0.001, **0.01**, **0.1** and **1** ng/mL) rhBMP-2 (**0.001**, **0.01**, **0.1** and **1** ng/mL) rxBMP-4 (0.001, 0.01, 0.1 and **1** ng/mL)
Wang et. al., 2004 [10]	Boyden chamber, 5 h	hMSCs derived from BM	MCP-1 (37.5, 75, **150**, **300** ng/mL) MIP-1α (5, 10, **20**, **40** ng/mL) IL-8 (25, 50, **100**, **200** ng/mL)
Wynn et. al., 2004 [11]	Transwell assay	hMSCs derived from BM	SDF-1 (0, 10, **30**, **50** and 100 ng/mL)
Sordi et. al., 2005 [14]	Boyden, overnight ——— human pancreatic islets	hMSCs derived from BM	MIP-1α (100, **300**, 1000 ng/mL) SDF-1β (100, **300**, **1000** ng/mL) CXCL21 (100, **300**, 1000 ng/mL) CX3CL1 (fractalkine) (**100**, **300**, **1000** ng/mL) MIP-3β/CCL19 (100, **300**, 1000 ng/mL) CXCL16 (**100**, **300**, **1000** ng/mL)
Son et. al., 2006 [13]	Trans-matrigel, 24 h	hMSCs derived from BM or CB	HGF (**40** ng/mL) SDF-1 (**100** ng/mL) HGF (**0**, **5** and **40** ng/mL) + SDF-1 (**50** ng/mL)
Ponte et. al., 2007 [26]	Transwell, overnight	hMSCs derived from BM	SDF-1 (**150** ng/mL) MCP-1 (100 ng/mL) MIP-1α (50 ng/mL) RANTES (**150** ng/mL) Eotaxin-1 and -2 (100 ng/mL) MDC (**100** ng/mL) GRO-α (50 ng/mL) Fractalkine (300 ng/mL) FGF-2 (10 ng/mL) PDGF-AB (**10** ng/mL) HGF (**50** ng/mL) EGF (**10** ng/mL) VEGF (10 ng/mL) IGF-1 (**30** ng/mL) Ang-1 (10 ng/mL)

Ringe et. al., 2007 [19]	96-multiwell format ChemoTx plates, 20 h	hMSCs derived from BM	SDF-1 (1, 10, 75, 100, **250**, **500**, **1000** nM) IL8 (10, 75, **100**, **500**, **1000** nM) MCP-1 (1, 10, 100, 500, 1000 nM)
Croitoru-Lamoury et. al., 2007 [21]	Transwell, 48 h	hMSCs derived from BM	SDF-1 (**50** and **500** ng/mL) CX3CL1/ fractalkine (20 and **100** ng/mL) MCP-1 (100 and 1000 ng/mL) CXCL10/IP-10 (100 and **1000** ng/ mL)
Dwyer et. al., 2007 [15]	Transwell, 18 h	hMSCs derived from BM	MCP-1 (**150**, **300** and **600** pg/mL)
Ozaki et. al., 2007 [17]	Boyden chamber, 6 h	Rabbit and human MSCs	PDGF-BB (0.01, 0.1, **1**, **8**, **10**, **20**, **50** ng/mL) PDGF-AB (0.01, 0.1, 1, **8**, **10**, **50**, **100** ng/mL) HB-EGF (0.01, 0.1, **1**, **8**, **10**, **20**, **50** ng/mL) PDGF-AA (0.1-100 ng/mL) EGF (0.01, **0.1**, **1**, 8, 10, 50 ng/mL) TGF-A (0.01, **0.1**, **1**, **8**, **10**, **20**, 50 ng/mL) IGF-1 (0.1, 1, **10**, **50**, **100**, **200**, **300** ng/mL) HGF (**1**, **10**, **20**, **50**, **100** ng/mL) FGF-2 (0.01, **0.1**, **1**, **8**, **10**, **20**, **50** ng/mL) Thrombin (0.01, 0.1, **1**, **8**, **10**, **25** unit/mL) TGF-β1, TGF-β3 (0.01, 10, 50, 100 ng/mL) VEGF, BMP-2, SDF-1A, NGF-β, MCP-1 (0.1, 1, 10, 50, 100 ng/mL) IL-8 (0.01, 0.1, 1, 10, 50, 100, 200 ng/mL) Leptin (0.001, 0.01, 0.1, 1, 10, 50 ng/mL)
	Wound migration assay	Rabbit MSCs	PDGF-BB (**20** ng/mL) HB-EGF (**5** ng/mL) PDGF-AB (**50** ng/mL) TGF-A (5 ng/mL) EGF (1 ng/mL) FGF-2 (20 ng/mL) IGF-I (100 ng/mL) HGF (5 ng/mL) Thrombin (**10** unit/mL)
Rice et. al., 2010 [18]	Agarose drop model		SDF-1 (25, 50, **100, 250** ng/mL) MCP-1 (25, **50**, 100, 250, 500 ng/mL) RANTES (25, 50, **100, 250, 500** ng/mL) IP-10 (25, 50, **100,** 250 ng/mL) MIP-1α (25, 50, **100,** 250, 500 ng/mL) MIP-1β (25, 50, 100, 250 ng/mL)

Lee et. al., 2010 [38]	Bioscaffold of poly-ε-caprolactone and hydroxyapatite	Rabbit endogenous cells	TGF-β3 (**10** ng/mL)
Mendelson et. al., 2011 [24]	Gelatin microspheres into a porous collagen	Human BM-MSCs, ASCs and SSCs	TGF-β3 (**100** ng) SDF-1β (**100** ng) TGF-β3 (**100** ng) + SDF-1β (**100** ng)
Boomsma et. al., 2012 [20]	Mesencult	Mice MSCs	VEGF (30 ng/mL) MCP-1 (**30** ng/mL) MIP-1α (**100** pg/mL)
Zhang et. al., 2012 [37]	Transwell system, 6 h	hAC-MSCs and SM-MSCs	rhSDF-1α (**120** ng/mL)
	Col1 scaffold		rhSDF-1α (**120** ng/mL)
Lee et. al., 2012 [23]	Boyden chamber, 2 h	hMSCs derived from BM	IL-8 (**50** ng/mL) CCL20 (**50** ng/mL) CXCL12 (**50** ng/mL) MCP-1 (**50** ng/mL) PDGF-AA (**25**, **50**, **100**, **200** ng/mL) HGF (**50** ng/mL) IGF-1 (**50** ng/mL)
	Rat osteochondral defect		PDGF-AA (8.5 and 17 ng/μL) TGF-β1 (**8** ng/μL) + PDGF-AA (**8.5** and **17** ng/μL)
Phipps et. al., 2012 [25]	Boyden chamber, 20 h	GFP-expressing human MSCs	PDGF-AB (**10** ng/mL) PDGF-BB (**1**, **10** and **30** ng/mL) BMP-2 (10 and 100 ng/mL) Mixture of SDF1α, CXCL16, MIP-1α, MIP-1β and RANTES (1, 10 and 100 ng/mL)
	PCL/col/HA scaffold coated with PDGF-BB (bone-mimetic scaffold), 20 h		PDGF-BB (**10** ng/mL)

Vertelov et. al., 2013 [27]	48- and 96-well Corning Costar transwell chamber, 4 h	hMSCs derived from BM	HGF (**40** ng/mL) PDGF-AB (**10** ng/mL) EGF (**10** ng/mL) VEGF-121 (10 ng/mL) FGF-2 (10 ng/mL) IGF-1 (30 ng/mL) MIP-3β (10 ng/mL) MIP-1α (50 ng/mL) BCA-1 (5 ng/mL) RANTES (150 ng/mL) GRO-α (50 ng/mL) fractalkine (300 ng/mL) SDF-1α (**150** ng/mL) IL-1β (**10** ng/mL) IL-6 (**100** ng/mL) IL-8 (**50** ng/mL) TNF-α (**50** ng/mL)
Haasters et. al., 2014 [22]	Chemotaxis chamber coated with collagen IV, 15 h	hMSCs	BMP-2 (**100** ng/mL) and BMP-7 (100 ng/mL)
	Transwell invasion assay		BMP-2 (**30** ng/mL) and BMP-7 (**30** ng/mL)

*AC, articular cartilage; ASCs, adipose stem cells; BM, bone marrow; CB, cord blood; hMPCs, human mesenchymal progenitor cells; hMSCs, human mesenchymal stem cells; SM, synovial membrane; SSCs, synovium stem cells.

2.1.2 Chondrogenic differentiation factors

Chondrogenesis is a process of condensation of MSCs, followed by chondroprogenitor cell differentiation into chondrocytes. These stages are characterized by interactions between cell to cell and cell to matrix, respectively. In the condensation phase, it is evident a dense packing of MSCs that express N-cadherin and N-CAM. In the differentiation phase, chondroprogenitor cells interact with the ECM and ECM remodelling occurs: at early stages of chondrogenesis collagen type II and hyaluronian are synthesized, and at late stages collagen IX and XI, aggrecan, and COMP are produced. The final stage of chondrogenesis is the hypertrophy of chondrocytes, characterized by the formation of collagen type X, which leads to the remodelling of the cartilage into bone [36, 43, 44].

Growth factors play a significant role in chondrogenic differentiation of MSCs. In the field of cartilage tissue engineering, the most investigated chondrogenic factors belong to the TGF-β superfamily, such as TGF-(β1, β2, β3), BMP-(2, 4, 6, 7) and GDF-5. An overview of the used chondrogenic differentiation conditions to culture MSCs available in the literature is listed in Table 2. In these studies, the use of serum free culture, dexamethasone and ascorbic acid are common features.

Table 2 – Relevant literature concerning the chondrogenic differentiation of mesenchymal stem cells. Effects on gene expression and protein synthesis after chondrogenic differentiation culture of MSCs.

Reference	Method	Cell source	Factors	Gene expression and protein synthesis
Barry et. al., 2001 [45]	Pellets of 2×10^5 cells for 35 days	hBM-MSC	TGF-β1 (10 ng/mL) TGF-β2 (10 ng/mL) TGF-β3 (10 ng/mL)	Gene expression: Increase COL2A1, COL10A1 and AGCN Protein synthesis: Aggrecan, collagen II and collagen X
Sekiya et. al., 2005 [46]	Pellets of 2×10^5 cells for 21 days	hBM-MSC	BMP-2 (500 ng/mL) and TGF-β3 (10 ng/mL) BMP-4 (500 ng/mL) and TGF-β3 (10 ng/mL) BMP-6 (500 ng/mL) and TGF-β3 (10 ng/mL)	Gene expression: Increase COL2A1, COL10A1 and SOX9 Protein synthesis: proteoglycan and collagen II The combination of TGF-β3 and BMP-2 was more effective promoter of chondrogenesis than TGF-β3 with BMP-4 and -6.
Toh et. al., 2005 [47]	Pellets of 3×10^5 cells for 20 days	rabbit BM-MSCs	BMP2 (100 ng/mL) TGF-β1 (10 ng/mL) BMP2 (100 ng/mL) and TGF-β1 (10 ng/mL)	Gene expression: Increase COL2A1 Protein synthesis: proteoglycan The combination of TGF-β1 and BMP-2 was more effective promoter of chondrogenesis than TGF-β1 and BMP-2 alone.
Pelttari et. al., 2006 [48]	Pellets of 4-5×10^5 cells for 14, 28 and 42 days	hBM-MSCs	TGF-β3 (10 ng/mL)	Gene expression: Increase COL2A1, COL11A1, COL10A1 and MMP13 Protein synthesis: Collagen II and X
Noth et. al., 2007 [49]	Collagen type I hydrogel with 2.5×10^5 cells for 3 weeks	hBM-MSCs	BMP-2 (100 ng/ml) TGF-β1 (10 ng/ml)	Gene expression: High COL2A1 and AGCN and low COL10A1

Kim et. al., 2009 [29]	Pellets of 2.5×10^5 cells for 4 weeks	hBM- and AT-MSCs	BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), BMP-7 (100 ng/ml) TGF-β2 (5 ng/ml) BMP-2 (100 ng/ml) + TGF-β2 (5 ng/ml) BMP-6 (100 ng/ml) + TGF-β2 (5 ng/ml) BMP-7 (100 ng/ml) + TGF-β2 (5 ng/ml)	Gene expression: Increase COL2A1 and stable COL10A1 Protein synthesis: proteoglycan The best chondrogenic promoter was the combination of TGF-β2 and BMP-7
Ronzière et. al., 2010 [50]	Pellets of 2.5×10^5 cells, 24 days	hBM- and AT-MSCs	BMP-2 (50 ng/mL) TGF-β3 (10 ng/mL)	The chondrogenic differentiation of BM- and AT-MSCs was increased in the presence of BMP-2 and TGF-β3: Increase in COL2A1, COL10A1 and MMP-13
Weiss et. al., 2010 [32]	Pellets of 4-5×10^5 cells, 45 days	hBM-MSCs	BMP-2, -4, -6, -7 (10 ng/mL) aFGF (10 ng/mL) IGF-1 (10 ng/mL) TGF-β3 (10 ng/mL) PTHrP (10 ng/mL) TGF-β3 (10 ng/mL) in combination with the previous factors	No chondrogenic differentiation was present when BMP-2, -4, -6, -7, FGF-2, IGF-1 were alone TGF-3 in combination with each factor increase gene expression: COL2A1 and COL10A1, and protein synthesis: proteoglycan and collagen II and X
Lee et. al., 2010 [38]	Bioscaffold of poly-ε-caprolactone and hydroxyapatite	Host endoge nous cells	TGF-β3 (10 ng/mL)	In the TGF-β3 infused bioscaffold there was an increase in collagen II and AGCN and a higher matrix density and articular cartilage thickness
Yoon et. al., 2011 [30]	Pellet and HA scaffold culture of 3×10^5 cells for 4, 7, 10, and 14 days	hAT-MSCs	TGF-β1 (10 ng/mL) and BMP2 (50 ng/mL) TGF-β1 (10 ng/mL) and PDGF (50 ng/mL)	Better chondrogenesis was verified in HA scaffold culture than in pellet culture; Significant chondrogenesis of hAT-MSCs in the presence of TGF-β1 and BMP-2: increase in collagen II, SOX9 and aggrecan, and decrease in collagen I Decrease in chondrogenesis of hAT-MSCs in response to TGF-β1 and PDGF

Shintani et. al., 2013 [51]		bovine synovial explants	BMP-2 (200 ng/mL)	Gene expression: collagen II Protein synthesis: GAG expression
Murphy et. al., 2015 [31]	Aggregate culture, 7 days	hBM-MSCs and hAC	BMP-2 (100 ng/mL) GDF-5 (100 ng/mL) TGF-β1 (10 ng/mL) Combinations of the previous factors	hBM-MSCs and hAC showed significant chondrogenic differentiation in the presence of BMP-2 and TGF-β1 or BMP-2, GDF-5 and TGF-β1: higher expression of COL2A1, COL2A1/COL10A1, SOX-9 and AGCN, and GAG and collagen II staining
Felicia et. al., 2016 [52]	Aggregate culture of 2.5×10^5 cells for 14 days	BM-MSCs	Inhibit PDGF, FGFR and TGF-β signalling using AG-370, SU5402 and SB431542, respectively	Inhibition of PDGF signalling did not change the chondrogenic differentiation compared with no inhibitor control. On the other hand, inhibition of TGF-β and FGFR signalling resulted in a complete lack or reduced chondrogenic differentiation, respectively.

Although some studies investigated the stimulation of migration and chondrogenic differentiation of MSCs in response to certain factors, just a few tried to analyse the effect of those factors in both properties. For instance, Mendelson et al. [24] demonstrated the stimulation of the migration and the chondrogenic differentiation of hMSCs in gelatin microspheres supplemented with TGF-β3, and the combination of TGF-β3 and SDF-1β. Lee et. al. [23] has shown an increment in hMSC migration within the marrow cavity and the healing of cartilage in osteochondral defects of athymic nude rat after implantation of heparin-conjugated fibrin (HCF) loaded with PDGF-AA and TGF-β1. In another study, [38], a bioscaffold of poly-ε-caprolactone and hydroxyapatite supplemented with TGF-β3 was used to replace a rabbit joint. After 4 months, endogenous cell migration was stimulated by TGF-β3 and chondrocytes were uniformly distributed in a matrix with collagen II and aggrecan. In addition, the formed cartilage tissue had higher matrix density and thickness, and the shear properties were similar to those of the normal cartilage.

2.2 Endogenous Cell Source for Cartilage Repair

In this section, a summary of the sources of progenitor/stem cells within or nearby the joint, such as articular cartilage, bone marrow, synovium and synovial fluid, and infrapatellar fat pad, which might migrate to injured sites and to regenerate cartilage is presented (Figure 1).

2.2.1 Bone Marrow

Bone marrow (BM) is a tissue in the inner part of the long bones. *In vitro* and *in vivo* studies showed that BM is the niche of hematopoietic and mesenchymal stem cells. For cartilage tissue engineering, BM-MSCs are cells of great interest due to some characteristics, such as long term self-renewal capacity while retaining stemness properties, easy and efficient isolation, migration potential to injured/inflamed sites and ability to give origin to chondrocytes. Microfracture is a common used procedure that creates multiple holes in the subchondral bone to allow migration of marrow components to the joint defects [3, 6, 7]. However, this technique is mainly used to focal defects rather than spread lesions like in osteoarthritis. The infusion of BM-MSCs to target sites, (for which, so far, efficient homing has not yet been achieved) and more recently the recruitment of endogenous BM-MSCs are promising applications in Orthopaedics for cartilage repair [3].

2.2.2 Articular Cartilage

Chondrocytes are the main cell type residing in articular cartilage (AC) and they are responsible for tissue viability. Although chondrocytes are evolved in a dense matrix of collagens and proteoglycans, chondrocyte migration has been demonstrated in *in vitro* and *ex vivo* systems. On the other hand, *in vivo* studies still remain to be determined [53].

An additional population that can be found in equine [54] and human [55] AC is called AC progenitor cells. This population have some properties in common with MSCs, such as the colony forming ability, the high telomerase activity, and maintenance of telomere length [55] and of the migration capacity to damaged cartilage [56]. Compared to BM-MSCs, which resulted in hypertrophic cartilage phenotype after pellet culture, AC progenitor cells lacking the presence of collagen type X, an hypertrophic marker, might be a potential source for cartilage regeneration under appropriate conditions [54].

2.2.3 Synovium and synovial fluid

Synovium (SYN), also referred to synovial membrane, is a tissue that lines the inner surface of the joint, except where cartilage is present. This tissue is constituted of two layers: the surface layer (intima) and the underlying layer (subintima). The former is loose organized and avascular, whereas the latter is a meshwork with cells and blood vessels interspersed. Two types of cells are present in intimal layer, the macrophage-like (type A) and fibroblast-like (type B) synoviocytes. The macrophage-like synoviocytes are involved in removing undesirable substances from the synovial fluid, whereas fibroblast-like synoviocytes are responsible for the production of hyaluronan, collagen and fibronectin which give origin to the intimal interstitium and the synovial fluid. Synovia, commonly known as synovial fluid, is a viscous fluid present in the synovial cavity which lubricates and nourishes the cartilage and the bone of the joints [57].

Synovium and synovial fluid are promising cell sources for cartilage regeneration due to the presence of a reservoir of MSCs with higher chondrogenic potential compared to BM, adipose tissue, muscle, periosteum and infrapatellar fat pad [58] and the migration potential of those cells [59].

2.2.4 Infrapatellar fat pad

The infrapatellar fat pad, also known as Hoffa's fat pad, is an adipose tissue very vascularized and innervated which is located in the anterior knee compartment. Its function is still not clear, but some studies support that the infrapatellar fat pad has a role in the biomechanics of the knee or as a cell storage for joint regeneration after injury [60].

The current findings indicate that the infrapatellar fat pad is a source of MSCs that exhibit chondrogenic, adipogenic, and osteogenic phenotypes in the presence of the appropriate media [61]. In addition, these cells demonstrated high proliferation and multipotent capacity even in late stages of osteoarthritis. This characteristic, the accessibility of this source, and the large number of cells at harvesting could make this source appropriate for cartilage therapy [62]. However, Wei et. al. have shown that the release of inflammatory factors from infrapatellar fat pad inhibit chondrogenic differentiation of MSCs and the production of hyaline cartilage matrix [63].

BM [3], AC [56] and SYN [59] progenitor/stem cells, due to their migratory potential to damage cartilage tissue and chondrogenic differentiation ability, raise new possibilities for the future treatment of cartilage defects.

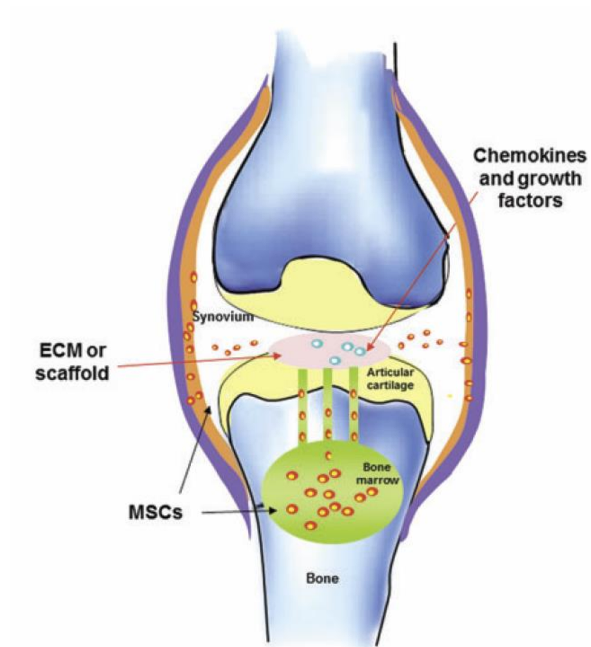


Figure 1 – Bone marrow, articular cartilage and synovium as sources of MSCs adjacent to the joint. (Adapted from [9]).

3. Materials and Methods

3.1 Cell isolation, culture and characterization

3.1.1 Human MSCs derived from bone marrow

Bone marrow derived MSCs (BM-MSC) were acquired by ethical approval and informed consent of four 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 counted in 3% acetic acid with methylene blue leave and platted at 30×10^6 nc/T175 to 30×10^6 nc/T175 in 20 mL of alpha-minimum essential medium (α-MEM, 1:1, Invitrogen) supplemented with 10% (vol/vol) 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. This medium is designated culture medium hereafter. After 24h, all nonadherent cells were washed off by changing the culture medium and BM-MSCs, the adherent cells, were further expanded when sub-confluent (approximately 90% confluent). BM-MSCs were used in passage 2 or 3 for the migration assays and chondrogenesis.

3.1.2 Human MSCs derived from synovium

Synovium was acquired by ethical approval and informed consent of four OA patients who underwent joint replacement surgery (Orthopaedics Department, Erasmus MC, University Medical Centre Rotterdam, the Netherlands). The 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. Synovium derived MSCs (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.

3.2 Migration assays

3.2.1 Spheroids formation

The first step of migration assays is the formation of spheroids from BM- or SYN-MSCs in a 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 were collected for the next phase.

3.2.2 Collagen matrix

The migration of the spheroids was evaluated in a 3D collagen gel for five chemokines including MCP1/CCL2 (50 and 100 ng/mL), MCP-3/CCL7 (50 and 100 ng/mL), MIP-1α/CCL3 (50 and 100 ng/mL), SDF-α/CXCL12 (50 and 100 ng/mL), and RANTES/CCL5 (10, 25, 35, 50 and 100 ng/mL), five growth factors such as PDGF-BB (50 and 100 ng/mL), NGF-β (50 and 100 ng/mL), BMP-2 (50 and 100 ng/mL), IGF-1 (50 and 100 ng/mL), and TGF-β1 (10, 50 and 100 ng/mL), and three inflammatory cytokines including IL-1β (1 and 10 ng/mL), IFN-γ (1 and 10 ng/mL), and TNF-α (1 and 10 ng/mL). 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 µL of 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. Duplicate wells of each condition were evaluated for BM donor 3 and 4, and SYN donor 4 for more consistent results. The chemokines used were recombinant factors of human origin and purchased from PeproTech. Concentrations of chemotactic factors were chosen based on migratory activity described in literature.

3.2.3 Confocal laser scanning microscopy

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.

3.2.4 ImageJ data analysis

The migration of the spheroids in the collagen gel was measured using Fiji [64]. Fiji is a software that includes ImageJ, an image processing program, with several plugins for biological functionalities. The image processing starts with filtering the image to remove the noise and increase the contrast, followed by binarisation, i.e. a value of 1 pixel is attributed to cells and a value of 0 pixel to the background. The binary image was decomposed into two components, such as the main core of cells and the cells that move from their initial position but stay attached to the core, and the single or group of cells that detached from the main core. The former will be hereinafter named ´core and sprouting cells´, whereas the latter will be named ´migrating cells´. 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*.

3.2.5 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.

3.3 Chondrogenic differentiation

3.3.1 Pellet culture

For testing chondrogenic differentiation, a three dimensional pellet culture system was used. Pellets were formed by centrifugation of 0.2×10^6 BM- and SYN-MSCs at 1100 rpm for 8 minutes in 15 mL conical polypropylene tubes (VWR). hMSCs were cultured in 0.5 mL of medium containing Dulbecco's Modified Eagle's Medium, High (4.5 g/l) Glucose (DMEM-HG 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), 0.1 mM ascorbic-acid (1:500, Sigma) and 100 nM dexamethasone (1:10000, Sigma). Hereafter, this medium is named chondrogenic medium. The chondrogenic medium was supplemented with 50 ng/mL of NGF-β, SDF-1α, BMP-2, and PDGF-BB or the combination of those factors with 10 ng/mL of TGF-β1. An addition condition: 25 ng/mL of RANTES along with 10 ng/mL of TGF-β1, was introduced later on for the SYN donor. The tubes were incubated at 37°C with a humidified atmosphere of 5% CO_2. The 0.5 mL chondrogenic medium was carefully refreshed twice a week for 28-32 days.

3.3.2 Immunostaining

After 28-32 days, the cells were fixed in 4% formalin overnight at 4°C and then infiltrated in paraffin. For sectioning, the pellets were embedded in paraffin blocks. Sections of 6 µm were done using a microtome. For staining, slides were deparaffinised and stained with thionin for 5 minutes, and subsequently hydrated. Images were taken under an optical microscope. To quantify the chondrogenic differentiation a qualitative scale was used (Table 3).

Table 3 – Qualitative scale of chondrogenic differentiation used to quantify pellets 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

3.3.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the pellets using mechanical pressure and stored in 350 μL of RNAbee (Bioconnect) at -80°C until use. RNA isolation was performed using the RNeasy micro kit (Qiagen) and its quantification was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) at 260/280 nm. Then, total RNA was converted to cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis kit (Thermoscientific). Finally, RT-PCR was performed with a Bio-Rad CFX96 RT-PCR Detection System using Taqman Universal PCR mastermix (Applied Biosystems) and Q-PCR mastermix Plus for SYBR green (Eurogentec). The expression of genes, such as collagen type II (Col2A1), collagen type X (Col10A1), aggrecan (AGCN) and SRY (sex determining region Y)-box 9 (SOX9), were examined. Col2A1 and AGCN are markers of chondrogenic phenotype. Col10A1 is a marker of hypertrophic phenotype. SOX9 is a transcription factor modulating chondrogenic differentiation and matrix synthesis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT)-1 and ribosomal protein (RP)-S27a were used as a housekeeping genes. The relative gene expression was determined by the $2^{-\Delta CT}$ method [65].

4. Results

4.1 Chemokines and Growth Factors that Induce Migration of Bone Marrow and Synovial MSCs

The migration potential of BM (passage 2 and 3) and SYN (passage 1 and 2) MSCs was evaluated in a 3D collagen gel (0.58 ng/mL), supplemented with different factors, after 48 hours. A panel of thirteen molecules: three inflammatory cytokines (IL-1α, IFN-γ, and TNF-β), five chemokines (MCP-1/CCL2, MCP-3/CCL7, MIP-1α/CCL3, SDF-1α/CXCL12, and RANTES/CCL5), and five growth factors (PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1), were tested (Table 4). Concentrations of factors were selected according to literature, which showed that these factors at certain concentrations induce chemotactic activity of stem cells [10-27].

Table 4 – Factors and correspondent concentrations tested in the migration assay.

	Factors		Concentration (ng/mL)
Inflammatory cytokines	IL-1β	Interleukine-1β	1 and 10
	IFN-γ	Interferon-γ	1 and 10
	TNF-α	Tumour necrosis factor-α	1 and 10
Chemokines	MCP-1	Monocyte chemoattractant protein-1	50 and 100
	MCP-3	Monocyte chemoattractant protein-3	50 and 100
	MIP-1α	Macrophage inflammatory protein-1α	50 and 100
	SDF-1α	Stromal cell derived factor-1α	50 and 100
	RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted	10, 25, 35, 50 and 100
Growth factors	PDGF-BB	Platelet growth factor-BB	50 and 100
	NGF-β	Nerve growth factor-β	50 and 100
	BMP2	Bone morphogenetic protein-2	50 and 100
	IGF-1	Insulin-like growth factor-1	50 and 100
	TGF-β1	Transforming growth factor-β1	10, 50 and 100

After 48 hour of migration in the collagen gel, the spheroids were imaged under the confocal microscope (Figure 2 A). Both sprouting cells (cells that migrated from their initial position but remained attached to the main core, Figure 2 B, red) and migrating cells (individual or group of cells detached from the main core, Figure 2 B, green) were observed. Based on the image, the migrating cell area and the core and sprouting cell area were computed for each spheroid as functions of the distance reached by the migrating and sprouting cells, respectively (Figure 2 C); it is worth to point out that it could not be possible to decompose the measurement of the core cell area and of the sprouting cell area. Only migrating cells were quantified to evaluate the migratory potential of MSCs derived from BM (Figure 3) and SYN (Figure 4) in response to different factors. Two parameters were quantified: the migratory cell area (sum of the values of migrating cell area) and the maximum migratory cell 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 cell distance.

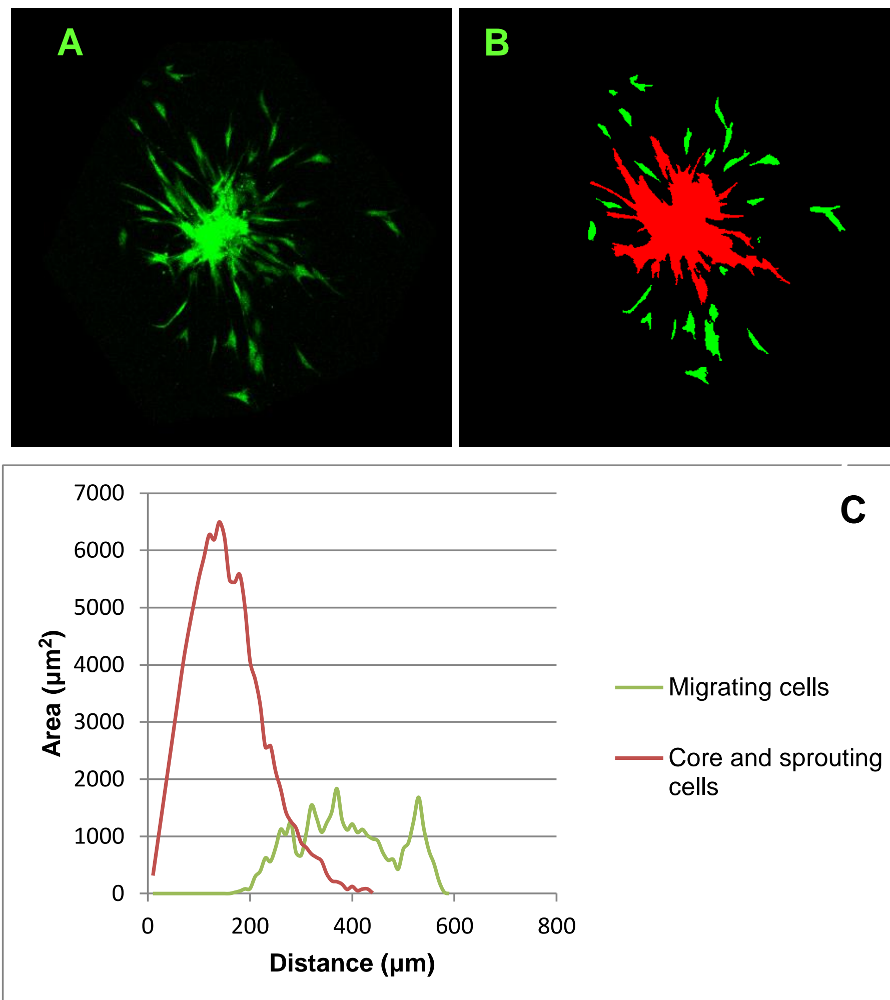


Figure 2 – Migration of a spheroid after 48 hours. A. Confocal microscope representation of a spheroid in response to BMP-2 (A) and the correspondent image (B) and migration data (C) after analysis (the red curve represents the core and sprouting cell; the green curve represents the migrating cells).

The results of the migratiory area of BM-MSCs (Figure 3 A) demonstrated that:

- The use of 50 and 100 ng/mL of SDF-1α (p<0.01 and p<0.05, respectively), and of 10, 25 and 50 ng/mL RANTES (p<0.05, p<0.05 and p<0.01, respectively) caused significant improvement of BM-MSCs migratory area compared to the condition without factors (negative control, NC). No significant differences were found between the two concentrations of SDF-1α (p>0.05), whereas among the concentrations of RANTES significant differences were evident between 50 and 100 ng/mL (p=0.1), and 10 and 100 ng/mL (p=0.05);
- Both concentrations (1 and 10 ng/mL) of IL-1β and TNF-α caused significant increase of migratory cell area compared to the NC (p<0.001 and p<0.01, respectively). No significant differences were evident in between the two concentrations for each factor (p>0.05);
- Among the growth factors, there was significant migration based on migratory cell area in the presence of 50 ng/mL of PDGF-BB (p<0.001), 50 and 100 ng/mL of BMP-2 (p<0.05), 50 ng/mL of NGF-β (p<0.05), and 10 ng/mL of TGF-β1 (p<0.05) compared to the NC.

The estimation of migration based on maximum migratory distance of BM-MSCs (Figure 3 B) revealed that:

- BM-MSCs migrated significantly ($p<0.001$) compared to the NC in the presence of 1 and 10 ng/mL of IL-1β; 1 ng/mL of TNF-α; 50 and 100 ng/mL of SDF-1α; 10, 25 and 50 ng/mL of RANTES; 50 ng/mL of PDGF-BB; 50 and 100 ng/mL of NGF-β; 50 and 100 ng/mL of BMP-2; and 10 and 50 ng/mL of TGF-β;
- A slight increase in the maximum migratory cell distance compared to the NC was seen in response to 100 ng/mL of MCP-1; 100 ng/mL of MCP-3; 100 ng/mL of RANTES; 10 ng/mL of TNF-α; and 50 and 100 ng/mL of MIP-1α;
- Both concentrations of IFN-γ demonstrated no differences in maximum migration cell distance compared to the NC.

A summary of the normalized migratory area and maximum migratory distance of BM-MSCs in the presence of different factors compared to the NC is presented in Table 5.

Based on the evaluation of the obtained migratory area and maximum migratory distance of BM-MSCs in the presence of the factors, the highest and significant migration potential of BM-MSCs in the presence of the different factors, the highest and significant migration potential of BM-MSCs was observed in response to both concentrations of IL-1β and 50 ng/mL of PDGF-BB, followed by 1 ng/mL of TNF-α, 50 and 100 ng/mL SDF-1α, 10, 25 and 50 ng/mL of RANTES, 50 ng/mL of NGF-β, and 50 ng/mL of BMP-2.

Furthermore, the migration of SYN-MSCs was carried out in the presence of the previous factors and the same parameters (migratory cell area and maximum migratory cell distance) were used to evaluate it (Figure 4).

For migratory area of SYN-MSCs (Figure 4 A):

- A very significant migration compared to the NC was measured in the presence of some chemokines, such as 50 ng/mL of MCP-1, 50 and 100 ng/mL SDF-1α, and 10 and 25 ng/mL of RANTES ($p<0.001$); Significant differences were found between the two concentrations of MCP-1 ($p<0.01$), and among the concentrations of RANTES (25 and 50 ng/mL, $p<0.001$; 25 and 100 ng/mL, $p<0.001$; 10 and 100 ng/mL, $p<0.05$);
- The presence of inflammatory cytokines, such as IL-1β, TNF-α and INF-δ did not show significant differences in migratory cell area comparing to the NC nor between the two different concentrations (1 and 10 ng/mL);
- The growth factors, 50 ng/mL of PDGF-BB ($p<0.001$), 50 ng/mL of NGF-β ($p<0.05$) and 50 ng/mL of BMP-2 ($p<0.001$) caused significant improvement.

Regarding maximum migratory distance of SYN-MSCs (Figure 4 B):

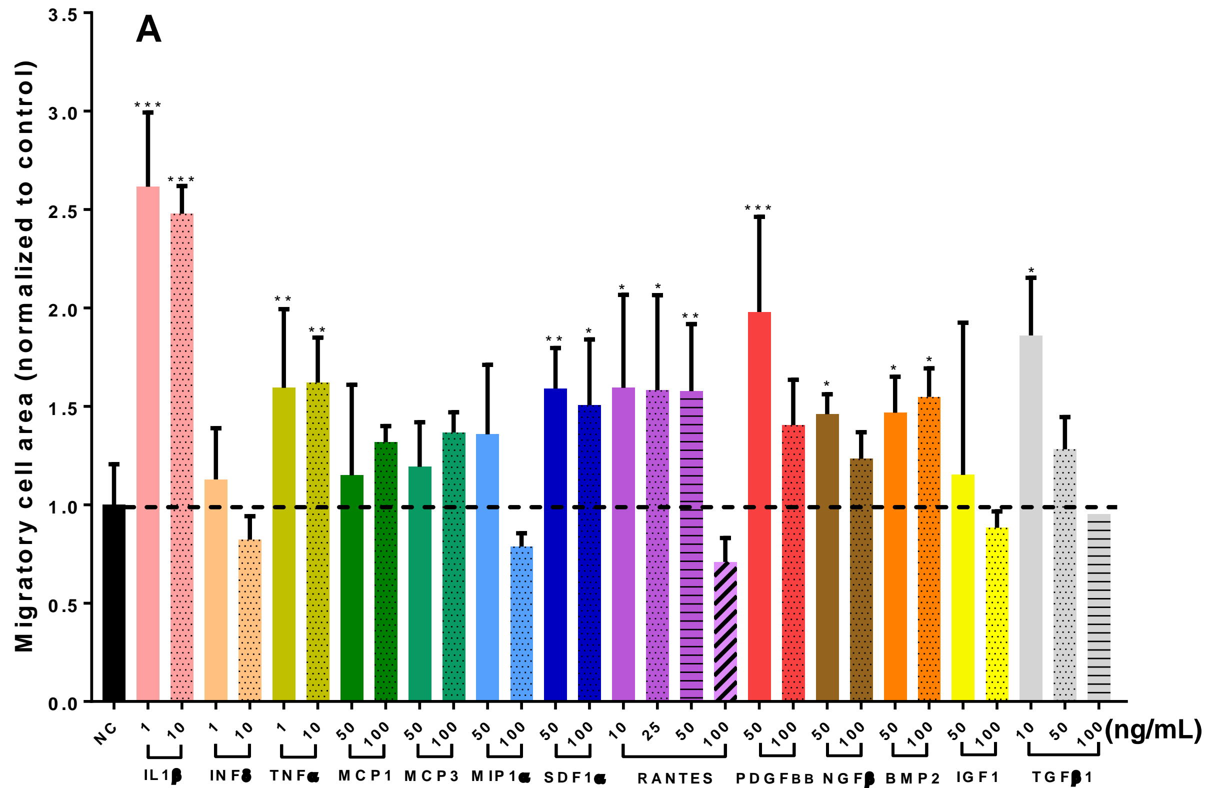
- TNF-α was the only inflammatory cytokine showing an increase in the maximum migratory cell distance ($p<0.05$);
- Most of the cytokines increased significantly the migration cell distance compared to the NC, except both concentrations of MIP-1α;

- The growth factors showed very significant increased ($p<0.001$) in the presence of both concentrations of PDGF-BB, NGF-β and BMP-2, and 10 and 50 ng/mL of TGF-β1.

A summary of the normalized migratory area and maximum migratory distance of SYN-MSCs in the presence of different factors with the reference to the NC is presented in Table 6.

For SYN-MSCs the factors that stimulated the highest and significant migration were 50 and 100 ng/mL of SDF-1α, 10 and 25 ng/mL of RANTES, 50 ng/mL of NGF-β, 50 ng/mL of BMP-2, and 50 ng/mL of PDGF-BB.

The comparison of migration of BM- and SYN-MSCs showed that IL-1β and TNF-α were recruitment factors for BM-MSCs, but not for SYN-MSCs. MCP-1 showed a significant increase in migratory area and distance of SYN-MSCs, 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.



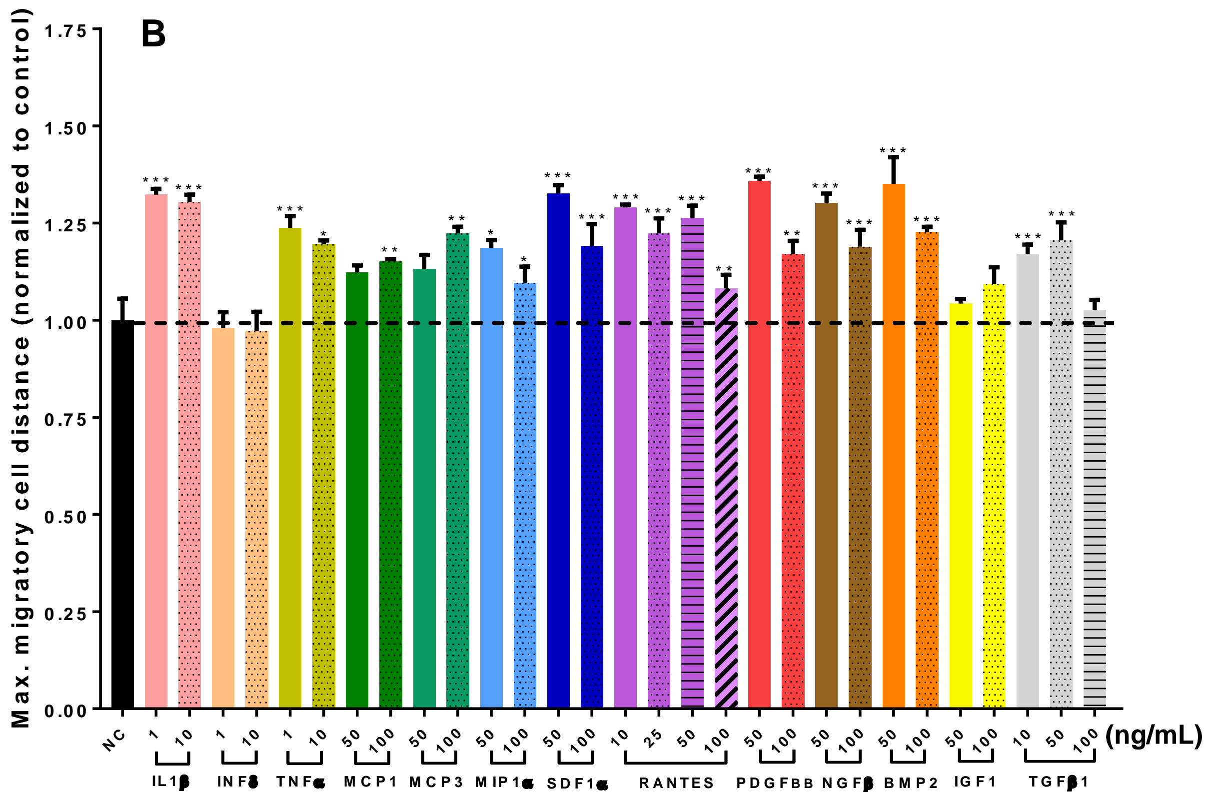


Figure 3 - Migration of MSCs derived from BM in response to different factors. *In vitro* migration of BM-MSCs in collagen gel supplemented with culture medium containing different factors was evaluated after 48 hours under confocal microscopy. Parameters such as migratory cell area (A) and maximum migratory cell distance (B) were 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. Data 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).

Table 5 – Normalised migratory cell area and maximum migratory cell distance of BM-MSCs in the presence of different factors with reference to the negative control. Values of significant migratory cell area and maximum migratory cell distance are represented as * *p*<0.05; ** *p*<0.01; *** *p*<0.001 where *p* is evaluated with reference to the negative control (n=4 independent experiments).

Factor and concentration		Migratory cell area	Max. migratory cell distance
Negative control		1±0.15	1±0.04
IL-1β	1 ng/mL	2.62±0.27 ***	1.32±0.01 ***
	10 ng/mL	2.48±0.10 ***	1.30±0.01 ***
INF-δ	1 ng/mL	1.13±0.18	0.98±0.03
	10 ng/mL	0.82±0.08	0.97±0.04
TNF-α	1 ng/mL	1.59±0.28 **	1.24±0.02 ***
	10 ng/mL	1.62±0.16 **	1.20±0.01 *
MCP-1	50 ng/mL	1.15±0.33	1.12±0.01
	100 ng/mL	1.32±0.06	1.15±0.00 **
MCP-3	50 ng/mL	1.19±0.16	1.13±0.03
	100 ng/mL	1.37±0.07	1.22±0.01 **
MIP-1α	50 ng/mL	1.36±0.24	1.19±0.01 *
	100 ng/mL	0.78±0.05	1.10±0.03 *
SDF-1α	50 ng/mL	1.59±0.15 **	1.33±0.01 ***
	100 ng/mL	1.51±0.24 *	1.19±0.04 ***
RANTES	10 ng/mL	1.59±0.33 *	1.29±0.01 ***
	25 ng/mL	1.58±0.34 *	1.22±0.03 ***
	50 ng/mL	1.58±0.24 **	1.26±0.02 ***
	100 ng/mL	0.71±0.09	1.08±0.02 **
PDGF-BB	50 ng/mL	1.98±0.34 ***	1.36± 0.01 ***
	100 ng/mL	1.4±0.16	1.17± 0.02**
NGF-β	50 ng/mL	1.46±0.07 *	1.30±0.02 ***
	100 ng/mL	1.23±0.10	1.19±0.03 ***
BMP-2	50 ng/mL	1.47±0.13 *	1.35±0.05 ***
	100 ng/mL	1.55±0.10 *	1.23±0.01 ***
IGF-1	50 ng/mL	1.15±0.55	1.04±0.01
	100 ng/mL	0.88±0.06	1.09±0.03
TGF-β1	10 ng/mL	1.86±0.7 *	1.17±0.02 ***
	50 ng/mL	1.28±0.20	1.21±0.03 ***
	100 ng/mL	0.95±0.12	1.03±0.02

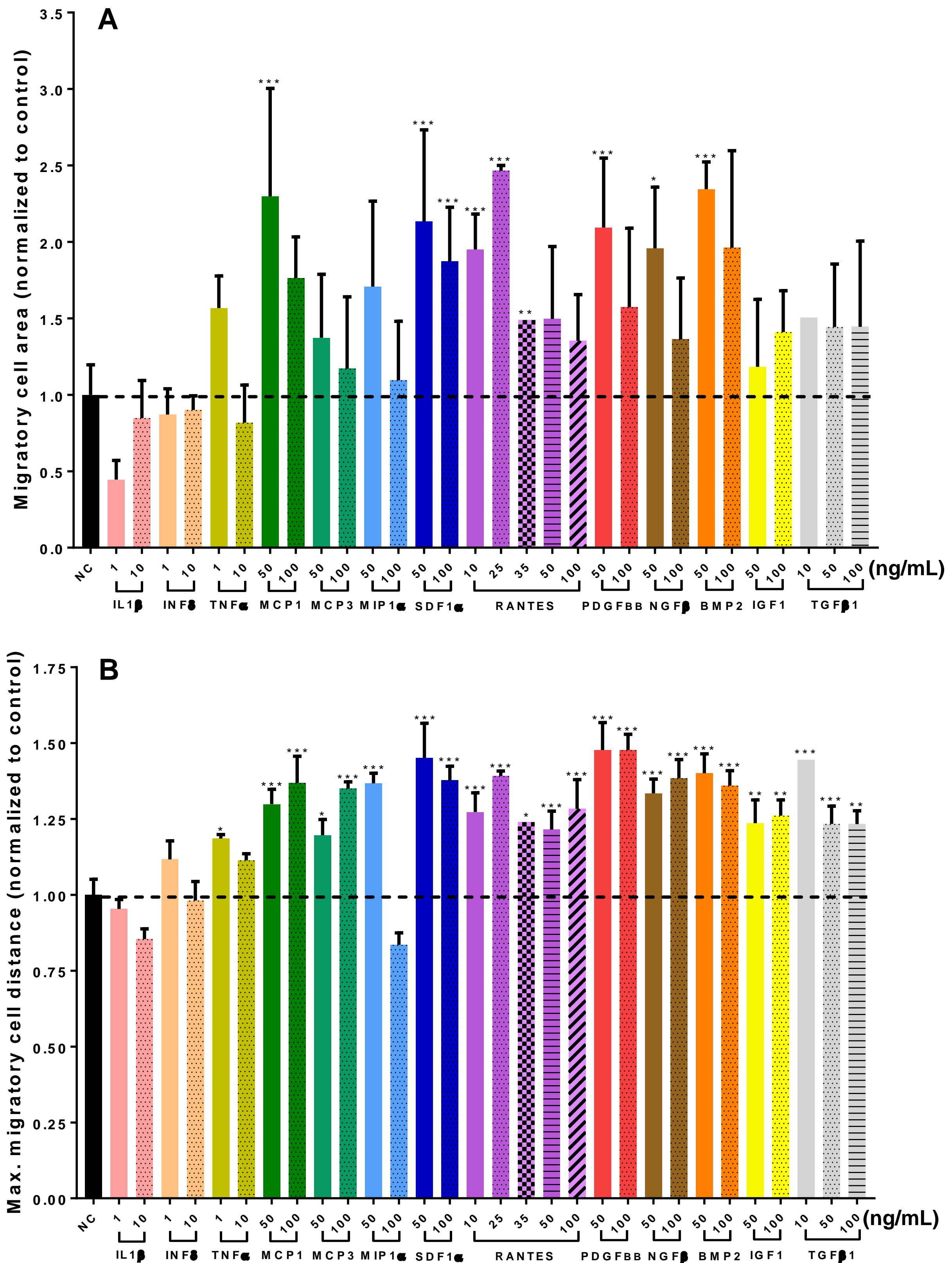


Figure 4 - Migration of MSCs derived from SYN in response to different factors. *In vitro* migration of SYN-MSCs in collagen gel supplemented with culture medium containing different factors was evaluated after 48 hours under confocal microscopy. Parameters such as migratory cell area (A) and maximum migratory cell distance (B) were 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. Data 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).

Table 6 – Normalised migratory cell area and maximum migratory cell distance of SYN-MSCs in the presence of different factors with reference to the negative control. Values of significant migratory cell area and maximum migratory cell distance are represented as * *p*<0.05; ** *p*<0.01; *** *p*<0.001 where *p* is evaluated with reference to the negative control (n=4 independent experiments).

Factor and concentration		Migration cell area	Max. migratory cell distance
Negative control		1±0.14	1±0.04
IL-1β	1 ng/mL	0.46±0.09	0.95±0.02
	10 ng/mL	0.85±0.17	0.85±0.02
INF-δ	1 ng/mL	0.87±0.12	1.11±0.04
	10 ng/mL	0.90±0.07	0.98±0.04
TNF-α	1 ng/mL	1.57±0.15	1.19±0.01 *
	10 ng/mL	0.82±0.17	1.11±0.02
MCP-1	50 ng/mL	2.30±0.50 ***	1.30±0.03 ***
	100 ng/mL	1.76±0.19	1.37±0.06 ***
MCP-3	50 ng/mL	1.37±0.29	1.20±0.04 *
	100 ng/mL	1.17±0.33	1.35±0.02 ***
MIP-1α	50 ng/mL	1.70±0.39	1.37±0.02 ***
	100 ng/mL	1.10±0.27	0.84±0.03
SDF-1α	50 ng/mL	2.13±0.42 ***	1.45±0.08 ***
	100 ng/mL	1.87±0.25 ***	1.38±0.03 ***
RANTES	10 ng/mL	1.95±0.16 ***	1.27±0.04 ***
	25 ng/mL	2.46±0.03 ***	1.39±0.01 ***
	35 ng/mL	1.49±0.00 **	1.24±0.00 *
	50 ng/mL	1.50±0.33	1.22±0.04 ***
	100 ng/mL	1.35±0.21	1.28±0.07 ***
PDGF-BB	50 ng/mL	2.09±0.32 ***	1.48±0.06 ***
	100 ng/mL	1.57±0.37	1.48±0.04 ***
NGF-β	50 ng/mL	1.96±0.28 *	1.33±0.03 ***
	100 ng/mL	1.36±0.28	1.38±0.04 ***
BMP-2	50 ng/mL	2.34±0.13 ***	1.40±0.04 ***
	100 ng/mL	1.96±0.45	1.36±0.03 ***
IGF-1	50 ng/mL	1.18±0.31	1.24±0.05 **
	100 ng/mL	1.41±0.19	1.26±0.04 **
TGF-β1	10 ng/mL	1.51±0.00	1.44± ***
	50 ng/mL	1.44±0.29	1.23±0.04 ***
	100 ng/mL	1.44±0.0.39	1.23±0.03 **

4.2 Chondrogenic Differentiation of Bone Marrow and Synovium MSCs

Cartilage regeneration not only requires the migration of mesenchymal stem cells to the defects but also the chondrogenic differentiation 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 chondrogenic differentiation. To achieve this, a 3D pellet culture was induced for 3 BM donors and 1 SYN donor for 28 or 32 days. The BM-MSCs of the first donor were cultured 32 days in chondrogenic medium supplemented with 10 ng/mL TGF-β1 (positive control, PC), 50 ng/mL NGF-β, 50 ng/mL PDGF-BB, 50 ng/mL BMP-2, and 50 ng/mL SDF-1α, or the combination of TGF-β1 with NGF-β (TGF-β1/NGF-β), PDGF-BB (TGF-β1/PDGF-BB), BMP-2 (TGF-β1/BMP-2), or SDF-1α (TGF-β1/SDF-1α); same concentration of factors as above. TGF-β1 has been proved in several works to be able to improve chondrogenic differentiation; specifically, 10 ng/mL was identified as the best concentration [30, 31, 45, 47, 49], and after it has been commonly adopted. Despite inducing significant migration of BM-MSCs, IL-1β and TNF-α were not considered in this assay as they are known to inhibit chondrogenesis [66].

Based on thionin staining, a qualitative scale as described in section 3.3.2 was used to quantify chondrogenic differentiation of the pellets of each donor (Table 7). Thionin binds to carbohydrates which are a main component of glycosaminoglycan (GAGs). Since GAGs are part of the cartilage matrix, they are used to measure the quality of the cartilage tissue.

Table 7 – Qualitative quantification of chondrogenic differentiation of BM-MSCs after 28 or 32 days of pellet culture in the presence of TGF-β1 (positive control), NGF-β, BMP-2, SDF-1α, PDGF-BB, the combination of TGF-β1 /NGF-β, TGF-β1/BMP-2, TGF-β1/SDF-1 and TGF-β1/PDGF-BB. (n=3 independent experiments. 0, +, ++ and +++ represent none, low, moderate and high chondrogenic differentiation, respectively).

Factor / Donor	TGF-β1	NGF-β	BMP-2	SDF-1α	PDGF-BB	TGF-β1/ NGF-β	TGF-β1/ BMP-2	TGF-β1/ SDF-1α	TGF-β1/ PDGF-BB
1	++	0	0	0	0	++	+++	++	+
2	+	-	-	-	0	++	+++	+	+
3	++	-	-	-	0	++/+++	+++	++	+

After 32 days of pellet culture, donor 1 revealed:

- No thionin staining in the presence of NGF-β, BMP-2, SDF-1α and PDGF-BB (Figure 5 B1, C1, D1 and E1, respectively);
- Very similar intensity of the thionin staining and chondrogenic-like morphology in response to TGF-β1/NGF-β and TGF-β1/SDF-1α compared to TGF-β1 (Figure 5 G1, I1 and A1 respectively);
- An increment in the proliferation of the cells with a fibroblast-like morphology in response to PDGF-BB and the combinatorial effect of TGF-β1/PDGF-BB (Figure 5 E1 and H1);
- The best thionin staining and highest chondrocyte-like morphology in the presence of TGF-β1/BMP-2 (Figure 5 H1).

As mentioned previously, chondrogenic differentiation was tested for two more BM donors. In these cases, pellet culture was carried out for 28 days in the presence of PDGF-BB and the combination of TGF-β1/NGF-β, TGF-β1/PDGF-BB, TGF-β1/BMP-2 or TGF-β1/SDF-1α. 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 alone, 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 main outcomes of the pellet culture performed on donor 2 are the following:

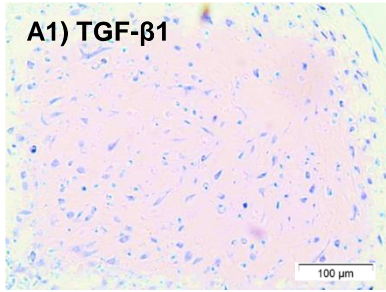
- A slight chondrogenic potential in the presence of TGF-β1 was shown by the low thionin staining (Figure 5 A2);
- No chondrogenic differentiation was seen in response to PDGF-BB; on the other hand an increase in the proliferation of cells with a fibroblastic-like morphology was present (Figure 5 B2). This latter result was also shown in the presence of TGF-β1/PDGF-BB (Figure 5 F2);
- No inhibition nor improvement in chondrogenesis was observed in the presence of TGF-β1/SDF-1α (Figure 5 E2);
- An increase in GAGs expresion compared to TGF-β1 was seen for the following combinations: TGF-β1/NGF-β, as well as TGF-β1/BMP-2 (Figure 5 C2 and D2, respectively).

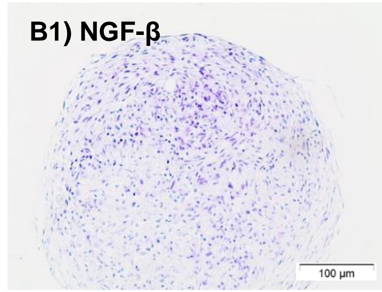
Furthermore, thionin staining of donor 3 after 28 days of pellet culture demonstrated:

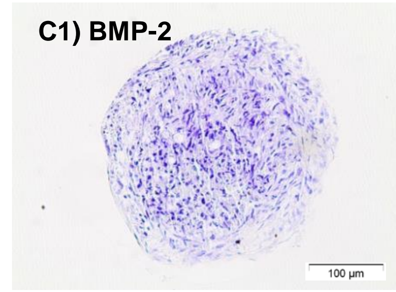
- Pellets in response to PDGF-BB showed no thionin staining (Figure 5 B3);
- A small number of chondrocyte-like morphology was seen in the structure of the pellets treated with TGF-β1/NGF-β; however no significant staining was observed with reference to TGF-β1 (Figure 5 C3 and A3, respectively);
- The highest accumulation of GAGs and chondrocyte-like morphology in the presence of TGF-β1/BMP-2 compared to TGF-β1 (Figure 5 D3 and A3, respectively);
- No notable differences in thionin staining in the pellets which were treated with TGF-β1/SDF-1α, and TGF-β1/PDGF-BB compared to TGF-β1 (Figure 5 E3 and F3, respectively); however, on the one hand TGF-β1/SDF-1α showed a small chondrocyte-like morphology and on the other hand TGF-β1/PDGF-BB demonstrated a fibroblast-like morphology.

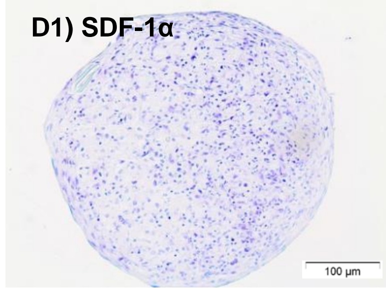
Altogether, pellets supplemented with TGF-β1/BMP-2 demonstrated for the three donors the highest thionin staining among the tested factors; beyond that, the existence of the chondrocyte morphology indicated close proximity to cartilage tissue. One more condition, TGF-β1/NGF-β, verified the same trend in the three donors: high expression of GAGs, however not higher than in the pellets from TGF-β1 (PC), and a small number of chondrocytes, which was also seen in the TGF-β1 treatment.

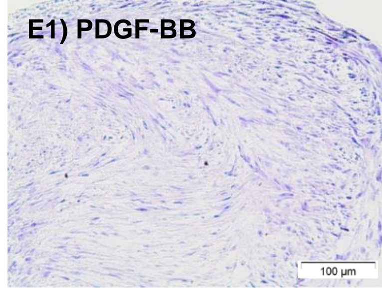
Donor 1

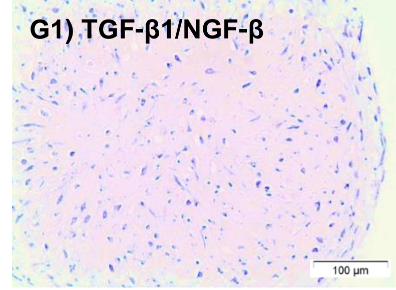


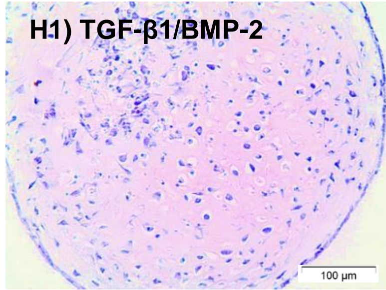


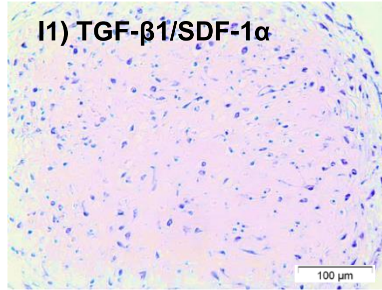


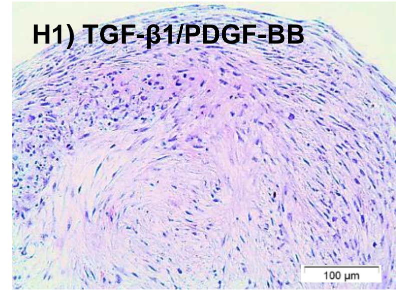




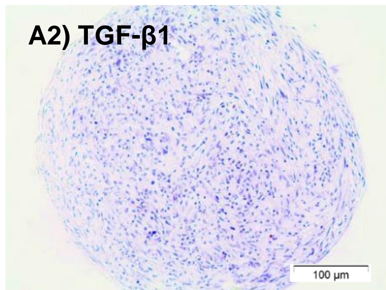


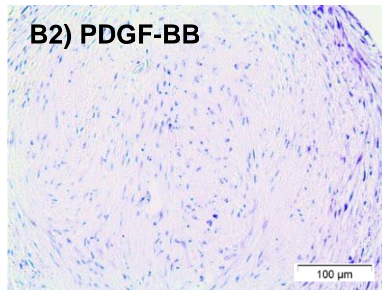


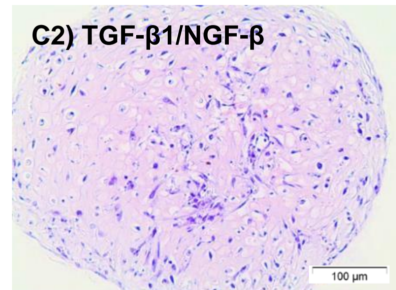


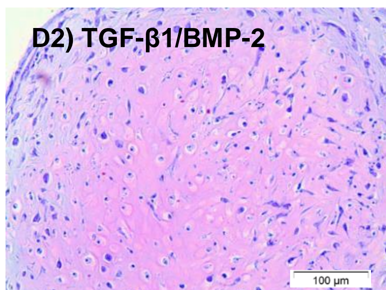


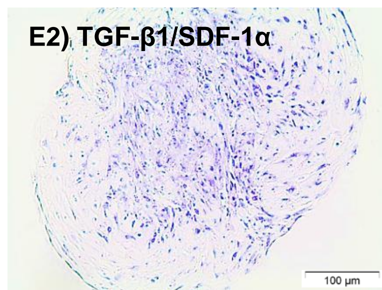
Donor 2

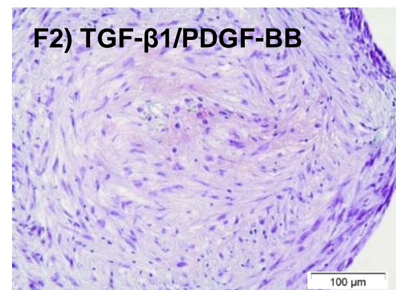












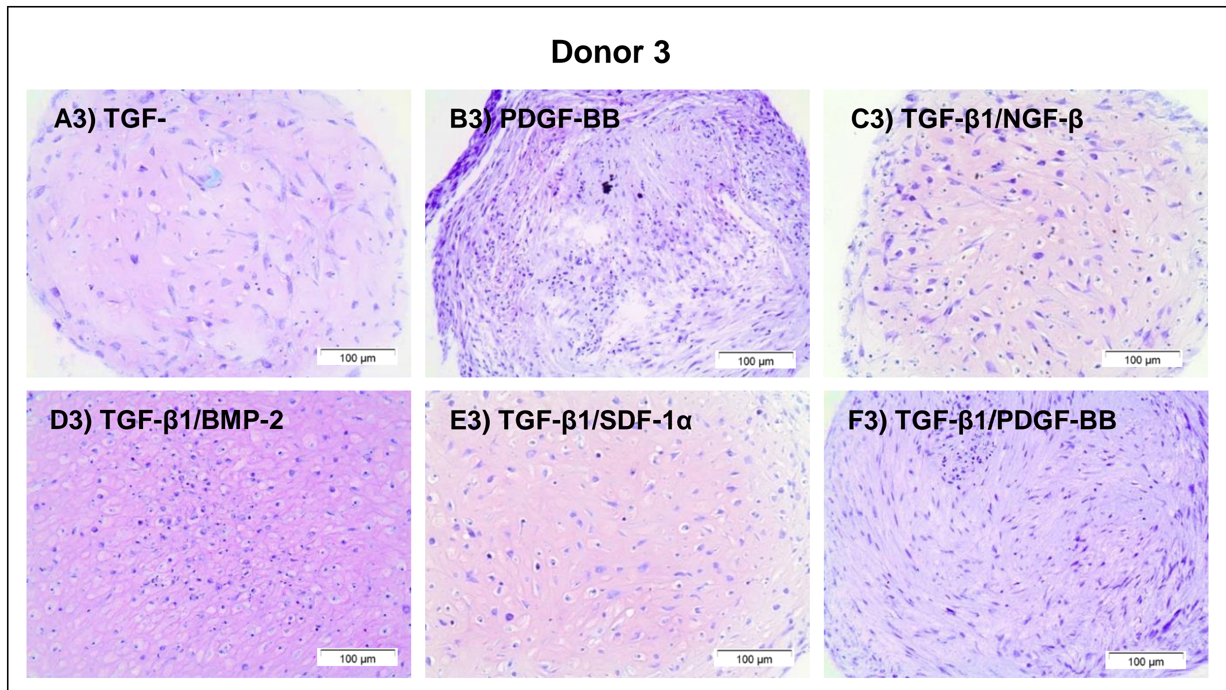


Figure 5 - GAGs analyses of BM-MSCs after 28 or 32 days of pellets culture treated in chondrogenic medium supplemented with additional factors. Thionin staining was performed to detect GAGs in the ECM of the pellets treated in chondrogenic medium supplemented with TGF-β1 (A1, A2, A3), NGF-β (B1), BMP-2 (C1), SDF-1α (D1) and PDGF-BB (E1, B2, B3), TGF-β1/NGF-β (F1, C2, C3), TGF-β1/BMP-2 (G1, D2, D3), TGF-β1/SDF-1α (H1, E2, E3), TGF-β1/PDGF-BB (I1, F2, F3) for 28 or 32 days. Images are representative of three pellets of three donors. Magnification 100×.

Later on, the gene expression of the pellets obtained from BM-hMSCs (donor 2) treated in six conditions, including TGF-β1 (PC), PDGF-BB and the combinations of TGF-β1/NGF-β, TGF-β1/BMP-2, TGF-β1/SDF-1α and TGF-β1/PDGF-BB, was examined for collagen type II (Col2A1) and X (Col10A1), aggrecan (AGCN) and SOX9 (Figure 6). Col2A1, a chondrogenic marker, was increased for TGF-β1/BMP-2 compared to the TGF-β1 (Figure 6 A). From the other chondrogenic genes (ACGN and SOX9), since very scattered results were found, a larger number of pellets should have been analysed in order to obtain reliable results (Figure 6 B and C). Moreover, Col10A1, the hypertrophic marker, was increased for TGF-β1/NGF-β, TGF-β1/BMP-2 and TGF-β1/SDF-1α compared to TGF-β1 (Figure 6 C). Based upon chondrogenic gene expression and GAG staining, TGF-β1/BMP-2 treatment induced the best chondrogenic differentiation on BM-MSCs. However more donors should be analysed for all conditions to take reasonable conclusions.

Additional studies of chondrogenic differentiation were carried out with MSCs derived from synovium in order to assess the response of the considered factors on these cells compared to the obtained outcomes on BM-MSCs, above presented. According to the migration analysis of SYN-MSCs described in the previous section, the pellet culture was performed with chondrogenic medium supplemented with 10 ng/mL TGF-β1 (PC) or the combination of TGF-β1 with 50 ng/mL NGF-β (TGF-β1/NGF-β), 50 ng/mL PDGF-BB (TGF-β1/PDGF-BB), 50 ng/mL BMP-2 (TGF-β1/BMP-2), 50 ng/mL SDF-1α (TGF-β1/SDF-1α) or 25 ng/mL of RANTES (TGF-β1/RANTES). The use of the factors alone was not considered, due to the absence of GAG expression in the previous experiments with BM donors. The thionin staining was qualitatively quantified after 32 days of pellets

culture (Table 8); the data showed the highest GAGs expression in pellets treated with TGF-β1/BMP-2 (Figure 7 C). In addition, certain conditions, such as TGF-β1/SDF-1α, and TGF-β1/PDGF-BB, did not differ much from the TGF-β1 (Figure 7 D, E and A). Finally, TGF-β1/NGF-β, and TGF-β1/RANTES decreased the chondrogenic differentiation compared to the PC (Figure 7 B, F and A). Based upon the matrix production, TGF-β1/BMP-2 was the best chondrogenic differentiation treatment.

In summary, the treatment of TGF-β1/BMP-2 in pellet culture of BM- and SYN-MSCs showed the highest GAG expression for all the donors and therefore better cartilage formation. In addition, BM- and SYN-MSCs cultured in chondrogenic medium supplemented with PDGF-BB alone and the combination of TGF-β1/PDGF-BB showed larger pellet size than the pellets of the remaining conditions (Figure 8). Also, an increase in cell number with a fibroblastic-like morphology was evident in these pellets compared to the other settings.

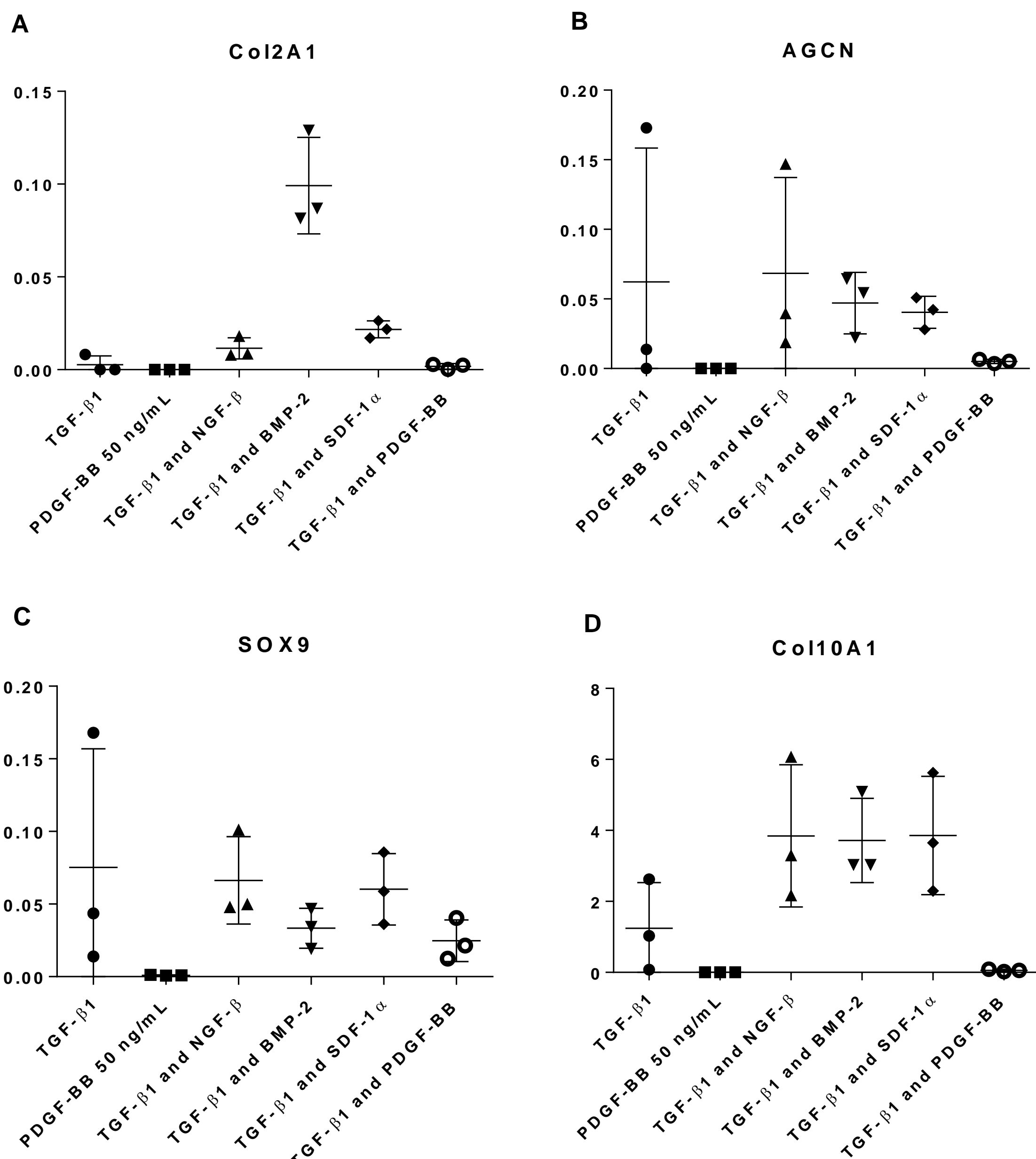


Figure 6 - Gene expression of pellet culture obtained from BM-MSCs. Data is expressed as mean ± SD of triplicates of bone marrow donor 2.

Table 8 - Qualitative quantification of chondrogenic differentiation of SYN-MSCs after 32 days of pellets culture in the presence of TGF-β1 (positive control), and the combination of TGF-β1/NGF-β, TGF-β1/BMP-2, TGF-β1/SDF-1, TGF-β1/PDGF-BB, and TGF-β1/RANTES. (n=1 independent experiment. 0, +, ++ and +++ represent none, low, moderate and high chondrogenic differentiation, respectively).

Factors / Donor	TGF-β1	TGF-β1/ NGF-β	TGF-β1/ BMP-2	TGF-β1/ SDF-1α	TGF-β1/ PDGF-BB	TGF-β1/ RANTES
1	++	0	+++	++	+/++	+

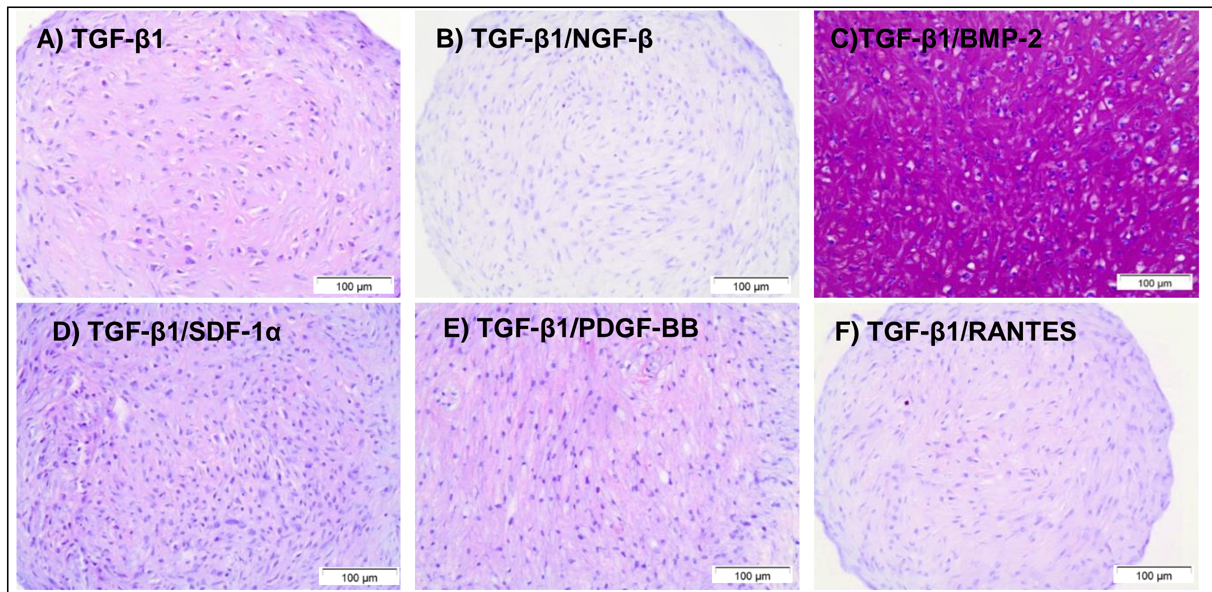


Figure 7 – GAGs analyses of SYN-MSCs after 32 days of pellets culture treated in chondrogenic medium supplemented with additional factors. Thionin staining was performed to detect GAGs in the ECM of the pellets treated in chondrogenic medium supplemented with TGF-β1 (A), TGF-β1/NGF-β (B), TGF-β1/BMP-2 (C), TGF-β1/SDF-1α (D), TGF-β1/PDGF-BB (E), and TGF-β1/RANTES (F) for 32 days. Images are representative of three pellets. Magnification 100×.

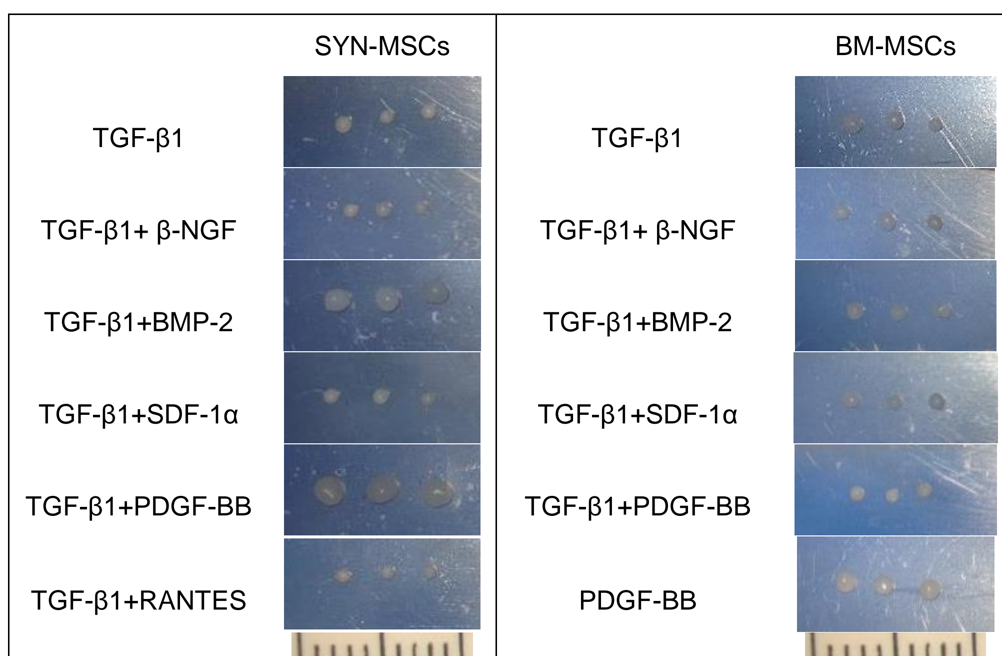


Figure 8 – MSCs cultured in pellet culture system. Macro pictures of MSCs from pellet culture at day 28 (BM-MSCs) and 32 (SYN-MSCs). Morphology ruler segments: 1 mm.

4.3 Chondrogenesis of fibrous and non-fibrous synovium sample

In this section, the chondrogenic differentiation for a sample of synovium with a fibrous and non-fibrous tissue is described. The sample was tested in order to understand whether the fibrous nature of synovium influences the chondrogenic differentiation of the MSCs. The MSCs were isolated separately from the fibrous and non-fibrous regions of the same synovium donor as described in section 3.1.2 and seeded as monolayer culture (Figure 9 A and B). The images of fibrous and non-fibrous regions are shown in Figure 9 C and D, respectively. The chondrogenesis of the fibrous and non-fibrous tissue (passage 1) was assessed in a 3D pellet culture for 28 days in chondrogenic medium supplemented with 10 ng/mL TGF-β1 (Figure 9 E and F). Thionin staining was present in the non-fibrous tissue and not in the fibrous tissue (Figure 9 E and F), showing that chondrogenic differentiation was influenced negatively in the fibrous tissue. However more donors have to be tested to take reliable conclusions whether chondrogenesis is affected by fibrous nature of synovium.

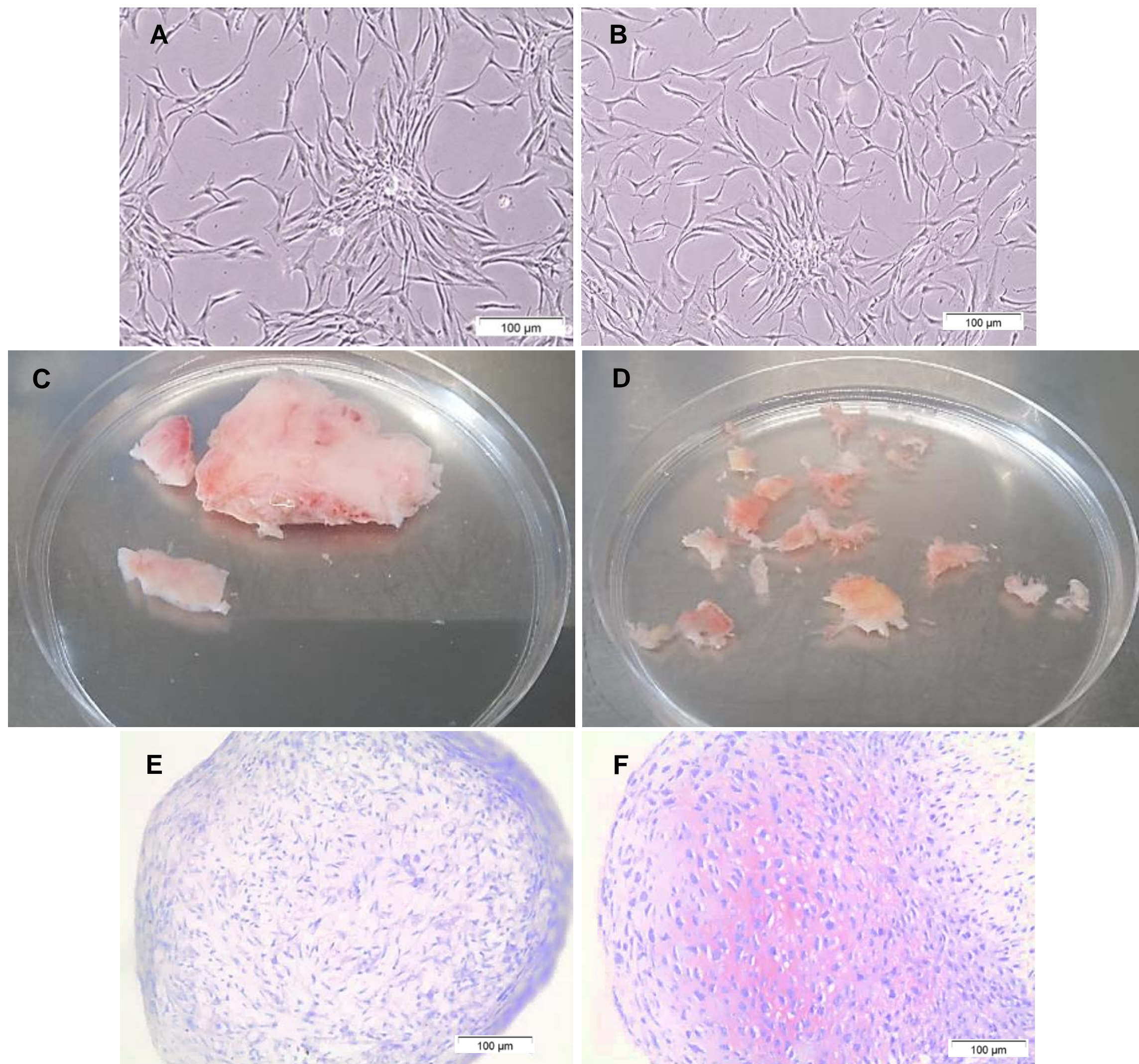


Figure 9 - MSCs derived from fibrous (left) and non-fibrous (right) synovium. Optical microscope images of monolayer culture of MSCs (A and B) derived from a fibrous (C) and non-fibrous (D) synovium pieces and the correspondent pellet after 28 days of culture (E and F). Magnification 100×.

5. 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 chondrogenic differentiation 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 [49] 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 [28]. In this study, a 3D collagen gel was used to assess the migration of bone marrow and synovium 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 the 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 [17, 25]; however, so far IL-1β had shown only moderate chemoattractant capacity [27]. 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 [11, 13, 21], TNF-α [27] and BMP-2 [22]. RANTES however has shown conflict within the findings, because some studies demonstrated that high concentrations of RANTES stimulated migration of MSCs [18, 26] and low concentrations of RANTES evidenced no chemotaxis [18, 25]; 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 [10, 14, 15, 18, 20, 23], 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 [19, 21, 26, 27].

An additional reservoir of MSCs is synovium. Its higher chondrogenic potential compared to BM, AT, muscle, periosteum and infrapatellar fat pad [58] 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 matrix. 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 [24, 37].

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, and the presence of chondrocyte-like morphology cells. Previous studies corroborated this finding for specific cell sources, such as BM [31, 47, 49], AT [30] and AC [31]. Moreover, the obtained findings demonstrated that chondrogenic differentiation of BM-MSCs appears to be decreased 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 [30]. 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. It is important to mention that only one synovium donor was evaluated, so more donors should be analysed for all conditions to confirm the results. 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.

The study of recruitment and chondrogenic differentiation factors is crucial for orienting MSCs fate through cartilage regeneration, nonetheless the administration and retention of these factors remains a challenge, because of factors short life and limitations crossing the biological barriers. Therefore, some technologies based on the controlled release of factors are now available [67] or under investigation [68] for cartilage repair. However, the development of a scaffold that combines a fast release of recruitment factors with a controlled and sustained release of chondrogenic differentiation factors would benefit a fast migration of MSCs to cartilage defected areas followed by chondrogenic differentiation of the recruited cells, thus leading to successful cartilage repair.

In this study two different systems were used to evaluate cell migration and chondrogenic differentiation: the 3D collagen matrix and the 3D pellet culture, respectively. In the former system, recruitment factors, including BMP-2, PDGF-BB, NGF-β, SDF-1α and RANTES, stimulated migration of MSCs, and in the latter system, differentiation factors, such as TGF-β1 and BMP-2, promoted the chondrogenesis of MSCs. Then, BMP-2 not only recruits MSCs but also induce their chondrogenic differentiation.

6. Overall summary, conclusion and future work

The inability of cartilage self-repair after injury requires techniques that induce cartilage regeneration. MSCs are considered promising candidates for cartilage repair due to their ability to migrate to damaged tissues and to differentiate into chondrocytes. Transplantation of exogenous MSCs alone or within a scaffold to injured sites has been adopted as a strategy to repair articular cartilage defects. However, the results have demonstrated that lower number of cells remain in the joint or in the damaged tissue after transplantation. More recently, a new approach that tries to overcome the above problem is the recruitment of a large number of MSCs existent within or nearby the joint (i.e. endogenous MSCs) to damaged areas and the differentiation of those migrated cells into chondrocytes. The use of endogenous MSCs avoids then the need of administration of exogenous MSCs. For the migration and the chondrogenic differentiation of endogenous MSCs, several chemokines and growth factors were studied commonly in 2D assays. In the present study, the *in vitro* migration of bone marrow and synovium MSCs, two promising cell sources in cartilage repair due to their location adjacent to the joint, were evaluated in a 3D collagen matrix supplemented with different factors. Furthermore, the chondrogenic differentiation of BM- and SYN-MSCs was tested in a 3D pellet culture.

The findings demonstrated that PDGF-BB, BMP-2, NGF-β, SDF-1α, and RANTES stimulated migration of BM- and SYN-MSCs and the combination of TGF-β1/BMP-2 induced their chondrogenesis. These results 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. Freyria, A.M. and F. Mallein-Gerin, *Chondrocytes or adult stem cells for cartilage repair: the indisputable role of growth factors.* Injury, 2012. **43**(3): p. 259-65.
2. Clouet, J., et al., *From osteoarthritis treatments to future regenerative therapies for cartilage.* Drug Discov Today, 2009. **14**(19-20): p. 913-25.
3. 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.
4. Ma, V.Y., L. Chan, and K.J. Carruthers, *Incidence, prevalence, costs, and impact on disability of common conditions requiring rehabilitation in the United States: stroke, spinal cord injury, traumatic brain injury, multiple sclerosis, osteoarthritis, rheumatoid arthritis, limb loss, and back pain.* Arch Phys Med Rehabil, 2014. **95**(5): p. 986-995 e1.
5. Magnussen, R.A., et al., *Treatment of focal articular cartilage defects in the knee: a systematic review.* Clin Orthop Relat Res, 2008. **466**(4): p. 952-62.
6. Steadman, J.R., W.G. Rodkey, and J.J. Rodrigo, *Microfracture: surgical technique and rehabilitation to treat chondral defects.* Clin Orthop Relat Res, 2001(391 Suppl): p. S362-9.
7. Richter, D.L., et al., *Knee Articular Cartilage Repair and Restoration Techniques: A Review of the Literature.* Sports Health, 2016. **8**(2): p. 153-60.
8. Goyal, D., et al., *Evidence-based status of osteochondral cylinder transfer techniques: a systematic review of level I and II studies.* Arthroscopy, 2014. **30**(4): p. 497-505.
9. Im, G.I., *Endogenous Cartilage Repair by Recruitment of Stem Cells.* Tissue Eng Part B Rev, 2016. **22**(2): p. 160-71.
10. 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.
11. 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.
12. Fiedler, J., et al., *BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells.* J Cell Biochem, 2002. **87**(3): p. 305-12.
13. 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.
14. Sordi, V., et al., *Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets.* Blood, 2005. **106**(2): p. 419-27.
15. Dwyer, R.M., et al., *Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells.* Clin Cancer Res, 2007. **13**(17): p. 5020-7.
16. Honczarenko, M., et al., *Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors.* Stem Cells, 2006. **24**(4): p. 1030-41.

17. Ozaki, Y., et al., *Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells.* Stem Cells Dev, 2007. **16**(1): p. 119-29.
18. 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.
19. Ringe, J., et al., *Towards in situ tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2.* J Cell Biochem, 2007. **101**(1): p. 135-46.
20. Boomsma, R.A. and D.L. Geenen, *Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis.* PLoS One, 2012. **7**(4): p. e35685.
21. 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.
22. 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.
23. 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.
24. 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.
25. 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.
26. 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.
27. 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.
28. Kramer, N., et al., *In vitro cell migration and invasion assays.* Mutat Res, 2013. **752**(1): p. 10-24.
29. 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.
30. 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.
31. 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.

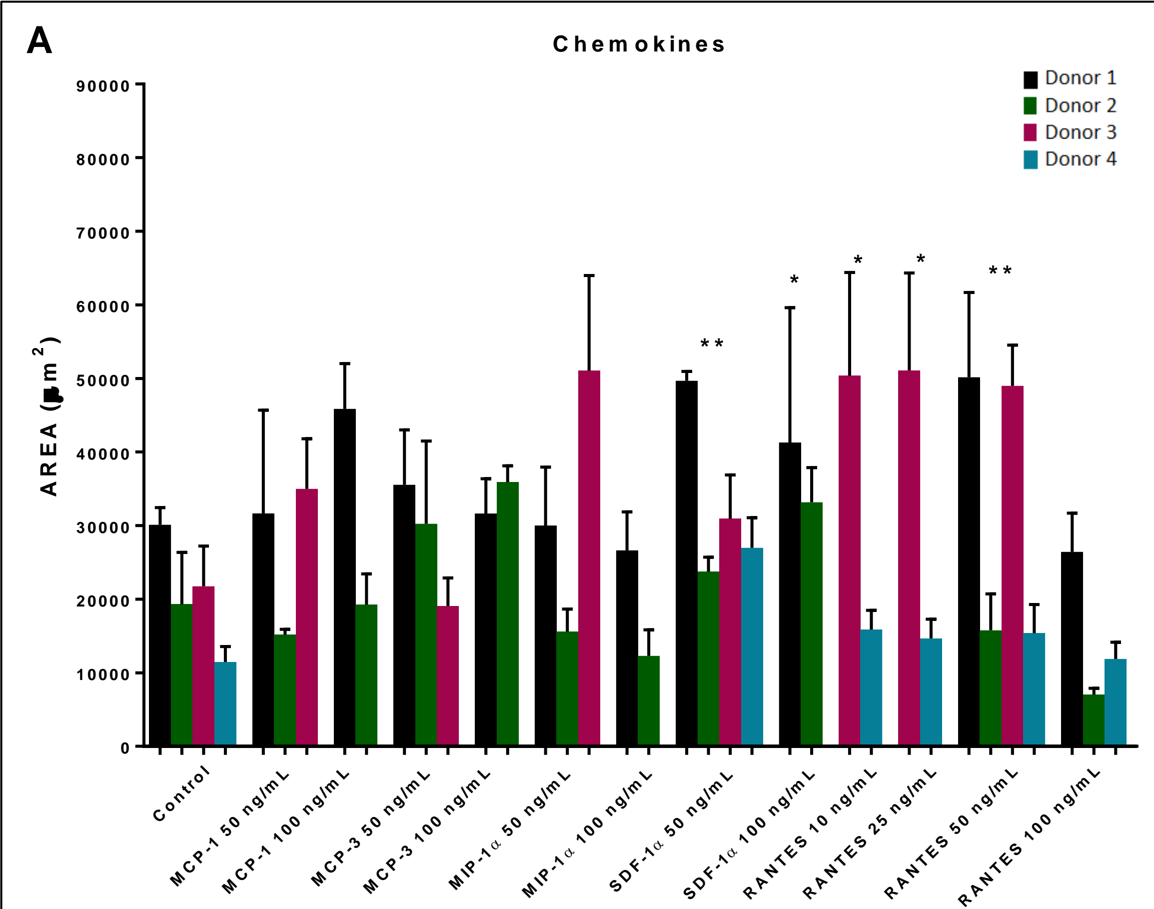
32. Weiss, S., et al., *Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells.* J Cell Physiol, 2010. **223**(1): p. 84-93.
33. Hart, D.A., *Perspectives on endogenous and exogenous tissue engineering following injury to tissues of the knee.* Biomedical Science and Engineering, 2014. **7**: p. 58-66.
34. Taub, D.D., *Cytokine, growth factor, and chemokine ligand database.* Curr Protoc Immunol, 2004. **Chapter 6**: p. Unit 6 29.
35. Mazzetti, I., et al., *A role for chemokines in the induction of chondrocyte phenotype modulation.* Arthritis Rheum, 2004. **50**(1): p. 112-22.
36. Mazor, M., et al., *Mesenchymal stem-cell potential in cartilage repair: an update.* J Cell Mol Med, 2014. **18**(12): p. 2340-50.
37. Zhang, W., et al., *The use of type 1 collagen scaffold containing stromal cell-derived factor-1 to create a matrix environment conducive to partial-thickness cartilage defects repair.* Biomaterials, 2013. **34**(3): p. 713-23.
38. Lee, C.H., et al., *Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study.* Lancet, 2010. **376**(9739): p. 440-8.
39. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.
40. Maurer, M. and E. von Stebut, *Macrophage inflammatory protein-1.* Int J Biochem Cell Biol, 2004. **36**(10): p. 1882-6.
41. Crawford, A., et al., *A role for the chemokine RANTES in regulating CD8 T cell responses during chronic viral infection.* PLoS Pathog, 2011. **7**(7): p. e1002098.
42. Ackermann, P.W., P.T. Salo, and D.A. Hart, *Neuronal pathways in tendon healing.* Front Biosci (Landmark Ed), 2009. **14**: p. 5165-87.
43. Lefebvre, V. and P. Smits, *Transcriptional control of chondrocyte fate and differentiation.* Birth Defects Res C Embryo Today, 2005. **75**(3): p. 200-12.
44. Augustyniak, E., et al., *The role of growth factors in stem cell-directed chondrogenesis: a real hope for damaged cartilage regeneration.* Int Orthop, 2015. **39**(5): p. 995-1003.
45. Barry, F., et al., *Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components.* Exp Cell Res, 2001. **268**(2): p. 189-200.
46. Sekiya, I., et al., *Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma.* Cell Tissue Res, 2005. **320**(2): p. 269-76.
47. 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.
48. Pelttari, K., et al., *Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice.* Arthritis Rheum, 2006. **54**(10): p. 3254-66.
49. 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.

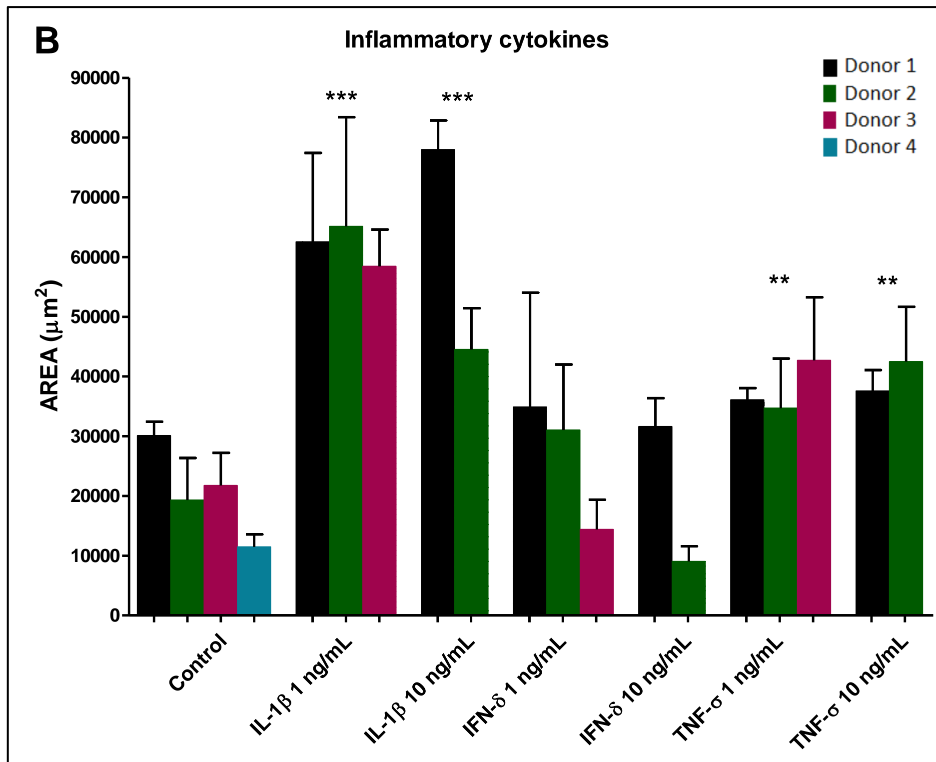
50. Ronziere, M.C., et al., *Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells.* Biomed Mater Eng, 2010. **20**(3): p. 145-58.
51. Shintani, N., E.B. Siebenrock Ka Fau - Hunziker, and E.B. Hunziker, *TGF-ss1 enhances the BMP-2-induced chondrogenesis of bovine synovial explants and arrests downstream differentiation at an early stage of hypertrophy.* PLoS One, 2013. **8**(1): p. e53086.
52. Ng, F., et al., *PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages.* Blood, 2008. **112**(2): p. 295-307.
53. Morales, T.I., *Chondrocyte moves: clever strategies?* Osteoarthritis Cartilage, 2007. **15**(8): p. 861-71.
54. McCarthy, H.E., et al., *The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse.* Vet J, 2012. **192**(3): p. 345-51.
55. Williams, R., et al., *Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage.* PLoS One, 2010. **5**(10): p. e13246.
56. Koelling, S., et al., *Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis.* Cell Stem Cell, 2009. **4**(4):324-35.
57. Harvanova, D., et al., *Isolation and characterization of synovial mesenchymal stem cells.* Folia Biol (Praha), 2011. **57**(3): p. 119-24.
58. 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.
59. Hunziker, E.B. and L.C. Rosenberg, *Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane.* J Bone Joint Surg Am, 1996. **78**(5): p. 721-33.
60. Dragoo, J.L., J. Johnson C Fau - McConnell, and J. McConnell, *Evaluation and treatment of disorders of the infrapatellar fat pad.* Sports Med, 2012. **42**(1): p. 51-67.
61. Wickham, M.Q., et al., *Multipotent stromal cells derived from the infrapatellar fat pad of the knee.* Clin Orthop Relat Res, 2003. **412**: p. 196-212.
62. English, A., et al., *A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis.* Rheumatology (Oxford), 2007. **46**(11): p. 1676-83.
63. Wei, W., et al., *The infrapatellar fat pad from diseased joints inhibits chondrogenesis of mesenchymal stem cells.* Eur Cell Mater, 2015. **30**: p. 303-14.
64. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nat Methods, 2012. **9**(7): p. 676-82.
65. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
66. Wehling, N., et al., *Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways.* Arthritis Rheum, 2009. **60**(3): p. 801-12.

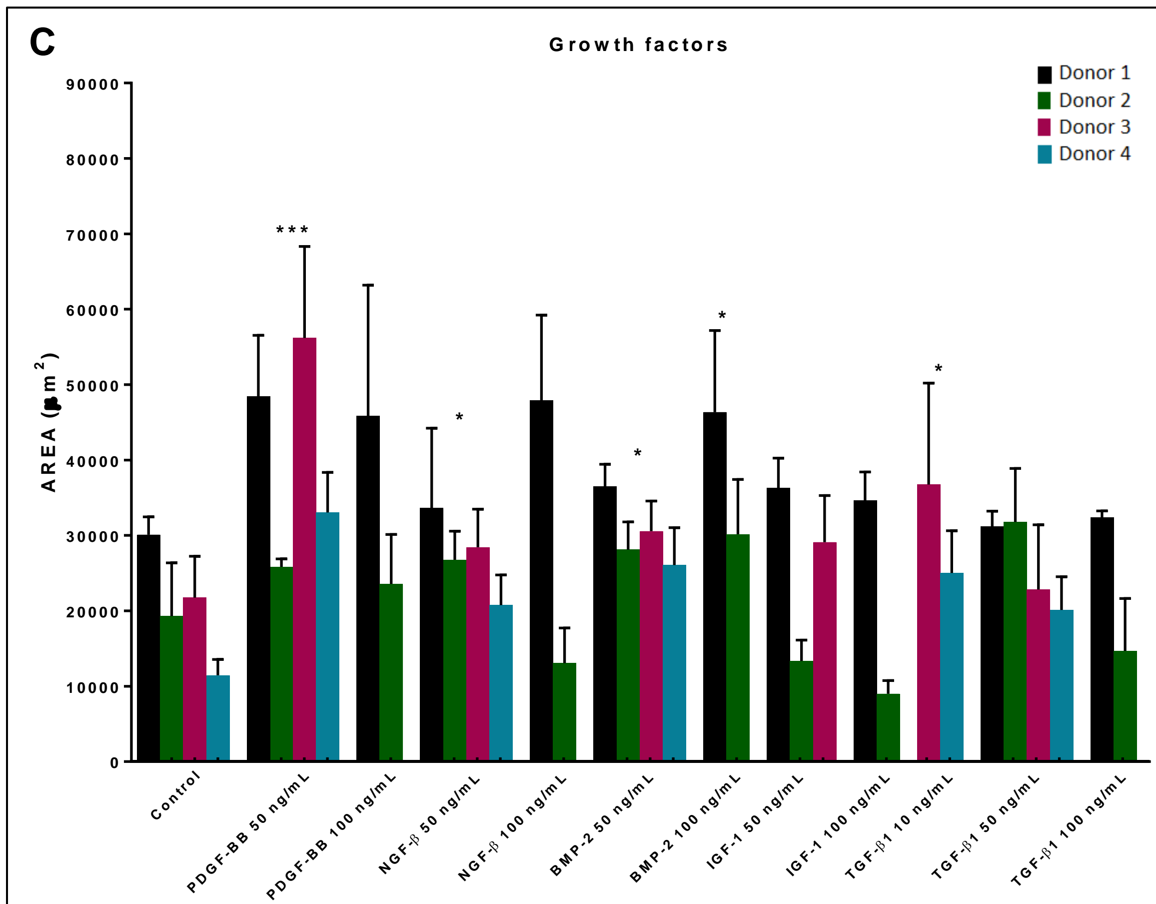
67. Censi, R., et al., *Bioactive hydrogel scaffolds - advances in cartilage regeneration through controlled drug delivery.* Curr Pharm Des, 2015. **21**(12): p. 1545-55.
68. Morille, M., et al., *Scaffolds for Controlled Release of Cartilage Growth Factors.* PLos One, 2013. **8**: p. e53086.

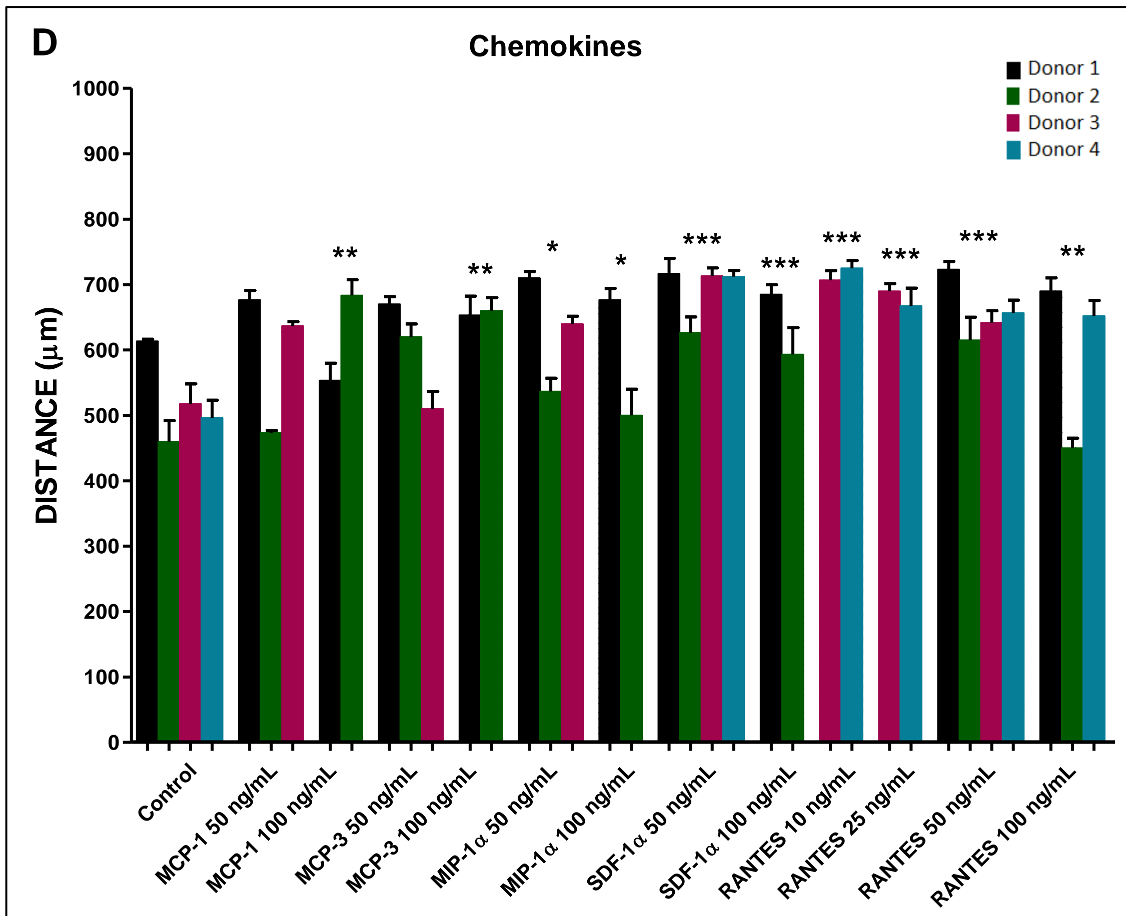
Annex

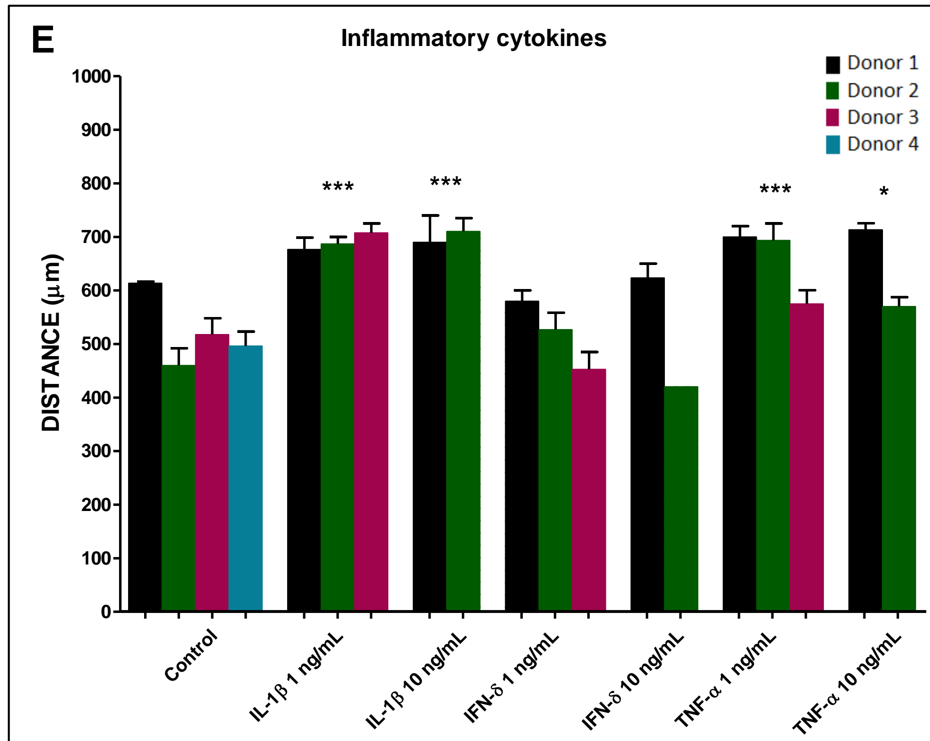
In this section additional plots of the migratory cell area and maximum migratory cell distance of four BM donors (Figure 10) and four SYN donors in the presence of chemokines (MCP-1, MCP-3, MIP-1α, SDF-1α, and RANTES), inflammatory cytokines (IL-1α, IFN-γ, and TNF-β) and growth factors (PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1) are present. The data is expressed as mean of 3 to 6 spheroids per condition per donor and the correspondent standard deviation. Differences were analysed as described in 3.2.5 and they were considered significant when p value was *<0.05, **<0.01 and ***<0.001.




B
Inflammatory cytokines
Donor 1
Donor 2
Donor 3
Donor 4
AREA (µm2)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
***
***
**
**
Control
IL-1β 1 ng/mL
IL-1β 10 ng/mL
IFN-δ 1 ng/mL
IFN-δ 10 ng/mL
TNF-σ 1 ng/mL
TNF-σ 10 ng/mL


C
Growth factors
Donor 1
Donor 2
Donor 3
Donor 4
AREA (µm2)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
***
*
*
*
*
Control
PDGF-BB 50 ng/mL
PDGF-BB 100 ng/mL
NGF-β 50 ng/mL
NGF-β 100 ng/mL
BMP-2 50 ng/mL
BMP-2 100 ng/mL
IGF-1 50 ng/mL
IGF-1 100 ng/mL
TGF-β1 10 ng/mL
TGF-β1 50 ng/mL
TGF-β1 100 ng/mL


D
Chemokines
Donor 1
Donor 2
Donor 3
Donor 4
DISTANCE (µm)
1000
900
800
700
600
500
400
300
200
100
0
**
**
*
*
***
***
***
***
***
**
Control
MCP-1 50 ng/mL
MCP-1 100 ng/mL
MCP-3 50 ng/mL
MCP-3 100 ng/mL
MIP-1α 50 ng/mL
MIP-1α 100 ng/mL
SDF-1α 50 ng/mL
SDF-1α 100 ng/mL
RANTES 10 ng/mL
RANTES 25 ng/mL
RANTES 50 ng/mL
RANTES 100 ng/mL


E
Inflammatory cytokines
Donor 1
Donor 2
Donor 3
Donor 4
DISTANCE (µm)
1000
900
800
700
600
500
400
300
200
100
0
***
***
***
*
Control
IL-1β 1 ng/mL
IL-1β 10 ng/mL
IFN-δ 1 ng/mL
IFN-δ 10 ng/mL
TNF-α 1 ng/mL
TNF-α 10 ng/mL

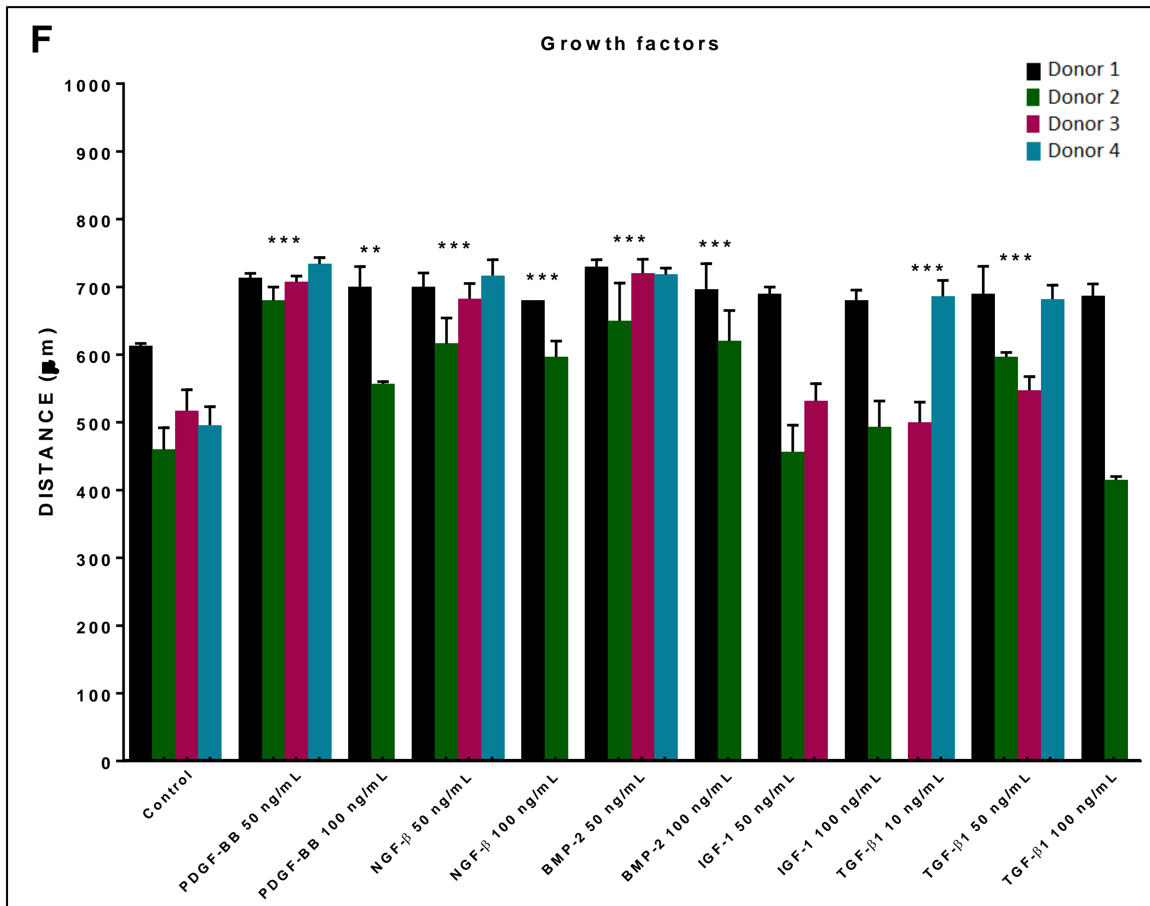
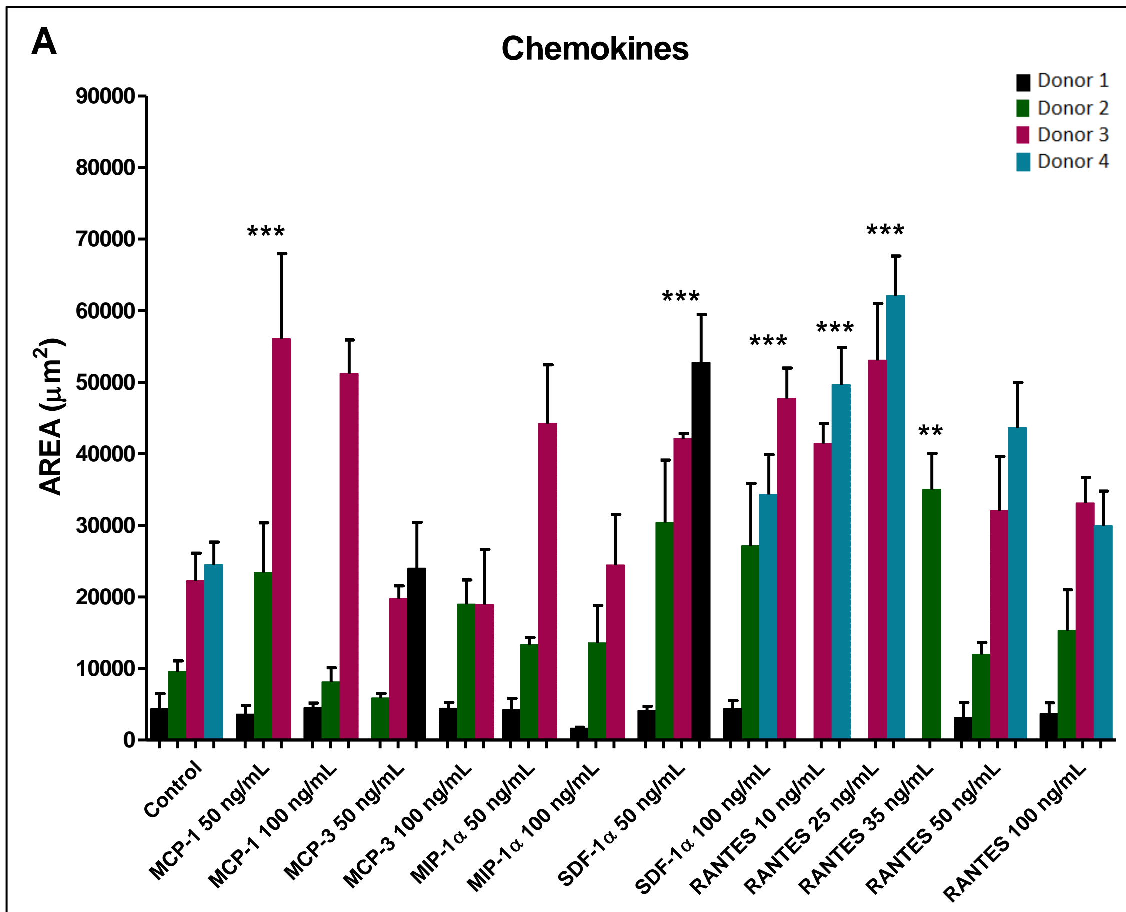
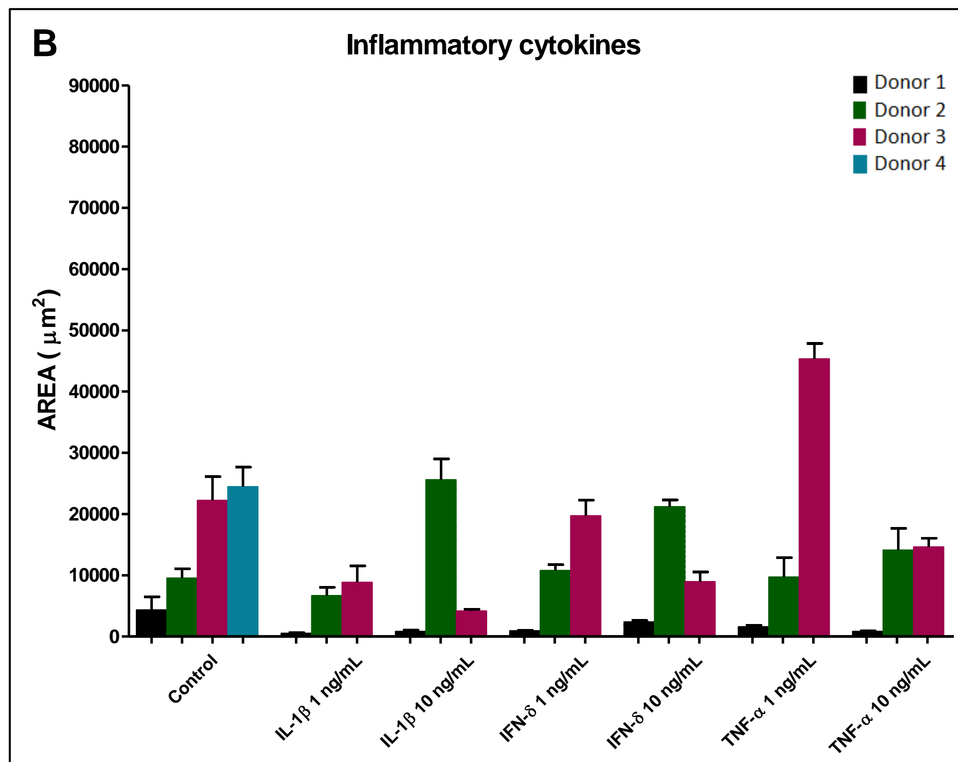
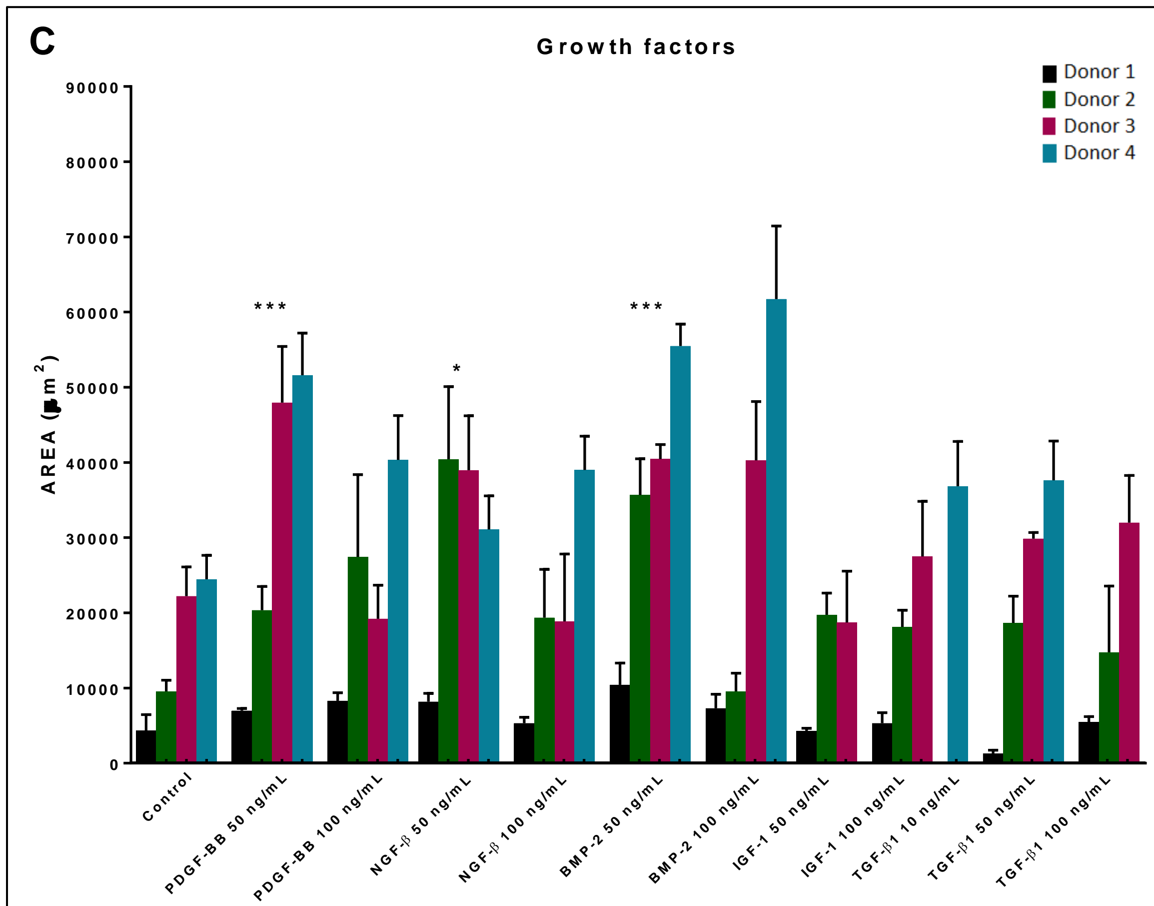


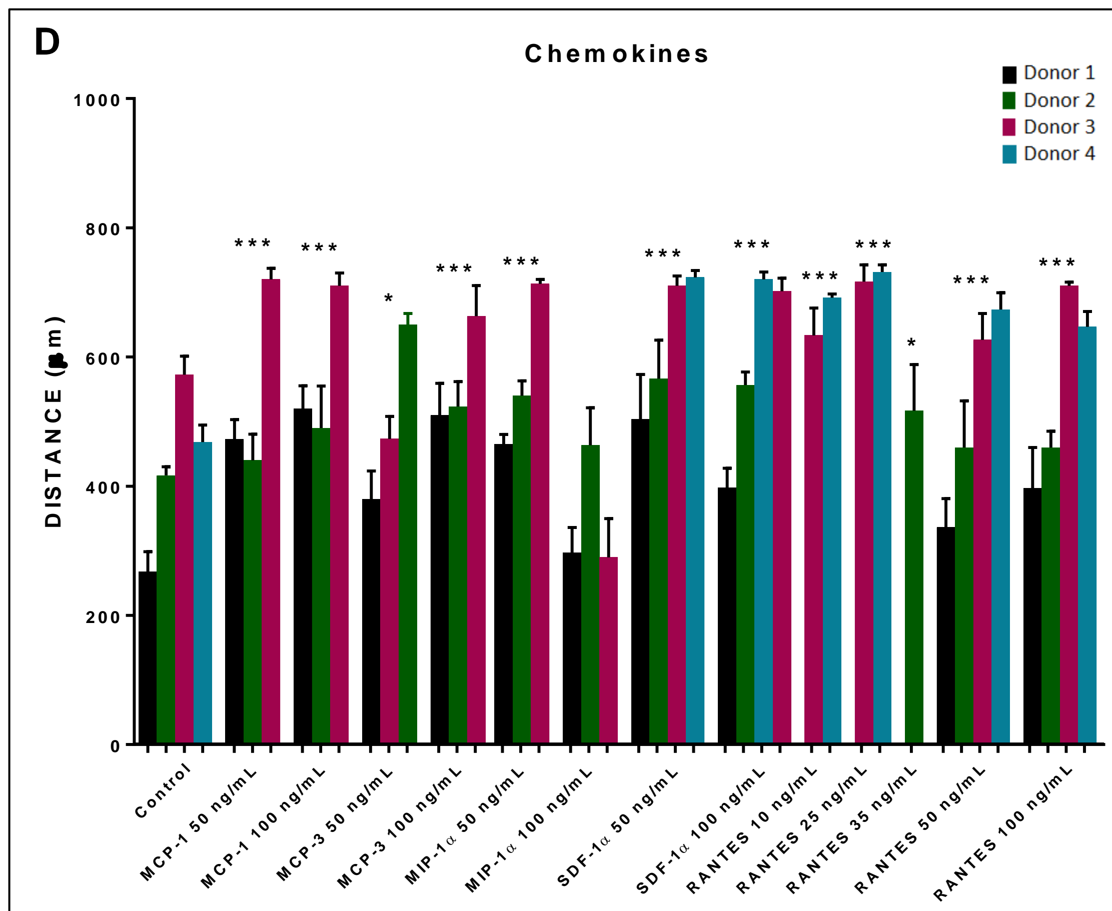
Figure 10 - Migration of MSCs derived from BM in response to different factors. A-C) Migratory cell area and D-E) maximum migratory cell distance was calculated for IL-1α, IFN-γ, TNF-β, MCP-1, MCP-3, MIP-1α, SDF-1α, RANTES, PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1. Data is expressed as mean ± SD of 4 independent experiments. * p<0.05; ** p<0.01; *** p<0.001 where p is evaluated with reference to the negative control.



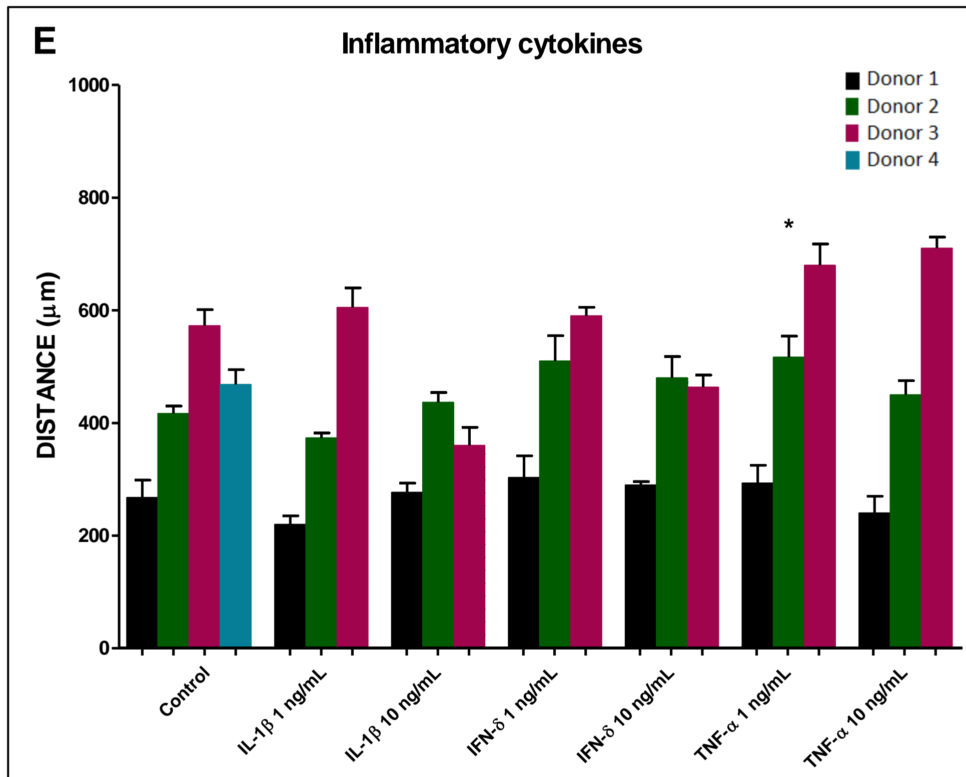


E





F



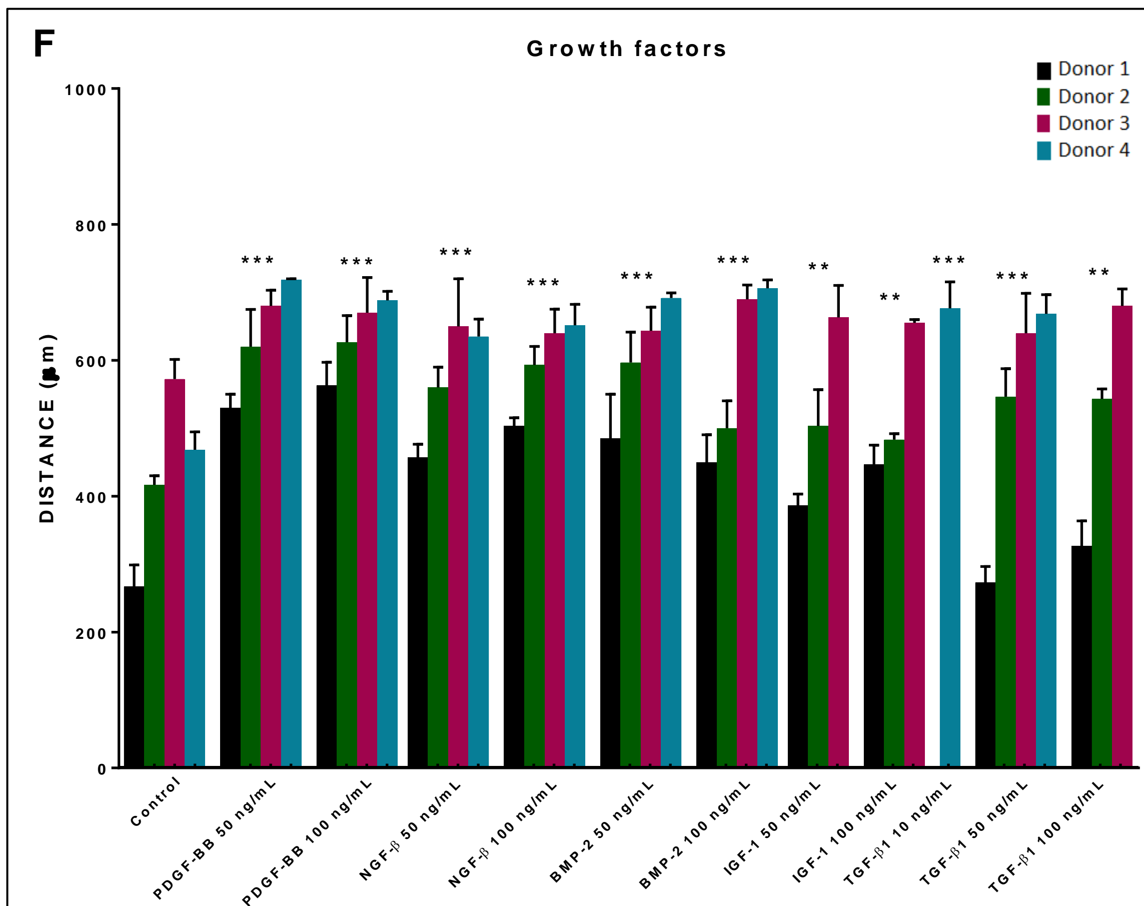


Figure 11 - Migration of MSCs derived from SYN in response to different factors. A-C) Migratory cell area and D-E) maximum migratory cell distance was calculated for IL-1α, IFN-γ, TNF-β, MCP-1, MCP-3, MIP-1α, SDF-1α, RANTES, PDGF-BB, NGF-β, BMP-2, IGF-1, and TGF-β1. Data is expressed as mean ± SD of 4 independent experiments. * p<0.05; ** p<0.01; *** p<0.001 where p is evaluated with reference to the negative control.