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Pre-treatment of human mesenchymal stem cells with inflammatory factors or hypoxia does not influence migration to osteoarthritic cartilage and synovium

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*Maarten J.C. Leijts,*y MD, Gerben M. van Buul,* MD, PhD, Jan A.N. Verhaar,* MD, PhD, Martin J. Hoogduijn,z PhD, Pieter K. Bos,* MD, PhD, and Gerjo J.V.M. van Osch,*§// PhD*
Investigation performed at the Erasmus MC Rotterdam, the Netherlands

Abstract

Background: Mesenchymal stem cells (MSCs) are promising candidates as a cell-based therapy for osteoarthritis (OA), although current results are modest. Pre-treatment of MSCs before application might improve their therapeutic efficacy.

Hypothesis: Pre-treatment of MSCs with inflammatory factors or hypoxia will improve their migration and adhesion capacities towards OA affected tissues.

Study Design: Controlled Laboratory Study; *in vitro* and *in vivo*.

Methods: We determined effects of different fetal calf serum (FCS) batches, platelet lysate (PL), hypoxia, inflammatory factors, factors secreted by OA tissues and OA synovial fluid (SF) on the expression of twelve genes encoding chemokine or adhesion receptors by RT-PCR. Migration of MSCs towards factors secreted by OA tissues was studied *in vitro*, and attachment of injected MSCs was evaluated *in vivo* in healthy and OA male Wistar rat knees.

Results: Different FCS batches, PL or hypoxia did not influence the expression of the migration and adhesion receptor genes. Exposure to inflammatory factors altered the expression of *CCR1*, *CCR4*, *CD44*, *PDGFR α* and *PDGFR β* . MSCs migrated towards factors secreted by OA tissues

in vitro. Neither pre-treatment with inflammatory factors nor the presence of OA influenced MSC migration *in vitro* or adhesion *in vivo*.

Conclusion: Factors secreted by OA tissues increase MSC migration *in vitro*. *In vivo* no difference in MSC adhesion was found between OA and healthy knees. Pre-treatment with inflammatory factors influenced the expression of migration and adhesion receptors of MSCs, but not their migration *in vitro* or adhesion *in vivo*.

Clinical Relevance: To improve the therapeutic capacity of intra-articular injection of MSCs, MSCs need to remain intra-articular for a longer period of time. Pre-treatment of MSCs with hypoxia or inflammatory factors did not increase their migration or adhesion capacity and will therefore not likely prolong their intra-articular longevity. Alternative approaches to prolong intra-articular presence of MSCs should be developed to increase the therapeutic effect of MSCs in OA.

Key Terms: Mesenchymal stem cells, migration, osteoarthritis, cell therapy, inflammation

Introduction

Osteoarthritis (OA) is characterized by an inflammatory and catabolic intra-articular environment, which causes progressive degeneration and inflammation of multiple intra-articular tissues. Sport-related injuries like anterior cruciate ligament ruptures or meniscal tears increase the incidence of OA.^{2, 50} To date, only symptomatic treatments are available for OA. A successful strategy to cure OA would consist of long term modulation of the degenerative joint environment by simultaneously reducing inflammation and promoting tissue regeneration. Mesenchymal stem cells (MSCs) are promising candidates for this therapeutic approach of OA,

They have been shown capable of suppressing immune-mediated cartilage destruction while contributing to cartilage repair via their chondrogenic differentiation and paracrine stimulation of endogenous repair processes.^{8, 16, 27, 33} The use of MSCs as cell-therapy for OA by intra-articular injection has been reported to be safe.^{4, 34} Clinical trials and pre-clinical studies did, however, not show consistent data on healing or regeneration of damaged tissues after MSC treatment.^{4, 48} A possible explanation for these inconsistent results could be the fast decrease in number of viable MSCs after injection in the joint; No viable MSCs were detected in the joint one to two weeks after injection.^{44, 46} A small proportion of intra-articularly injected MSCs attached to synovial surfaces after these injections, but almost none to the affected cartilage.³² During their rather short local intra-articular presence, MSCs can only have a small effect. In cardiology, it has been shown that providing a longer interplay between the diseased tissue and applied MSCs, increased the therapeutic effect.^{31, 49, 53} We hypothesized that pre-treatment of MSCs with inflammatory factors or hypoxia would improve their migration and adhesion capacities towards OA affected tissues.

The migration and adhesion of injected MSCs in OA joints will depend on multiple factors including the secreted chemotactic factors by OA joint tissues, the expression of chemotactic receptors for these factors and the expression of cell adhesion receptors by the MSCs.^{3, 13, 14, 16, 21, 23, 24} In OA joints, the presence of multiple chemokines that can attract MSCs have been described.^{3, 6} Furthermore, multiple chemokine receptors and adhesion receptors have already been indicated as possible migration receptors and adhesion receptors of MSCs.^{3, 16, 19, 35, 39, 43, 51, 54} Previous studies showed that the expression of these migration receptors can be influenced by exposure to inflammatory factors^{16, 18, 35, 37} or hypoxia,^{10, 22} but none of these studies evaluated the effect on intra-articular migration or adherence.. Moreover, exposure of MSCs to

inflammatory factors increases their immunomodulatory capacities^{15, 28, 38, 41} and was previously shown by us to increase the anti-inflammatory and anti-catabolic effects of MSCs on inflamed osteoarthritic joint tissues in culture.⁴⁷ In this study we evaluated whether OA synovium and cartilage produce chemotactic factors that can stimulate MSCs to migrate and adhere to the OA affected tissues after injection and whether we could influence MSC migration capacities *in vitro* and adhesion capacities *in vivo*.

TABLE 1
Abbreviations Used

Abbreviation	Definition
aMEM	Minimum Essential Medium Eagle–alpha modification
<i>CCR1</i>	C-C chemokine receptor type 1
<i>CCR4</i>	C-C chemokine receptor type 4
<i>CCR5</i>	C-C chemokine receptor type 5
cDNA	complementary DNA
CM	conditioned medium
CT	cycle threshold
<i>CXCR1</i>	CXC chemokine receptor 1
<i>CXCR3</i>	CXC chemokine receptor 3
<i>CXCR4</i>	CXC chemokine receptor 4
CX3CL1	fractalkine
<i>CX3CR1</i>	CX3C chemokine receptor 1/fractalkine receptor
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FGF2	fibroblast growth factor 2
GAG	glycosaminoglycan
<i>HPRT</i>	hypoxanthine phosphoribosyltransferase
IFN- γ	interferon γ
IP-10 (CXCL10)	interferon γ -induced protein 10
IL-8 (CXCL8)	interleukin 8
<i>ITGβ1</i>	integrin β -1
<i>ITGβ2</i>	integrin β -2
ITS	insulin-transferrin-selenium
MCP-1 (CCL2)	monocyte chemotactic protein 1
MCP-3 (CCL7)	monocyte chemotactic protein 3
MDC (CCL22)	macrophage-derived chemokine
MIA	monoiodoacetate
MIP-1a (CCL3)	macrophage inflammatory protein 1a
MIP-1b (CCL4)	macrophage inflammatory protein 1b
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSC	mesenchymal stem cell
OA	osteoarthritis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
<i>PDGFRα</i>	platelet-derived growth factor receptor α
<i>PDGFRβ</i>	platelet-derived growth factor receptor β
PL	platelet lysate
SF	synovial fluid
SPIO	superparamagnetic iron oxide
TNF- α	tumor necrosis factor α

Methods

Harvesting OA synovial fluid and conditioned medium of OA synovium and cartilage

Human synovial fluid (SF), cartilage explants and synovial explants were obtained as surgical waste material from patients undergoing total knee replacement surgery for OA. All patients implicitly consented to the use of these tissues for scientific research (with approval of medical ethical committee, MEC-2004-322).

To remove debris, SF samples were centrifuged (1600 rpm, 5 min) and supernatant was subsequently stored at -80°C. Full thickness layers of uncalcified cartilage of macroscopically unaffected areas were taken from weight bearing and non-weight bearing parts of the tibial plateau and femur condyles. The synovial layer was carefully dissected from Hoffa's fat pad, taking care to include as little fat tissue as technically possible. The cartilage and synovium explants were cut in 1-2 x 1-2 mm pieces. To generate conditioned medium (CM), 1 g of tissue was cultured per 5 ml for 24 h in medium we used before⁵ consisting of Dulbecco's Modified Eagle Medium (DMEM) low glucose (Invitrogen, Carlsbad, CA, USA) containing 1% ITS (Insulin-Transferrin-Selenium; BD Bioscience, Bedford, MA, USA), 1.5 µg/ml fungizone (Invitrogen, Carlsbad, CA, USA), 50 µg/ml gentamicin (Invitrogen, Carlsbad, CA, USA), and 0.1 mM of L-ascorbic acid 2-phosphate (vitamin C) (Sigma, St. Louis, MO, USA). CM was harvested after 24 h, centrifuged (1200 rpm, 8 min) and the supernatant was stored at -80°C.

Chemokine detection in OA synovial fluid and conditioned media of OA tissues

Selected chemokines in SF of OA patients (n=6 patients, 2 male/4 female, 65.5 (59-73) years old), OA synovium CM (n=6 patients, 1 male/5 female, 63.3 (59-73) years old) and OA cartilage CM (n=6 patients, 2 male/4 female, 62.7 (54-70) years old) were measured by using a custom made 10-plex MILLIPLEX[®] multiplex assay (Merck Millipore, Temecula, CA, USA). Technical duplicates of the samples were used for the measurements. Ten factors were selected by matching known chemokine and adhesion receptors of MSCs with chemokines known to be present in in OA SF.^{3, 6, 18, 23, 35, 39} The multiplex assay was performed according to the manufacturer's protocol, with the human chemokine magnetic bead panel kit for Fractalkine (CX3CL1), Monocyte Chemotactic Protein 3 (MCP-3, CCL7), Macrophage-Derived Chemokine (MDC, CCL22), Platelet-Derived Growth Factor (PDGF)-AA, PDGF-BB, InterLeukin 8 (IL-8, CXCL8)), Interferon gamma-induced Protein 10 (IP-10, CXCL10), Monocyte Chemotactic Protein 1 (MCP-1, CCL2)), Macrophage Inflammatory Protein-1a (MIP-1a, CCL3) and Macrophage Inflammatory Protein-1b (MIP-1b, CCL4) (EMD Millipore Corporation, Billerica, MA, USA). Assay read-out was performed by using Bio-Plex[®] 200 multiplex array reader and Bio-Plex software (Bio-Rad Laboratories, Veenendaal, the Netherlands). Values below the minimum detectable concentration were set at 50% of the minimum detectable concentration.

Isolation and culture of MSCs

MSCs were isolated from heparinized femoral-shaft marrow aspirate of patients undergoing total hip arthroplasty, after signed informed consent and with approval of the local ethical committees. 5-10 ml marrow was harvested with sterile Jamshidi needles and sterile 10 ml syringes

containing 0.5 ml of heparin (1000 U/ml). $30-100 \times 10^6$ mononuclear cells were plated in T175 flasks in 25 ml expansion medium consisting of aMEM (Minimum Essential Medium Eagle - Alpha Modification) (Invitrogen, Carlsbad, CA, USA) containing 10% heat inactivated fetal calf serum (FCS) (Lonza, Verviers, Belgium, selected batch), 1.5 $\mu\text{g/ml}$ fungizone, 50 $\mu\text{g/ml}$ gentamicin, 1 ng/ml fibroblast growth factor-2 (FGF2) (InstruChemie B.V., Delfzijl, The Netherlands) and 0.1 mM of vitamin C. After 24 h, non-adherent cells and erythrocytes were removed by washing three times with 2% FCS in 1x PBS (Invitrogen, Carlsbad, CA, USA). Remaining adherent cells were cultured in expansion medium at 37°C and 5% carbon dioxide (CO₂). Expansion media were renewed twice a week. Cells were trypsinized at subconfluency with 0.25% trypsin solution containing 0.01% ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, CA, USA) and re-plated at a density of 2300 cells/cm².

To evaluate whether different FCS batches or PL, generally used for MSC culturing, influence the migration or adhesion factor expression in MSCs, cryopreserved passage two MSCs (N = 3 donors) were used. MSC were expanded for one passage in three different 10% FCS batches with three different lot numbers of the same company (Lonza, Verviers, Belgium, selected batches) and PL (>50 pooled human donors prepared as described previously³³) in expansion medium. Before usage, PL was thawed and centrifuged at 2,000 g for 10 min to remove remaining platelet fragments. After centrifugation, PL was stored at 4°C and used for up to 1 week. After passaging the MSCs remained in their specific serum condition for another 72 h with a final medium change 24 h prior to harvesting for gene expression analysis.

To study the effect of OA SF and the effect of exposure to inflammatory factors and hypoxia, passage two MSCs were thawed and expanded for one passage. MSCs (N = 3 donors) were plated in six well plates and at 70% confluence washed with PBS and cultured for 48 h in

DMEM low glucose containing 20% OA SF, 1% ITS, 1.5 µg/ml fungizone, 50 µg/ml gentamicin and 0.1 mM of vitamin C.²⁹ After 48 h MSCs were harvested for gene expression analyses. To evaluate the effect of hypoxia and inflammatory factors, MSCs were plated in six well plates (2×10^5 cells/well) and cultured in expansion medium for 48 h under 20% of oxygen (normoxic) or 1% of oxygen (hypoxic) conditions. After 48 h, cells were washed with PBS and cultured for 24 h in DMEM low glucose containing 1% ITS, 1.5 µg/ml fungizone, 50 µg/ml gentamicin and 0.1 mM of vitamin C with additional 1 ng, 20 ng or 50 ng of IFN γ and TNF α (PeproTech, London, UK) per millilitre. MSCs were kept in their normoxic or hypoxic situation and subsequently were harvested for gene expression analyses, CM was harvested, centrifuged and stored at -80 °C. The choices for concentrations of inflammatory factors and exposure time to hypoxia and inflammatory factors have been made based on literature and own experiments.^{10,}

11, 40, 47

Gene expression analysis

Total RNA from MSCs was isolated using RNeasy® microkit with RNeasy MinElute spin columns (Qiagen, Hilden, Germany). After nucleic acids quantification by spectrophotometry (NanoDrop 2000, Thermo Scientific, IJsselstein, The Netherlands), RNA was reverse transcribed using a first strand cDNA synthesis kit (RevertAid™, MBI Fermentas, St. Leon-Rot, Germany). Amplifications were performed as 10 µL reactions in CFX96 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Thermocycler conditions comprised an initial holding at 95°C for 10 min, followed by one step at 95°C for 15 s and 60°C for 60 s for 40 cycles. A dissociation stage was added at the end using 95°C for 15 s, 60°C for 20 s and 95°C for 15 s. For

HPRT TaqMan Master Mix (ABI, Branchburg, NJ, USA) was used. For receptor expression for chemotactic factors TaqMan universal Master Mix (Life Technologies, Bleiswijk, the Netherlands) was used for receptors C-C chemokine receptor type 1 (*CCR1*), C-C Chemokine Receptor type 4 (*CCR4*), C-C Chemokine Receptor type 5 (*CCR5*), Platelet-Derived Growth Factor Receptor alpha (*PDGFR α*), Platelet-Derived Growth Factor Receptor beta (*PDGFR β*), CXC Chemokine Receptor 1 (*CXCR1*), CXC Chemokine Receptor 3 (*CXCR3*), CXC Chemokine Receptor 4 (*CXCR4*), CX3C Chemokine Receptor 1/fractalkine receptor (*CX3CR1*) and adhesion factors Integrin beta-1 (*ITG β 1*), Integrin beta-2 (*ITG β 2*) and homing cell adhesion molecule *CD44*. Primers and probes used in this study are depicted in table 1. Genes were detected using TaqMan assays on demand (Life Technologies, Bleiswijk, the Netherlands). Data were quantitatively analysed on CFX manager™ software (version 3.1, Bio-Rad). Gene expressions in MSCs were calculated by cycle threshold (CT) values. CT values of ≥ 36 were considered non-expressed and set to 100 for further calculations. CT values were corrected for housekeeper gene *HPRT* by using the $2^{-\Delta CT}$ formula.

TABLE 1
Primers and probes used for Real-Time PCR

Genes	Gene accession number	Predesign assay number	Primer/probe
<i>HPRT</i> (housekeeper)	NM_000194.2	-	Fw:TATGGACAGGACTGAACGTCTTG Rv:CACACAGAGGGCTACAATGTG FamAGATGTGATGAAGGAGATGGGAGGCCA
<i>CCR1</i>	NM_001295.2	Hs00928897_s1	Assay on demand
<i>CCR4</i>	NM_005508.4	Hs00747615_s1	Assay on demand
<i>CCR5</i>	NM_001100168.1	Hs99999149_s1	Assay on demand
<i>CD44</i>	NM_000610.3	Hs01075861_m1	Assay on demand
<i>PDGFRα</i>	NM_006206.4	Hs00998018_m1	Assay on demand
<i>PDGFRβ</i>	NM_002609.3	Hs01019589_m1	Assay on demand

<i>ITGβ1</i>	NM_002211.3	Hs00559595_m1	Assay on demand
<i>ITGβ2</i>	NM_000211.3	Hs00164957_m1	Assay on demand
<i>CXCR1</i>	NM_000634.2	Hs01921207_s1	Assay on demand
<i>CXCR3</i>	NM_001142797.1	Hs01847760_s1	Assay on demand
<i>CXCR4</i>	NM_001008540.1	Hs00607978_s1	Assay on demand
<i>CX3CR1</i>	NM_001171171.1	Hs01922583_s1	Assay on demand

Migration assay

The migration assays was performed with 24 well plate transwell inserts (Millicell®, inserts 8.0 μm PET, cat# PIEP12R48, Merck Millipore, Temecula, CA, USA). OA cartilage CM (pooled from 6 donors), OA synovium CM (pooled from 6 donors) and control medium consisting of DMEM low glucose, 1% ITS, 1.5 μg/ml fungizone, 50 μg/ml gentamicin, and 0.1 mM of vitamin C, were placed in the wells (600 μl/well). Each condition was performed in six wells. Transwell inserts were placed in the wells and 6*10³ passage 2 MSCs in 200 μl control medium were plated in the transwell insert and incubated for 18 h. Then transwell inserts were harvested and the medium was discarded. The bottoms of the wells were checked for adherent MSCs to exclude missed migrated cells. The inserts were washed with PBS and cells on the insert membrane (inside and outside) were fixed with 10% formalin for 30 min, permeabilized with 0.1% Triton X-100 for 5 min and washed with milliQ. Cells were stained by placing the inserts in toluidine blue for 60 s followed by a quick rinse in milliQ. The inner side of the membrane was swapped with a cotton wool swab to remove non migrated MSCs. Microscopic pictures of migrated MSCs to the outer side of the transwell membrane were taken directly after the swab. Migrated cells were counted at a 200x magnification using an inlay raster and nine pre-set standardized fields

for the membranes. Based on these counts the total number of cells per square centimetres could be calculated and extrapolated to the total surface of the membrane. Hereby the number of migrated cells per condition could be determined. .

In vivo cell adhesion in OA knees and healthy rat knees

The animal experiments were approved by the animal ethical committee (EMC2285 116-11-03). The animals were housed in groups of 2 animals per IVC cage (21 x 37 cm) in sawdust, enriched with nesting material. The animals had food and water ad libitum with a light/dark cycle of 12 h. Discomfort and welfare were evaluated and scored daily by the animal caretakers. Animals were allocated to OA or control group at random. OA was induced bilaterally in the knees of 13 week old male wild-type Wistar rats (N = 5) (Harlan Netherlands BV, Horst, the Netherlands) by an intra-articular injection of 20 µl saline containing 1 mg mono-iodo acetate (MIA). Healthy controls, 13 week old male wild-type Wistar rats (N = 5) were bilaterally injected intra-articularly in the knee with 20 µl saline. All injections and imaging procedures were applied under isoflurane inhalation anaesthesia (Pharmachemie BV, Haarlem, the Netherlands) while monitoring cardiac function, with additional subcutaneous injections of 0.01 mg/kg buprenorphine during and one day after intra-articular injections. MIA injections cause structural damage after three weeks⁴² and at that point MSCs were injected intra-articular.

Passage 3 MSCs were labelled at 80-90% confluency with superparamagnetic iron oxide (SPIO) by incubating the MSCs for 24 h in aMEM with 5% FCS, ferumoxides 100 µg/ml (11.2 mg Fe/ml, Endorem, lot/batch 08GE601A) and protamine sulphate 5 µg/ml (LEO Pharma).⁴⁵ Labelled cells were trypsinized and plated at a density of $2 \cdot 10^4$ MSCs/cm². After 48 h, MSCs

were exposed to 50 ng/ml IFN γ and TNF α in expansion medium or cultured in normal expansion medium (non-activated) for 24 h. After 24 h, MSCs were washed with PBS and trypsinized. 1×10^6 MSCs in 20 μ l saline were injected intra-articularly by using a 50 μ l syringe (Hamilton, Bonaduz, Switzerland). This number of 1×10^6 MSCs was selected based on literature that reported effect of this cell number and side effects of higher number of cells.^{1, 20, 46} 24 h after injection SPIO-MSCs were visualized with a preclinical 7.0T MRI scanner (MR 901 Discovery, Agilent/GE Healthcare, Milwaukee, Wisconsin, USA) by using a fast spoiled gradient echo (FSPGR) sequence. TR/TE = 30/4 ms, slice thickness 0.4 mm, matrix 192x192, FOV 5 cm, 2 averages, flip angle 16. Cell attachment and migration were evaluated after 24 h of injection since we expected attachment and migration processes to take place in these 24 hours. Directly after the scan, the rats were sacrificed by a pentobarbital overdose and the knees were harvested for histology. 24 hours to evaluate cell migration and attachment to the tissue. These processes are expected to take place in the first 24 hours

Tissue Harvest and Histologic Evaluations

All knees (N= 20 knees) were fixed in formalin 4% (v/v) at room temperature for 1 week, decalcified in 10% EDTA at room temperature for 3 weeks and embedded in paraffin. Coronal sections were stained with Thionin to evaluate cartilage damage.⁴⁶ Perl's iron staining was done for localization of SPIO-MSCs. All samples were blinded before scoring and two different histology sections of each knee were scored by two researchers. Disagreements of greater than 1 point were resolved by consensus. Consultation of a third researcher when disagreement persisted, appeared unnecessary. The average score of MSC attachment of both researchers is

depicted in figure 4. To confirm osteoarthritis, cartilage quality was scored on the patella, trochlea, tibia plateau and femoral condyles with a modified Pritzker score.^{36, 46} An average score for GAG loss and structural damage was calculated. To score the amount of injected MSCs that adhered to different tissues in the joint we used a semi quantitative scoring system on the Perl's iron histology. 1: <10 MSCs; 2:10-50 MSCs; 3: 50-250 MSCs; 4: 250-1000 MSCs; 5:>1000 MSCs. Locations of the injected MSCs were divided in synovium, cartilage and non-adherent in the joint space.

Statistics

Effects of different FCS batches, PL, inflammatory factors, hypoxia and OA SF on gene expression of chemotactic receptors genes were analyzed with a mixed model ANOVA, which takes correlation within donors into account. Analysis of cell migration was done with a separate ANOVA linear regression model for MSCs with and without being exposed to inflammatory factors. Posthoc, independent two-tailed T-tests were performed to compare conditions within both groups. The effect of MIA on MSC adhesion to different OA tissues *in vivo* was done by independent two-tailed T-tests. P-values of ≤ 0.05 were considered statistical significant. Analyses were performed using SPSS 21.0 Statistics (SPSS Inc., Chicago, IL, USA).

Results

Chemokines in the synovial fluid and secreted by OA tissues in vitro

To evaluate chemokine secretion by cartilage and synovium, we did a 10-plex milliplex assay on CM of OA synovium and cartilage, and on OA SF. The ten selected factors were detected in all conditions (Table 2). High levels of MCP-1 and IL-8 were secreted by synovium and cartilage. Furthermore, synovium secreted high amounts of MIP-1a and MIP-1b, whereas cartilage secreted high amounts of PDGF-AA and IP-10. mRNA of the receptors for these chemokines were expressed in cultured MSCs. *CD44*, *PDGFR α* and *PDGFR β* were highly expressed (CT \leq 25), *CCR1*, *CCR4*, *CCR5*, *CXCR3* and *CX3CR1* were intermediately expressed (CT = 26 – 36) and *CXCR1* was not expressed (CT \geq 36).

TABLE 2

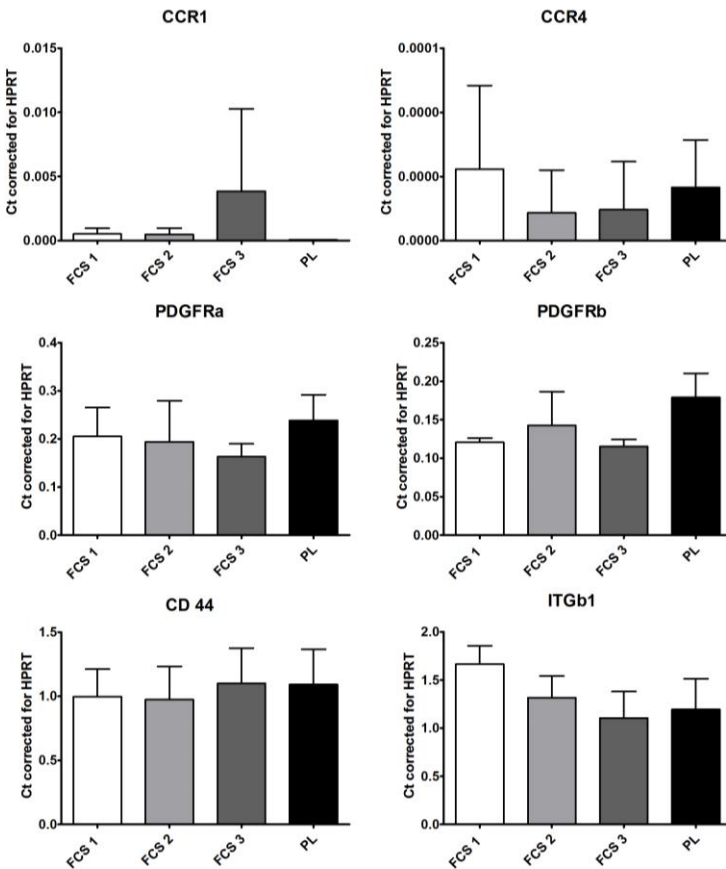
Chemokine secretion by osteoarthritic synovium and cartilage

Chemokine	OA synovium CM (mean and range)	OA cartilage CM (mean and range)	OA synovial fluid (mean and range)	Minimum DC (pg/ml)
MCP-1	31,753 (15,689 – 63,264)	3,499.4 (1,344.6 – 6,040.8)	3,276.6 (847.4 – 4,935.5)	1.9
IL-8	116,848 (63,634 – 197,030)	3,548.3 (114.0 – 11,826)	170.2 (27.4 – 463.5)	0.4
MIP-1a	309.1 (48.6 – 1,077.4)	12.7 (3.2 – 23.9)	7.6 (1.5 – 11.8)	2.9
MIP-1b	367.2 (73.4 – 718.0)	29.7 (5.8 – 57.1)	72.6 (24.8 – 197.1)	3.0
PDGF-AA	14.7 (4.9 – 32.2)	258.3 (115.1 – 513.5)	104.9 (26.7 – 229.0)	0.4
IP-10	139.1 (4.3 – 425.5)	1,014.0 (171.5 – 3,297.1)	4,661.8 (2,223.9 – 7,907.7)	8.6
MDC	74.7 (29.4 – 197.4)	146.4 (104.7 – 168.4)	234.8 (137.0 – 369.5)	3.6
MCP-3	27.7 (1.9 – 47.2)	6.7 (1.9 – 14.7)	66.4 (1.9 – 263.6)	3.8
PDGF-AB/BB	97.6 (1.1 – 318.9)	3.3 (1.1 – 6.1)	799.0 (417.4 – 1,706.2)	2.2
Fractalkine	434.8 (213.9 – 795.2)	19.8 (11.4 – 34.8)	111.6 (11.4 – 208.3)	22.7

Chemokine secretion by osteoarthritic synovium and cartilage for 24 hours in medium and chemokine content in osteoarthritic synovial fluid in pg/ml. Undetectable and below minimum detectable concentrations values were set at 50 % of the minimum detectable concentration value. Data are indicated as mean with range. N= 6 donors. Minimum detectable concentrations pg/ml (Minimum DC), osteoarthritis (OA), conditioned medium (CM).

Expression of migration and adhesion factors in MSCs is independent of serum used in culture

During culture the MSC are generally exposed to serum. Different laboratories use different batches of fetal calf serum (FCS). Moreover, the use of pooled batches of human platelet lysate (PL) becomes increasingly popular. Serum could potentially influence cell behaviour and could make comparison between results obtained in different laboratories difficult. We evaluated the expression of known migration receptors *PDGFR α* , *PDGFR β* , *CCR1* and *CCR4*, and adhesion receptors *ITG β 1*, *ITG β 2*, and *CD44*, in MSCs expanded in 3 different batches of FCS and a



batch of pooled human PL. Different culture sera did not significantly affect the expression of migration receptors *CCR1*, *CCR4*, *PDGFR α* and *PDGFR β* or the expression of the adhesion receptors *CD44* and *ITG β 1* in MSCs (Figure 1).

Figure 1. The expression of migration and adhesion receptors in MSCs is culture serum independent. Culturing MSCs (N=3 donors with triplicate samples for each donor) with 3

different batches of FCS in the expansion medium or with a batch of pooled human PL (N = >50 donors) in the expansion medium. Data shown as mean \pm SD; Fetal calf serum (FCS), platelet lysate (PL).

Influence of inflammation, hypoxia and OA synovial fluid on MSC migration/adhesion receptors

To evaluate whether a short treatment in culture could increase expression of migration or adhesion receptor genes in MSCs, we exposed the cells to inflammatory factors IFN γ /TNF α or cultured them in hypoxia (1% O₂). Furthermore, we determined whether OA SF would influence expression of the before mentioned genes. Exposure of MSCs to different concentrations of IFN γ /TNF α upregulated the expression of *CCR1*, *CCR4* and *CD44*, whereas *PDGFR α* and *PDGFR β* were downregulated by the inflammatory factors (Figure 2). The other migration receptor genes, *CCR5*, *CXCR1*, *CXCR3*, *CXCR4* and *CX3CR1*, and adhesion receptor genes *ITG β 1* and *ITG β 2*, were not influenced by inflammatory factors (data not shown). Hypoxia did not have any effect on the gene expression of the migration receptors and adhesion receptors studied, neither in the presence or absence of IFN γ /TNF α (Supplementary figure 1). Finally, we only observed an upregulation of gene expression of *PDGFR β* (P = 0.006) in MSCs by OA SF (supplementary figure 2)

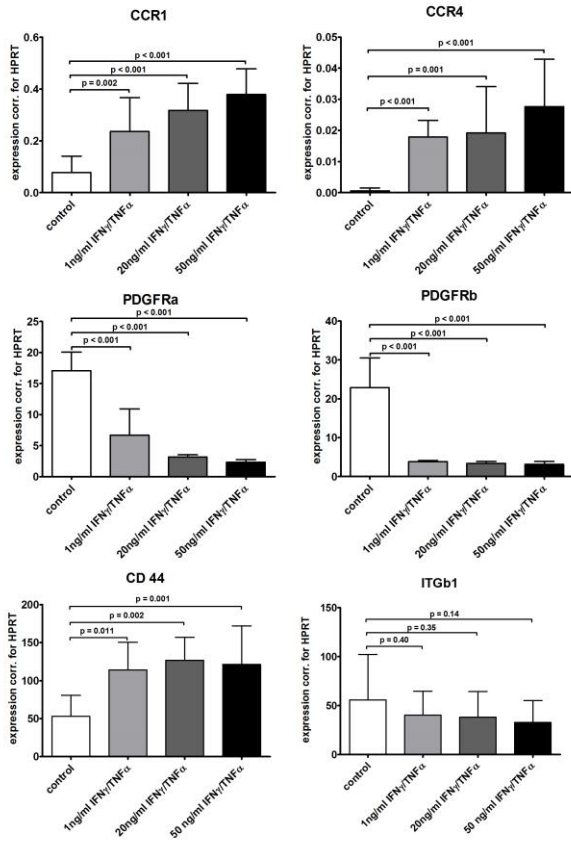
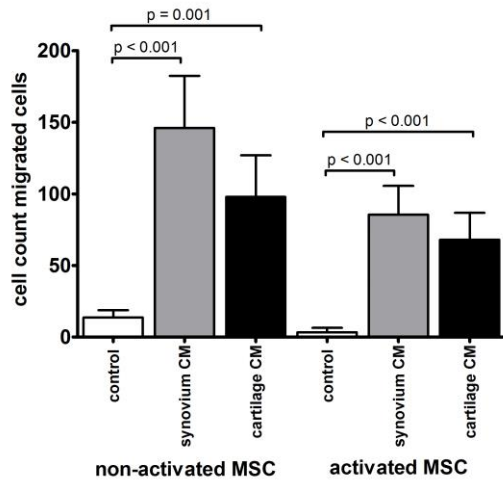


Figure 2. Gene expression of migration and adhesion receptors in MSCs after exposure to inflammatory factors IFN γ /TNF α for 24h. N=3 MSC donors with triplicate samples for each donor. Data shown as mean \pm SD.

MSC migration towards factors secreted by OA tissues

We evaluated whether MSCs would migrate to factors secreted by OA synovium or cartilage and whether this was influenced by pre-treatment of MSCs with IFN γ /TNF α . MSCs migrated more towards OA synovium CM and OA cartilage CM compared to control medium for both non-



activated ($P \leq 0.001$) and activated MSCs ($P < 0.001$), indicating that OA cartilage and synovium secreted factors which stimulated MSC migration (Figure 3). No significant effect of pre-treatment of MSCs was found ($P = 0.060$). Non-activated MSCs migrated more towards synovium CM compared to cartilage CM ($P = 0.031$), a comparable trend was observed for MSC activated by inflammatory factors ($P = 0.065$).

Figure 3. MSC migration towards factors secreted by OA synovium and OA cartilage in a transwell migration assay. Data shown as mean \pm SD; Conditioned medium (CM), non-activated MSCs = 24 h refreshment of medium; activated MSCs = MSCs 24 h activated with 50 ng/ml IFN γ /TNF α in medium.

In vivo adhesion of non-activated MSCs and activated MSCs in healthy knees or OA knees.

An *in vivo* experiment was performed to study MSC migration and attachment to synovium and cartilage. All animals used were healthy wild-type Wistar male rats prior to the study and no side

effects or health problems were found during the study. We evaluated if there was a difference between activated and non-activated MSCs in their migration and adherence patterns and whether this was influenced by the presence or absence of OA. MSCs were found attached to synovium, cartilage or freely in the joint space (Fig 4 E). Cartilage damage and synovial inflammation were clearly visible after OA induction by the MIA model (modified Pritzker score 20.4 ± 4.84) and absent in control knees (modified Pritzker score 4.4 ± 1.27) ($P < 0.001$) (Figure 4A, 4B). 24 h after injection MSCs were present in all control and OA knees. MSCs were mostly found attached to synovium (semi quantitative score 3.13 ± 1.19) or freely in the joint space (semi quantitative score 3.95 ± 1.25), this was approximately equally distributed based on our semi-quantitative scoring system. Since very small amounts of cells were found attached to cartilage (semi quantitative score 1.03 ± 0.11) (Figure 4D), we only evaluated MSC attachment to synovium to compare healthy vs OA knees and to evaluate the effect of MSC pre-treatment with inflammatory factors. We could not detect significant differences in MSC attachment to synovium between OA and healthy knees nor an effect of pre-treatment with inflammatory factors (Figure 4F, 4G).

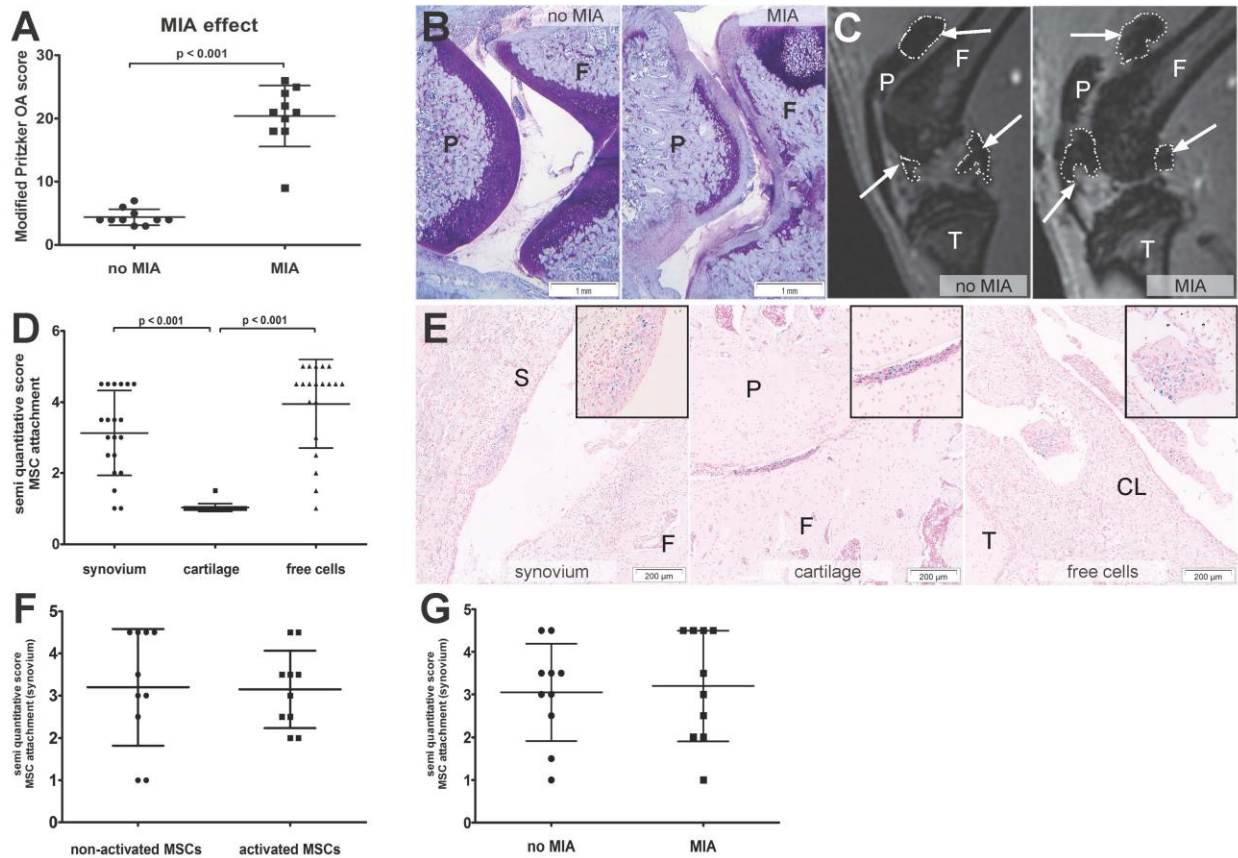


Figure 4. In vivo adherence of non-activated MSCs and activated MSCs in healthy knees or OA knees. OA was bilateral induced in rat knees (N=10 knees) with a MIA injection. Healthy rat knees (N=10 knees) were used as control. (A) Modified Pritzker OA score on histology of 20 rat knees. (B) Thionin staining on coronal knee histology sections with a control knee (left side) and MIA induced OA knee (right side). (C) Arrows indicate SPIO labelled MSC on MRI in a control knee (left side) and OA knee (right side). (D) Semi quantitative score of MSC attachment on synovium and cartilage, (control and OA knees combined). (E) Perl's iron staining on coronal knee histology was done to localize intra-articular injected MSCs. From left to right: histological samples of attached MSCs to synovium, cartilage and free cells in the joint space. The upper right corner shows an enlargement of the attached cells. (F) Comparison between the attachment of activated and non-activated MSCs to synovium in all knees. (G) Difference between control

and OA knees in the migration of MSCs towards synovium . Data is shown as mean \pm SD; patella (P), femur (F), tibia (T), synovium (S), cruciate ligament (CL), osteoarthritis (OA), non-activated = MSCs 24 h refreshment of medium, activated = MSCs 24 h activated with 50 ng/ml IFN γ /TNF α in medium, mono-iodo acetate (MIA).

Discussion

We investigated whether MSCs migrate to OA tissues and if this migration could be influenced by adapting the culture conditions before application of MSCs. Both OA synovium and OA cartilage secreted substantial amounts of chemokines. The profile of the secreted chemokines varied between both tissues. We showed that MSCs express migration and adhesion receptor genes and migrate to factors secreted by both OA synovium and cartilage *in vitro*. *In vivo* more MSCs attached to synovium than to cartilage. The expression of migration and adhesion receptors was altered by adding inflammatory factors during culture, albeit this did not influence migration *in vitro* nor adhesion *in vivo*.

Throughout the world, laboratories use different batches of FCS to culture MSCs. Nowadays human PL is often used and is described to have a positive effect on proliferation and differentiation of MSCs.²⁵ However, contradictory results have been published regarding the effect of culturing with PL on the migration/adhesion capacity of MSCs and the migration of MSCs towards PL.^{17, 33} We compared the effects of 3 different batches of FCS and a batch of pooled PL on gene expression of migration and adhesion receptors in MSCs. Our results indicate that different FCS batches or PL will not likely have a differential effect on migration and attachment of MSCs, making different sera in expansion culture less likely to be a confounder

for migration and adhesion in the comparison of different studies with intra-articularly injected MSCs.

Next to MSC capacities to differentiate and regenerate damaged tissue, their immunomodulatory and trophic properties are very important for their therapeutic function in OA. MSCs need to be activated with inflammatory factors like IFN γ and TNF α to secrete immunomodulatory and trophic factors.^{15, 28, 38, 41} This immunomodulatory and trophic capacity is maintained under hypoxic conditions,^{30, 40} which is important because MSCs as cell therapy for OA will be injected in a hypoxic joint environment. We showed no effect of hypoxia on the expression of migration or adhesion receptor genes in MSCs. It is not clear what the levels of hypoxia are in joints, but for culture, hypoxia levels range from 1-5% of oxygen. We chose 1% of oxygen based on previous studies and experiments in our laboratory.^{10, 40} When using a different oxygen level this might have different effects. It is known that inflammatory factors can upregulate chemokine receptors in MSCs *in vitro*.^{16, 18, 35, 37} We hypothesized that these inflammatory factors could also improve their migration to and adhesion on OA tissues. Indeed, inflammatory factors IFN γ /TNF α upregulated gene expression of *CCR1* and *CCR4*, although *PDGFR α* and *PDGFR β* were downregulated. Chemokines that bind to CCR1 and CCR4 (i.e. MIP-1a, MIP1b, MCP-1, MCP-3) were found in a higher concentration in synovium CM compared to cartilage CM. These chemokines can increase MSC migration.⁷ Since the receptors that bind these chemokines are upregulated in MSCs by activation with IFN γ /TNF α , we would expect better attachment and migration to synovium after MSC activation. Nevertheless, this was not confirmed *in vitro* or *in vivo* where cell activation with IFN γ and TNF α did not improve functional migration of MSCs to synovium. PDGF β , a chemokine that binds to PDGFR β and indirectly interacts with PDGFR α , was more abundantly found in cartilage CM. There was less MSC attachment and migration to

cartilage, although activation with IFN γ and TNF α did not decrease the cell attachment and migration. These results are in line with a recent report on MSC homing in a mouse hypoxic gut model.²⁶ We cannot exclude that longer or shorter exposure to culture conditions or different concentrations of inflammatory factors or oxygen might have had an effect. In our study, the absolute migration was even slightly decreased *in vitro*, albeit the activated cells still migrated towards factors secreted by OA tissues. We excluded that cell death due to treatment with 50 ng/ml IFN γ /TNF α could be an explanation of the decreased absolute migration found, since exposure to these factors did not decrease cell survival after 24 h. We have, however, not evaluated whether the immunomodulatory properties of MSCs pre-treated with IFN γ /TNF α were indeed increased *in vivo*. This will need careful consideration before future applications since, Kavanagh et al. recently found a decreased vasculoprotective effect *in vivo* of MSCs pre-treated with IFN γ or TNF α .²⁶

There are several limitations to our study. We have used human MSCs from relatively aged OA donors who underwent total hip arthroplasty. Although these MSCs might be considered less potent *in vivo* compared to rat MSC from young donors, we consider this cell type to be more clinically relevant, being better predictive for expected results in future clinical application. We have used cartilage and synovium of six different donors and MSCs of three donors. Donor variability should always be taken into account. The level and the variation we have seen in the chemokine data are comparable with the variation we have seen in previous studies.^{6,9} The effect of inflammatory factors or hypoxia on chemokine and adhesion receptor expression was not large on all MSC donors. Therefore, we consider our conclusions valid. Furthermore, cells were labelled using SPIO for tracking purposes by MRI and histology. SPIO labelling could have an effect on cell migration and adhesion capacities.^{12, 52} Although we did not find effects of our

labelling procedure of cell viability, differentiation and secretion of immunomodulatory factors in previous studies ⁴⁷, and we confirmed the cells were viable and adherent when cultured on tissue culture plastic, we cannot completely rule out an effect. Migration and adherence of the cells was evaluated 24 hours after injection. These processes are expected to take place in the first 24 hours. We realize that this time window is not sufficient to study the many processes that might happen afterwards, such as cell proliferation, cell invasion into tissues and cell death. We assume that cells will not attach when they have died during injection or during the first hours after injection. The attached cells were scored on histology by a semi quantitative score which can be not accurate enough to quantify eventual differences between the groups.

MSCs migrated more towards factors secreted by OA synovium compared to cartilage. In line with this, more cells attached to synovium than to cartilage *in vivo*. This could be explained by both chemo-attractive and mechanic processes. Overall, chemotactic factors were found in a higher concentration in synovial CM compared to cartilage CM. Care should be taken however, to directly compare the levels of factors secreted by synovium and cartilage in our studies. Although synovium CM and cartilage CM were made in a standardized way, based on wet weight tissue per volume, synovial tissue contains substantially more cells per gram wet weight than cartilage tissue. Nevertheless, we can conclude that both synovium and cartilage secrete high amounts of chemokines, and the profile of the secreted chemokines seems to differ between synovium and cartilage. The factors secreted by cartilage and synovium were also found in synovial fluid. In a previous study we found that the levels of MDC, PDGF-AA, IP-10, CCL5, MIP-1b were higher in OA than in control SF ⁶, which would be indicative for higher chemotactic effects of osteoarthritic tissues. Nevertheless, no differences in migration or adhesion was seen between rat knees with and without OA. The synovial fluid did not affect the

receptor gene expression in MSCs (supplementary figure 2), but we did not evaluate whether the secreted factors individually could affect the gene expression or function of MSCs. Another explanation for better adhesion of MSCs to synovium compared to cartilage could be the continuous movements of the joint that might evoke shear stresses that MSCs cannot resist, especially those who primarily attached to cartilage. Furthermore, cells are more likely to attach to a rough surface like synovium than to a smooth surface like cartilage. Nevertheless, in our OA model with marked cartilage damage, no improvement of cell adhesion to cartilage was seen. We assume that intra-articular cell attachment is influenced by a combination of chemo-attractive processes, tissue surface characteristics and biomechanics.

Conclusion

Intra-articular tissues affected by OA secrete chemotactic factors that can attract MSCs. Exposure to inflammatory factors did influence MSC gene expression of several chemokine receptors while these inflammatory factors did not affect the migration of MSCs *in vitro* or MSC attachment *in vivo*.

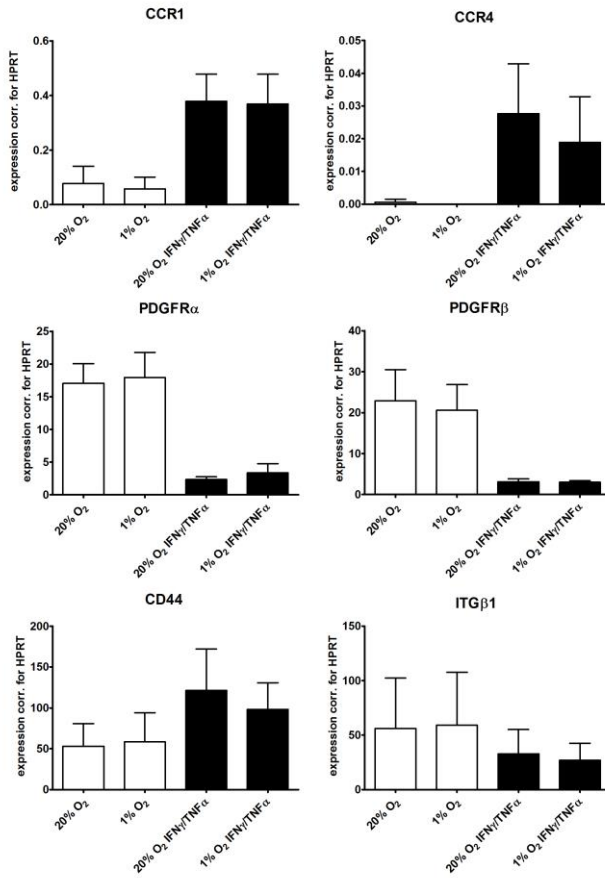
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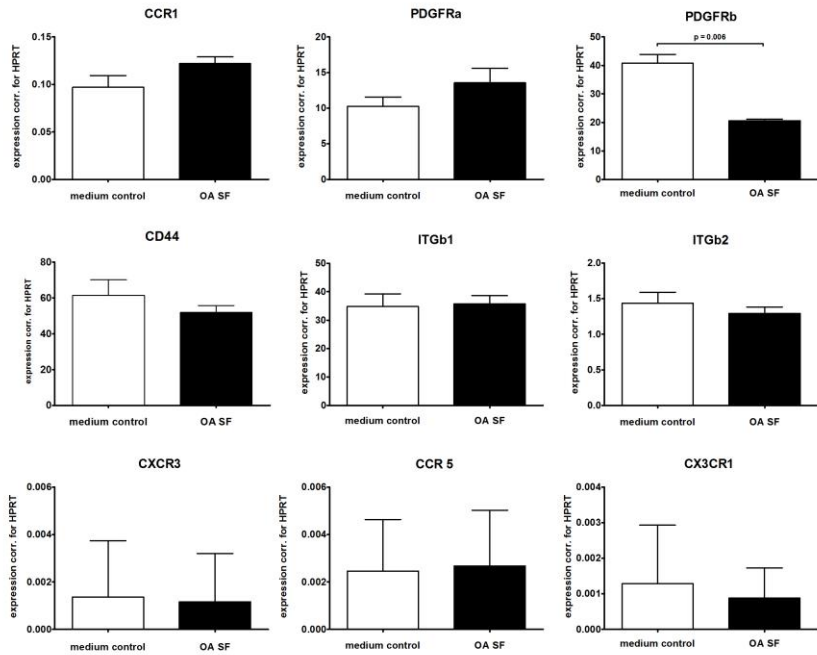
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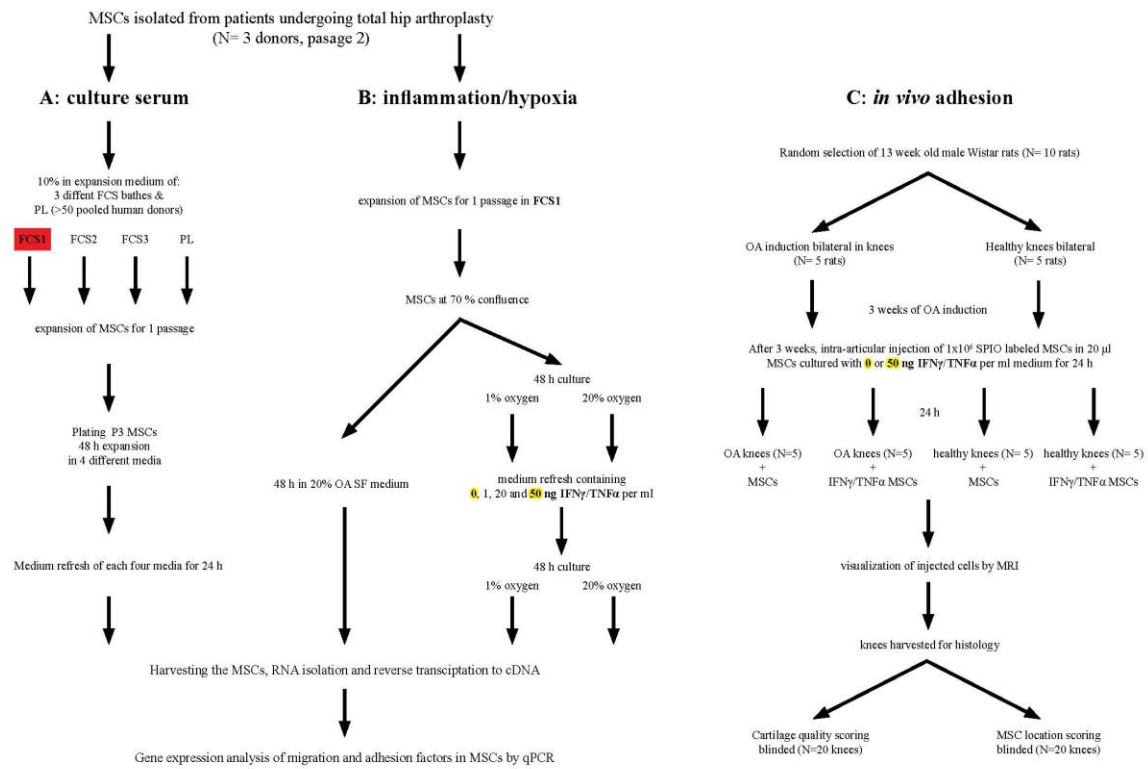
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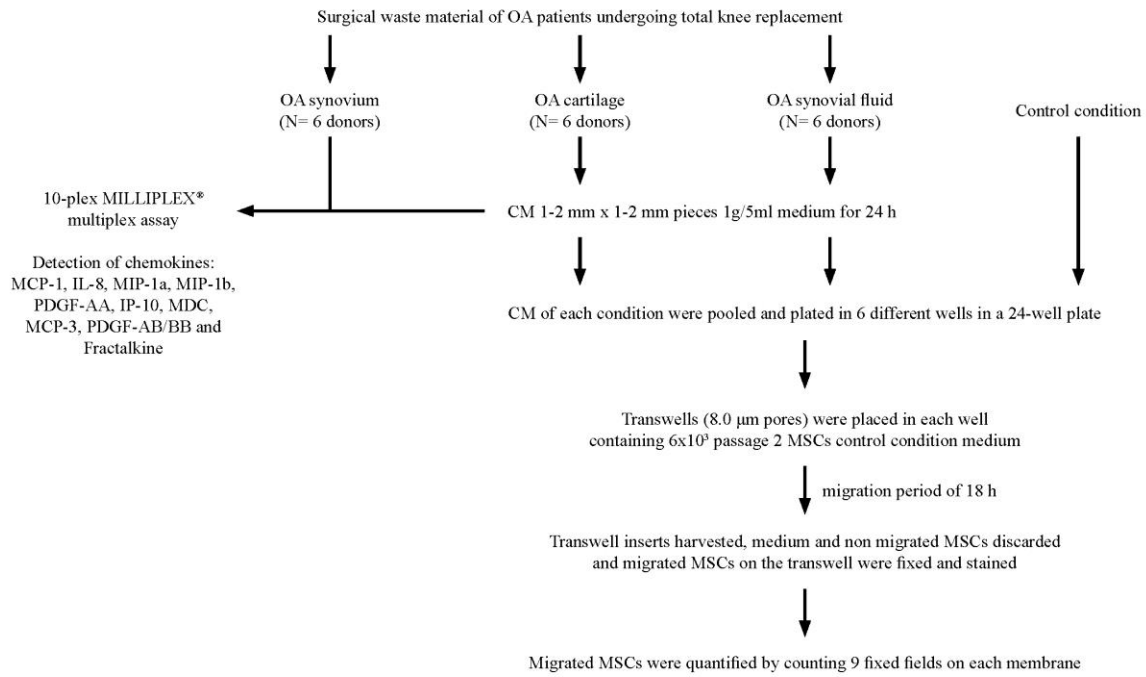
Supplementary figure 1. MSC culture under hypoxia and the effect on expression of migration and adhesion receptors. MSCs (N=3 donors with triplicate samples for each donor) were cultured for 48 h under 20 % of oxygen (normoxic) or 1 % of oxygen (hypoxic) conditions with additional 24 h culturing in combination with or without 50 ng/ml of IFN γ /TNF α . Data is shown as mean \pm SD.



Supplementary figure 2. MSC culture with OA SF and the effect on expression of migration and adhesion receptors. MSCs (N=3 donors) were cultured for 48 h with 20% OA SF in serum free medium. Data shown as mean \pm SD.



Supplementary figure 3.



Supplementary figure 4.