Towards Cell Therapy for Osteoarthritis

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Towards Cell Therapy for Osteoarthritis

Richting Celtherapie voor Artrose

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag de
rector magnificus

Prof.dr. H.A.P Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

donderdag 28 november 2013 om 15.30 uur

door
Gerben Matthijs van Buul
geboren te Leiderdorp

The printing of this thesis was financially supported by:
Nederlandse Orthopaedische Vereniging, Erasmus MC afdeling orthopaedie, Erasmus MC afdeling radiologie, Erasmus Universiteit Rotterdam, Reumafonds, Annafonds.

The research described in this thesis was financially supported by the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science.

Cover, layout and design: G.M. van Buul and A.W. Everaers, Erasmus MC, Rotterdam.

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Chapter 1

General introduction

Osteoarthritis (OA) is a disabling joint disease affecting over 10% of individuals aged 60 years or older [1]. In a study from 2007, prevalence of OA in the Netherlands was estimated to be around 650,000 patients [2]. OA involves the entire joint and is characterized by inflammation and catabolic processes, leading to progressive cartilage damage [3]. Current treatments include pain medication, lifestyle changes such as weight loss and regular exercise, and ultimately joint replacement. Pain medication and lifestyle changes are symptomatic treatments which are not able to cure the disease. Joint replacement is a good option for many OA patients, but unfortunately joint prostheses have a limited lifespan and as a result they do not provide a final solution for OA. Regenerative medicine therapies are promising approaches to modify the pathologic processes associated with OA and other musculoskeletal diseases [4]. Regenerative medicine aims at replacing or regenerating human cells, tissues or organs to restore or establish normal function. This goal can be achieved by replacing damaged tissue or by stimulating the body's own repair mechanisms to heal tissues or organs with poor self-repair capacity. Regarding regenerative medicine, a lot can be learned from other organisms. Probably the most striking example of musculoskeletal regeneration is the regrowth of an entire limb, after it has been traumatically amputated, in a salamander [5]. This classic example unfortunately doesn't apply to humans, but growth factors and stem cells are indispensable in physiological tissue homeostasis and regeneration after musculoskeletal tissue injury

In this thesis we studied the efficacy and working mechanisms of biological treatments for OA. Two biological treatments that are currently under extensive investigation are platelet-rich plasma (PRP), a preparation of concentrated, platelet-derived bio-active factors isolated from blood, and mesenchymal stem cells (MSCs). Both these treatments have possibilities to influence the degenerative joint environment characteristic of OA. Therefore, the aim of this thesis is to evaluate and improve the effects of biological treatments to inhibit progressive joint damage. To this aim, we studied the effects of PRP and MSCs on multiple inflammatory, anabolic and catabolic processes throughout the joint in in vitro models as well as small animal models.

Osteoarthritis

Osteoarthritis (OA) represents the most widespread cause of physical morbidity and impaired quality of life throughout the industrialized world [6]. The disease causes pain and joint stiffness that can severely impair daily activities. OA is a chronic and progressive disease of the joint with multifactorial etiology that amongst others can involve age, preceding trauma, obesity, female gender and genetic predisposition [7]. Many simultaneous processes take place in OA, including cartilage breakdown, subchondral bone alterations and joint inflammation. These processes influence each other by means of

direct cell-cell contact or via the cellular secretion of bioactive factors. Many of these processes are not only reactive to the degenerative joint environment, but they result in degradation products or compounds which induce further inflammatory and catabolic effects by itself [8]. Thereby, these processes contribute to a vicious degenerative circle, which characterizes progressive joint damage [9,10].

Cartilage

Articular cartilage limits friction and allows for efficient load bearing and distribution in synovial joints. Cartilage contains one cell-type, the chondrocyte, which produces the extracellular matrix (ECM). Chondrocytes account for approximately 5% of the wet weight of cartilage, the ECM for 25% and water for the remaining 70% [11]. The most abundant protein in the ECM is collagen type II, which represents around 60-80% of the dry weight of cartilage, followed by approximately 20-30% of glycosaminoglycans (GAGs). The collagen molecules form an organized network, defining the shape and tensile strength of the cartilage. The hydrophilic proteoglycans are intertwined in this collagen network and are responsible for the high water content of articular cartilage. Cartilage is avascular, aneural and lacks lymphatic drainage [12]. These factors contribute to the fact that natural repair mechanisms are underprovided, giving cartilage a very limited intrinsic regenerative capacity [13]. As a result, cartilage lesions are prone to progress to OA and the cartilage damage in OA progresses over time [14,15]. Macroscopically, cartilage degeneration is displayed as fibrillations and fissures in the articular surface and partial or complete erosion of the tissue. Cartilage breakdown at the molecular level is mediated by the presence of inflammatory and catabolic mediators, together with a diminished synthetic function of chondrocytes, a reduced responsiveness to anabolic growth factors or even chondrocyte death [16,17,18].

Bone

Bone alterations present as osteophyte formation and subchondral bone sclerosis in late stage OA. Osteophytes are abnormal outgrowths of bone occurring in joints. The formation of osteophytes is not completely understood, but their presence is classically related to joint degeneration [19]. The involvement of the subchondral bone in the pathogenesis of osteoarthritis has become more evident during the last decade [10]. The underlying bone undergoes dynamic changes, leading to loss of bone in early stages and increased bone formation in late stages of the disease [20]. Although the interaction of cartilage and bone is not fully elucidated, it has been demonstrated that both mechanical interactions as well as the exchange of bioactive factors play a role [21].

Synovium

Synovium is a thin membrane lining the inside of the joint which produces synovial fluid, the “lubricant” of the joint. Next to this, synovium contains high numbers of macrophages, which are the main representative of the immune system in the joint. There-

by, the synovium is primarily involved in joint inflammation. Inflammation manifests as an elevation of inflammatory cytokines in synovial fluid and increased synovial thickness resulting from synovial hyperplasia together with infiltration of inflammatory cells [22,23,24]. The synovium produces multiple inflammatory and catabolic factors in the joint, including interleukin-1 and tumor necrosis factor alpha [25]. Factors secreted by osteoarthritic synovium have been shown to reduce chondrogenic differentiation of MSCs [26]. In cartilage explants, these factors increase GAG breakdown and diminish GAG production [25,27]. In later stages of OA the synovium can become fibrotic [23], contributing to stiffening of the joint.

Osteoarthritis therapies

Current OA therapies

Current treatments for OA mainly aim at treating symptoms of the disease, not at a durable way of modifying osteoarthritic pathologic processes. Therapeutic options include life-style changes, physical therapy, a variety of pain medications and ultimately surgical intervention like joint replacement. Hyaluronic acid, glucosamine sulfate and chondroitin sulfate have more recently been postulated as symptom relieving pharmaceuticals which might also have structural OA modifying effects, but efficacy of these drugs on pain reduction as well as on structural alterations remain debated [28].

Platelet-rich plasma

Due to the complexity and dynamic character of OA, biological treatments appear very appealing. Biological treatments can likewise provide a high complexity and versatility in effects, thereby engaging in many different processes simultaneously. Platelet-rich plasma (PRP) is blood plasma enriched with platelets. Platelets are irregularly shaped cell fragments derived from precursor megakaryocytes, and contain many growth factors. Thereby, PRP is an autologous preparation of concentrated bioactive factors, which can be easily derived from blood by centrifugation. Previous animal studies and some preliminary clinical studies have evaluated the efficacy of single growth factors in modifying OA processes [29,30,31,32]. PRP on the other hand, provides a multitude of growth factors which could orchestrate together to interfere with and adjust many pathological processes simultaneously. The growth factors in PRP encompass vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-beta) [33]. Initial clinical studies show promising effects of intra-articular PRP injections as an OA treatment, although these studies were very preliminary and not randomized double blind placebo-controlled trials [34,35,36]. Next to this, the basic fundamental evidence for PRP as an OA treatment is rather limited. Some studies have shown anabolic effects of PRP on cell proliferation [37,38], but possible anti-inflammatory effects of PRP or effects on cartilage matrix formation are currently

not studied. Further insight in the working mechanism and anti-osteoarthritic effects is warranted for additional optimization and broad clinical application.

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) represent another biological therapeutic option which is considered a promising alternative for an OA modifying treatment [39]. Stem cells play a vital role in physiological tissue homeostasis and regeneration after tissue injury [40]. MSCs were initially found in the bone marrow [41], but at present cells with a high resemblance to these MSCs have been found in many musculoskeletal and other tissues, such as fat tissue. MSCs have the potential to differentiate into multiple lineages, such as the adipogenic, osteogenic and chondrogenic lineage [42]. Thereby, they provide a very suitable source of cartilage forming cells. Next to this differentiation potential, more recent studies have indicated the capacity of MSCs to alter their microenvironment via secretion of so-called trophic factors, which might play an even more important role in tissue repair [43,44]. These factors could possibly counteract inflammatory and catabolic aspects of OA and attract endogenous repair cells. MSCs are able to survive in vivo and secrete trophic factors for a longer period of time, which could be advantageous over PRP which provides only a short term delivery of growth factors to the joint. By providing a more durable presence of growth factors, a long term modification of pathologic OA processes is possible. Next to the advantage of duration of therapeutic efficacy, MSCs are normally present in healthy and diseased joints. Moreover, they are able to react to the joint environment as shown before by the increased presence of MSCs in synovium and synovial fluid after mechanical joint injury in an animal OA model as well as a clinical study concerning intra-articular ligament injury [45,46]. This indicates that MSCs respond to stimuli after articular damage and suggests that the increased intra-articular presence of MSCs is part of a natural healing process. The administration of additional MSCs in an osteoarthritic joint could be a therapy for OA, mimicking and enhancing this healing process and thereby provide a natural, long term and tailor made treatment for OA. Animal studies have indicated some positive effects of intra-articularly injected MSCs on cartilage morphology in various joint damage models [47,48,49,50,51,52,53,54,55,56,57]. Even some clinical studies report the feasibility and safety of this therapy, although in these initial studies nothing could be said about efficacy [58,59]. In order to further optimize this therapy and to be able to translate it to a broad clinical OA setting, more insight in the fate and function of the MSCs and their effects on multiple OA processes is needed.

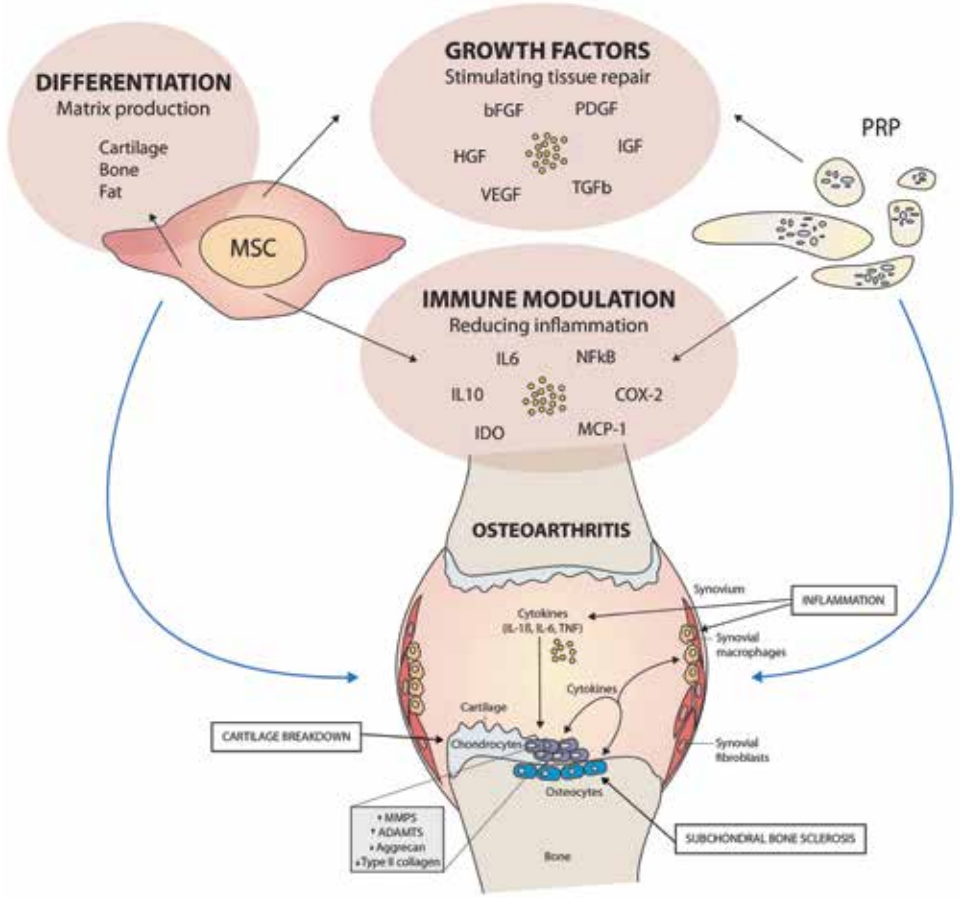

Figure 1: biological treatments for osteoarthritis

Evaluating cell fate and function of MSC therapy

Cell tracking is an invaluable tool for determining the efficacy and safety of cell based regenerative therapies. By using different techniques, cell-tracking could give insight into the distribution, survival and even function of applied cells. Intra-articularly applied cells are found up to four weeks after injection, but their numbers dramatically decrease in this time-period [50,51,56]. Unraveling their survival and working mechanism gives tremendous options for further optimization of cell-based therapies. Many modalities for cell visualization exist, including histology, optical imaging and magnetic resonance imaging (MRI) [60,61]. Cell labeling using superparamagnetic iron oxide (SPIO) particles allows in vivo cell tracking using MRI. This technique has the main advantage that only clinically applied compounds are used for cell labeling, and it has already entered the clinical arena in non-orthopedic fields [62,63,64]. Before implementing this approach to an orthopedic application, it has to be tailored to intra-articular use in terms of safety

and cell traceability. Relevant safety issues include the influence of SPIO on cell viability, cell differentiation and MSC secretion profile. Regarding cell traceability it is important to ensure accurate intra-articular visualization and distinction from other joint structures, together with quantification of labeled cells. If we would know where the cells go to in the joint and what they exactly do, we could improve for instance the mode or timing of intra-articular application. This way, these techniques could be used to further optimize cellular therapies by evaluating adjustments to increase cell survival or functionality.

Optimizing cellular OA therapies

Several aspects of MSCs as a regenerative therapy are already known from other fields, which could hamper the use of MSCs as a durable, disease modifying osteoarthritic drug (DMOAD). Isolating and culturing MSCs is a costly and time-consuming procedure requiring special expertise and facilities. This aspect is a slight drawback in an experimental setting, but is a huge shortcoming in order to accomplish broad clinical application once therapeutic efficacy has been established. By using allogeneic MSCs, cells from one single, well-characterized donor could be used for multiple patients. This would considerably reduce the total costs per patient, realizing a higher cost-effectiveness of the therapy. Another option would be to use freshly isolated bone marrow mononuclear cells, the cell fraction from bone marrow containing MSCs [65,66]. This cell fraction can be isolated in a one-step procedure, in that way making costly culturing procedures redundant.

Next to the cell-type being used, the mode of application could also be used to optimize cell therapies. Cells injected intravenously have been shown to be removed from the body within 24h, while MSC administered in tissues like muscle, spine, and fat pads remain present locally up to several weeks [67]. The joint is known to be an immune privileged and sealed-off compartment, which likely beneficially affects cell survival and their retention [68]. Still, expectedly, improving cell survival will increase the duration of advantageous therapeutic effects. Cell encapsulation in alginate beads could be a way to protect cells from the host immune system, but at the same time allow them to fulfill their regenerative role. This enables an increase in longevity and intra-articular presence of the cells and thereby an increase in durability of therapeutic efficacy. Evaluating these effects on joint integrity and various osteoarthritic processes is a necessary feed-back step to allow for further therapy development.

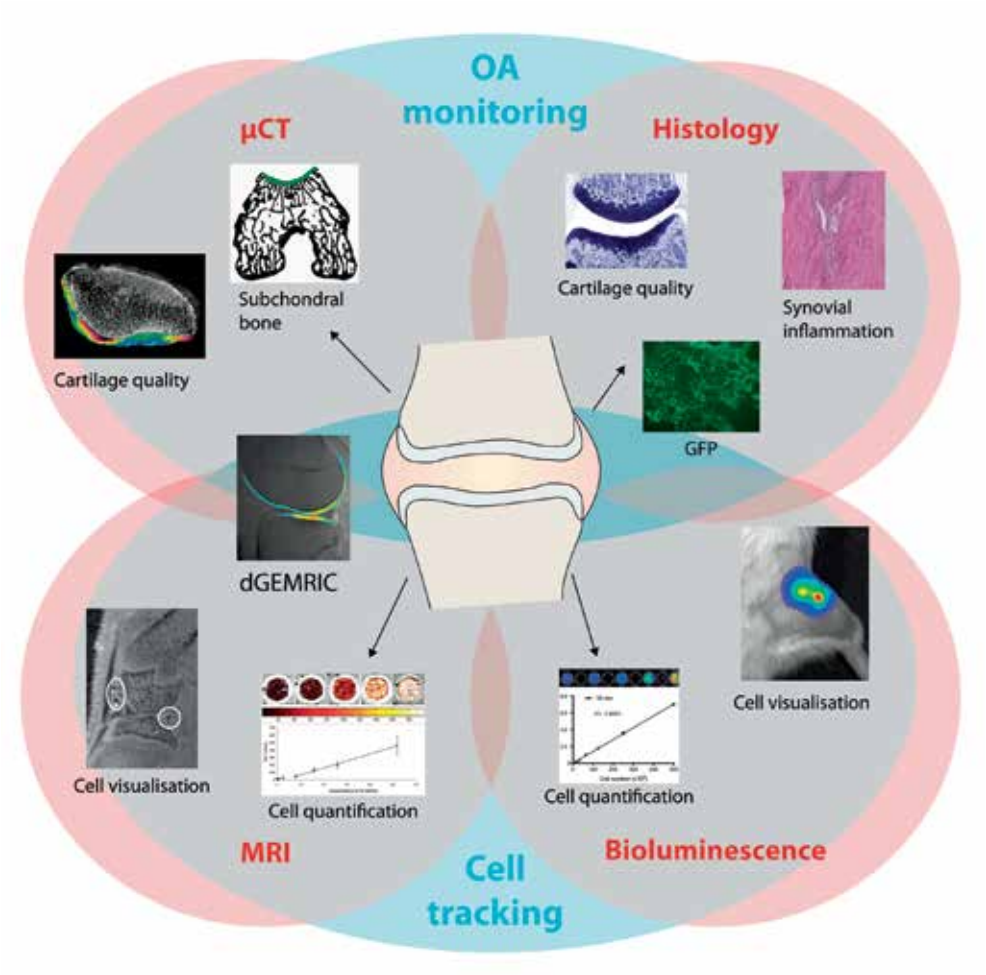

Figure 2: Different modalities and techniques to track cells and monitor joint structures together with osteoarthritic processes

Aim and outline of this thesis

The aim of this thesis was to elucidate the effects of PRP and MSCs on multiple OA related processes. Additionally, we aimed to develop a method to track cells in vivo to evaluate their fate in future studies.

In **chapter 2** we studied the capacity of an autologous preparation of bioactive factors to reduce the effects of an inflammatory mediator on osteoarthritic chondrocytes. The growth factor preparation we used was platelet-rich plasma releasate, which contains many growth factors. Platelets are small cell fragments derived from megakaryocytes and can be isolated from blood by means of centrifugation. In **chapter 3** we reviewed

the presence and characteristics of mesencymal progenitor cells in various musculoskeletal tissues. These cells provide a renewable cell source for physiological tissue homeostasis and regeneration after musculoskeletal tissue injury. The progenitor cells from different musculoskeletal tissues have small dissimilarities from one another, but in general are very alike the bone marrow derived MSCs further discussed in this thesis. In **chapter 4** we determined at which level MSCs were able to secrete a panel of factors and whether the total of MSC-secreted factors was able to exert anti-inflammatory and anti-catabolic effects on osteoarthritic cartilage and synovium in-vitro. In **chapter 5** we determined the effects of MSCs, as well as freshly isolated bone marrow mononuclear cells, on several OA aspects in a rat OA model in-vivo. We used these different cell-types in order to enhance clinical translatability, and evaluated their effects on pain, structural damage and inflammation. In **chapter 6** we compared ferucarbotran to ferumoxides, both superparamagnetic iron oxides (SPIOs), for cell labeling in order to track them with magnetic resonance imaging (MRI). Both SPIOs have different physical properties and different techniques for labeling non-phagocytic cells, resulting in different amounts of endocytosed SPIO. We selected the best technique and SPIO based on these studies and in **chapter 7** we determined several aspects regarding safety and MRI traceability in order to use this technique in-vivo in an intra-articular environment. Since limited cell survival might be one of the aspects compromising long term effects of cell therapies, we studied a method to increase their longevity after intra-articular application. In **chapter 8** we encapsulated MSCs in alginate beads to see if this would enable a long-lasting interplay between MSCs and an inflammatory environment. The alginate beads are permeable to small soluble factors as secreted by MSCs and diseased tissues, yet they can protect MSCs from hostile interactions with the immune system. In **chapter 9** and **10** we discuss and summarize the findings of this thesis and elaborate on directions for future studies in order to translate MSC application towards a clinical OA therapy.

Chapter 2

Platelet-Rich Plasma Releasate Inhibits Inflammatory Processes in Osteoarthritic Chondrocytes

Gerben M. van Buul, Wendy L.M. Koevoet, Nicole Kops, P. Koen Bos, Jan A.N. Verhaar, Harrie Weinans, Monique R. Bernsen, Gerjo J.V.M. van Osch.
American Journal of Sports Medicine, 2011;39(11):2362-70

Abstract

Background: Platelet-rich plasma (PRP) has recently been postulated as a treatment for osteoarthritis (OA). Although anabolic effects of PRP on chondrocytes are well documented, no reports are known addressing effects on cartilage degeneration. Since OA is characterized by a catabolic and inflammatory joint environment, we studied whether PRP was able to counteract the effects of such an environment on human osteoarthritic chondrocytes.

Methods: Human OA chondrocytes were cultured in the presence of IL-1 beta to mimic an osteoarthritic environment. Medium was supplemented with 0%, 1% or 10% PRP releasate ([PRPr] the active releasate of PRP). After 48 hours, gene expression of collagen type II alpha 1 (COL2A1), aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, ADAMTS5, matrix metalloproteinase (MMP)13 and prostaglandin-endoperoxide synthase (PTGS)2 was analyzed. Additionally, glycosaminoglycan (GAG) content, nitric oxide (NO) production and nuclear factor kappa B (NFkB) activation were studied.

Results: PRPr diminished IL-1 beta induced inhibition of COL2A1 and ACAN gene expression. PRPr also reduced IL-1 beta induced increase of ADAMTS4 and PTGS2 gene expression. ADAMTS5 gene expression and GAG content were not influenced by IL1-beta or additional PRPr. MMP13 gene expression and NO production were upregulated by IL-1 beta but not affected by added PRPr. Finally, PRPr reduced IL-1 beta induced NFkB activation to control levels containing no IL-1 beta.

Conclusion: PRPr diminished multiple inflammatory IL-1 beta mediated effects on human osteoarthritic chondrocytes, including inhibition of NFkB activation. NFkB activation is a major pathway involved in the pathogenesis of OA. These results encourage further study of PRP as a treatment for OA.

Introduction

Articular cartilage provides a low friction and efficient load bearing joint surface. Cartilage is an avascular and aneural tissue without lymphatic drainage, and has a very limited intrinsic regenerative capacity[13]. Osteoarthritis (OA) is a degenerative joint disease, characterized by an imbalance of anabolic and catabolic processes in synovial joints[69]. This imbalance results in progressive cartilage damage and ultimately patient disability. In order to stimulate repair processes by endogenous cells, platelet-rich plasma (PRP) has recently been under extensive investigation[70,71,72]. PRP is a plasma preparation that can be obtained from whole blood, having an increased platelet concentration above baseline[73]. Platelets contain various organelles, including α granules. These granules hold numerous growth factors and other bioactive proteins, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-beta), many of which play a pivotal role in hemostasis or tissue healing[33]. Upon activation, platelets are triggered to release these growth factors to the injured site to stimulate various healing processes. PRP can be produced by centrifugation of autologous blood, making PRP a highly concentrated, natural and autologous treatment option for a variety of regenerative medicine fields[74]. Several pre-clinical and clinical studies using PRP as a treatment for OA have been performed, reporting promising results[75,76,77,78]. Unfortunately, no randomized controlled clinical trials regarding this subject have been reported, thereby limiting the level of current evidence of benefit for PRP-based treatment of OA. Elucidating the working mechanisms of PRP in a standardized *in vitro* environment could provide tools to further optimize these therapies. Several studies have previously described the effects of PRP on chondrocytes in an *in vitro* model[37,38,79,80,81]. These studies did not evaluate PRP primarily as a treatment option, but mainly as a culture additive in standard *in vitro* conditions. They describe without exception a stimulating effect of PRP on cell proliferation, although the effects on the maintenance of a chondrogenic phenotype were less concordant. However, to evaluate PRP as a therapy for OA, the *in vitro* environment has to simulate the *in vivo* situation. The joint environment in OA has shifted towards the catabolic side at the moment patients require medical care. Therefore, the emphasis may well be better directed toward understanding not only the anabolic effects, but also the effects of PRP on prevention of degeneration in an osteoarthritic environment. Interleukin (IL)-1 beta is one of the most potent inflammatory factors in osteoarthritic joints. This cytokine has been described to induce the production of destructive proteases together with the inhibition of extracellular matrix formation[82,83]. Treating cells with IL-1 beta has been reported to be a useful model to reproduce the mechanisms involved in degenerative arthropathies[84]. IL-1 beta exerts its effects through a diverse spectrum of signaling cascades, including nuclear factor kappa B (NFkB) activation[84,85]. NFkB is normally located in an inactivated state in the cytosol bound to IκB, an inhibitory protein. Upon activation NFkB translocates to the nucleus and acts on various regulatory genes involved in apoptosis, inflammation and

other immune responses[86]. In order to determine the effects of PRP in a standardized inflammatory environment, we studied the capacity of PRP releasate ([PRPr] the active releasate of PRP) to counteract IL-1 beta induced effects on genes involved in matrix formation and degradation in osteoarthritic chondrocytes, including NFkB as a possible pathway through which these effects occurred.

Materials and methods

PRP releasate preparation

Anticoagulated whole blood from three healthy male donors was acquired (Sanquin Blood Supply Foundation, Amsterdam, the Netherlands) after informed consent. Whole blood was further processed within two hours after collection. PRP was prepared by means of a GPS® III System (kindly provided by Biomet Nederland BV, Dordrecht, the Netherlands) according to the manufacturer's protocol. Clotting upon addition of 22.8 mM $CaCl_2$ 1:10 (v/v) activated platelets to release their growth factors. Supernatant was collected and designated PRP releasate (PRPr). Baseline platelet, white blood cell and red blood cell concentrations of whole blood, PRP and PRPr were measured on a clinical Sysmex XE-2100 automated hematology system (Sysmex Europe, Norderstedt, Germany). PRPr was subsequently stored in aliquots of 1.5 ml at -80°C for further experiments and growth factor analyses. Cryopreservation has been reported before to maintain the ability of PRPr to influence chondrocyte behavior[37]. Baseline VEGF, PDGF-AA, PDGF-AB/BB in PRPr were measured by means of a MILLIPLEX (Millipore BV, Amsterdam, the Netherlands) and TGF-beta 1 using the human TGF-ß1 Immunoassay Quantikine® (R&D Systems, Abingdon, UK). Two dilutions of the samples were measured for growth factor analyses. The measurements that were best in the range of the standard are reported.

Chondrocyte culture with PRP releasate

Human OA cartilage from six donors was obtained from patients undergoing total knee replacement surgery (after implicit consent, as approved by the local ethical committee; MEC-2004-322). Full thickness cartilage was harvested, treated with 0.2% protease (Sigma-Aldrich,Zwijndrecht, Netherlands) in physiological saline solution for 90 minutes and subsequently digested overnight in standard culture medium (DMEM containing 10% FCS, 50 µg/mL gentamicine and 1.5 µg/mL fungizone) supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). Following digestion, cells were washed twice with physiologic saline and the harvested cell number was determined using a hemocytometer. Alginate beads were prepared as described previously[87]. In short, chondrocytes were suspended in 1.2% low viscosity alginate (Keltone LV; Kelco, Surrey, UK) at a concentration of four million cells/ml. Beads were created by dripping the alginate/cell suspension through a 23 gauge needle into a CaCl2

solution. Chondrocytes in alginate beads were cultured in standard culture medium as described above, supplemented with $1x10^{-4}$ M ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO). After pre-culturing chondrocytes in alginate beads for 3 weeks to allow extracellular matrix formation, beads were washed three times with serum-free DMEM. Subsequently, beads were placed in serum free culture medium (DMEM containing ITS+1 1:100 (v/v), 50 µg/ml gentamicin, 1.5 µg/ml fungizone and $1x10^{-4}$ M ascorbic acid-2-phosphate) with or without 10ng/ml IL-1 beta and the addition of PRPr at concentrations of 0, 1 and 10% (v/v). After 48 hours of culture, beads were harvested for gene expression analyses. 48 hours of culture has been described previously as a suitable time point to study the effects of IL-1 beta and various treatments on chondrocyte behavior[84,88]. Additionally, beads were harvested for glycosaminoglycan (GAG) and DNA analyses and medium was harvested for nitric oxide and GAG analysis.

Gene expression analysis

Alginate beads were dissolved in 55 mM sodium citric acid and spun down at 350G for 8 minutes at a temperature of 4 °C. Cell pellets were resuspended in RNA-BeeTM (TEL-TEST, Friendswood, TX). RNA was extracted with chloroform and purified from the supernatant using the RNAeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines with on-column DNA digestion. Nucleic acid content was determined spectrophotometrically (NanoDrop ND1000; Isogen Life Science, IJsselstein, The Netherlands). Complementary DNA (cDNA) synthesis was performed using a RevertAid™First Strand cDNASynthesis Kit (MBI Fermentas, St. Leon-Rot, Germany), and polymerase chain reactions (PCRs) were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, Capelle a/d IJssel, the Netherlands) as described earlier[89]. RT-PCR primers for collagen type II alpha 1 (COL2A1), aggrecan (ACAN), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, ADAMTS5 and matrix metalloproteinase (MMP)13 are reported by Uitterlinden et al[90]. Prostaglandin-endoperoxide synthase (PTGS)2 gene expression was determined using a commercial assay-on-demand set (Hs01573474.g1, Applied Biosystems, Capelle a/d IJssel, the Netherlands). GAPDH, HPRT and beta 2-microglobulin were compared as housekeeping genes. Relative expression levels, normalized to GAPDH as the most stably expressed of the three reference genes (data not shown), were calculated using the $2^{-\Delta\Delta Ct}$ method[91].

Glycosaminoglycan and DNA assay

Glycosaminoglycan (GAG) analysis was performed by a dimethylmethylene blue (DMB) assay. Alginate beads were digested overnight at 60 °C in papain buffer (250 µg/ml papain in 50 mM EDTA and 5 mM L-cysteine). Glycosaminoglycan (GAG) amount in the digest was quantified using a modified dimethylmethylene blue (DMB) assay[92], based on the original description by Farndale et al.[93]. The metachromatic reaction of GAG with DMB was monitored using a spectrophotometer, and the ratio A530 : A590 was used to determine the amount of GAG present, using chondroitin sulphate

C (Sigma-Aldrich, Zwijndrecht, the Netherlands) as a standard. The amount of DNA in each papain-digested sample was determined using ethidium bromide with calf thymus DNA (Sigma-Aldrich, Zwijndrecht, the Netherlands) as a standard.

Nitric oxide assay

Nitric oxide (NO) production was determined by quantifying its derived product, nitrite, in medium using a spectrophotometric method based upon the Griess reaction[94]. Briefly, 100 µl of culture medium was mixed with 100 µl of Griess reagent (0.5% sulphanilamide, 0.05% naphtyl ethylenediamine dihydrochloride, 2.5% H_3PO_4). A serial dilution of sodium nitrite ($NaNO_2$, Fluka, Buchs, Switzerland) was used as a standard. The absorption was measured at 540 nm.

NFkB

To evaluate the involvement of NFkB activation in the working mechanism of PRPr, freshly isolated chondrocytes were seeded in monolayer at a density of 20,000 cells/cm^2. Cells were precultured in basal medium in 48-well plates until subconfluency. A preliminary study revealed one hour of IL-1 beta incubation to be the optimal moment to evaluate NFkB activation (data not shown), confirming a previous study by Largo et al.[85]. Cells were cultured for one hour in the presence or absence of IL-1 beta and PRPr at concentrations of 0, 1 and 10% (v/v) and subsequently fixed in formalin 4%. All conditions were simultaneously fixed per donor. Fixation was followed by incubation with NFkB-p65 Antibody (Ab-276) 1:100 (Signalway Antibody, Leusden, the Netherlands) and goat-anti rabbit AP 1:20 (Sigma, St. Louis, MO, USA). Freshly prepared new fuchsin was used as substrate to achieve staining. Negative controls were performed using an IgG isotype control. Staining was performed synchronously for all conditions per donor. The percentage of positive cells was determined by two independent observers by scoring three times 100 cells/well. All separate values from both observers were used for statistical analysis.

Statistical analysis

Experiments were performed in triplicate samples for all three PRPr donors on two separate chondrocyte donors each (six chondrocyte donors in total). NFkB immunohistochemistry was performed for all three PRPr donors on single chondrocyte donors. Statistical analysis was performed using a mixed model ANOVA, in which treatment was considered a fixed factor and donor a random factor. The effects of IL-1 beta and PRPr were calculated in separate models. A p-value < 0.05 was considered statistically significant.

Table 1: Analyses of blood components, PRP and PRP releasates from three donors.

Blood component analyses					PRP analyses		PRPr analyses			
		Concentration (x10^6/ml)					Concentration (pg/ml)			
Blood components	Donor	PLT	WBC	RBC	Donor	N-Fold change PLT concentration	VEGF	PDGF-AA	PDGF-AB/BB	TGF-ß1
Whole blood	1	89	5.86	4260	1	7.75	290	73000	86000	226000
	2	190	5.45	4160						
	3	96	5.35	4970						
Platelet-rich plasma (PRP)	1	690	38.86	910	2	6.66	1380	103000	86000	391000
	2	1266	39.48	440						
	3	580	31.38	480						
Patelet-rich plasma releasate (PRPr)	1	13	5.41	290	3	6.04	< 5	117000	141000	386000
	2	76	6.93	140						
	3	80	2.56	130						

PLT: platelet; WBC: white blood cell; RBC: red blood cell; VEGF: vascular endothelial growth factor; PDGF: platelet derived growth factor; TGF-ß1: transforming growth factor-ß1. Two dilutions of each sample were measured, measurements that were best in the range of the standard are shown.

Results

Baseline platelet concentration and growth factor analyses

Whole blood samples contained physiological values of platelets, white blood cells and red blood cells for all three donors. PRP contained on average 6.04 – 7.75 times more platelets than whole blood (Table 1). White blood cells increased in a comparable manner (5.87 – 7.24 fold increase), while red blood cell counts decreased. After activating the PRP by clotting, platelet numbers were decreased to 0.15 – 0.83 times the baseline value of whole blood levels. Next to this, the clotting procedure decreased white blood cells to 0.48 – 1.27 times baseline and red blood cell count to 0.03 – 0.07 times baseline whole blood level. The growth factors PDGF-AA, PDGF-AB/BB, TGF-beta 1 were abundantly present in PRPr from all three donors (values listed in Table 1). VEGF was found in a lower amount on average and was below detection level in one donor.

PRPr diminishes IL-1 beta effects on chondrocyte gene expression

The addition of the inflammatory cytokine IL-1 beta diminished expression of COL2A1 and ACAN in chondrocytes, while it increased expression of ADAMTS4, MMP13 and PTGS2 (Fig. 1, $P<0.03$ for all genes). Upon addition of 10% PRPr, COL2A1gene expression increased compared to IL-1 beta only treated samples ($P=0.003$) as well as ACAN ($P=0.001$), while ADAMTS4 expression was reduced ($P=0.001$) as was PTGS2 ($P=0.004$). The addition of 1% PRPr to our cultures revealed a trend towards normalizing COL2A1 and ADAMTS4 expression, although these results did not reach statistical significance. MMP13 expression was not changed by PRPr, whereas ADAMTS5 was not influenced by the presence of IL-1 beta or additional PRPr.

Without the addition of IL-1 beta to our cultures, PRPr did not significantly affect COL2A1 gene expression compared to the control containing no PRPr, while ACAN showed a significant downregulation after the addition of 1% and 10% PRPr ($P \leq 0.003$ for both). ADAMTS4 and MMP13 were significantly upregulated by 10% PRPr ($P \leq 0.002$ for both), whereas ADAMTS5 was downregulated by 10% PRPr ($P = 0.002$). PTGS2 was not influenced by PRPr in the absence of IL-1 beta.

PRPr effects on GAG and NO production

Neither IL-1 beta nor simultaneous addition of PRPr showed an effect on amount of GAG per cell in our experiments (Fig. 2). Next to this, no effects were seen on GAG release in the medium, DNA content per bead or total GAG per bead (data not shown). Moreover, PRPr did not alter any of these parameters in the absence of IL-1 beta (data not shown). We also determined the effect of PRPr on nitric oxide (NO) production in IL-1 beta treated chondrocyte cultures. PRPr was unable to counteract the IL-1 beta induced production of this inflammatory mediator (Fig. 3). In the absence of IL-1 beta, PRPr alone did not induce production of NO by osteoarthritic chondrocytes (data not shown).

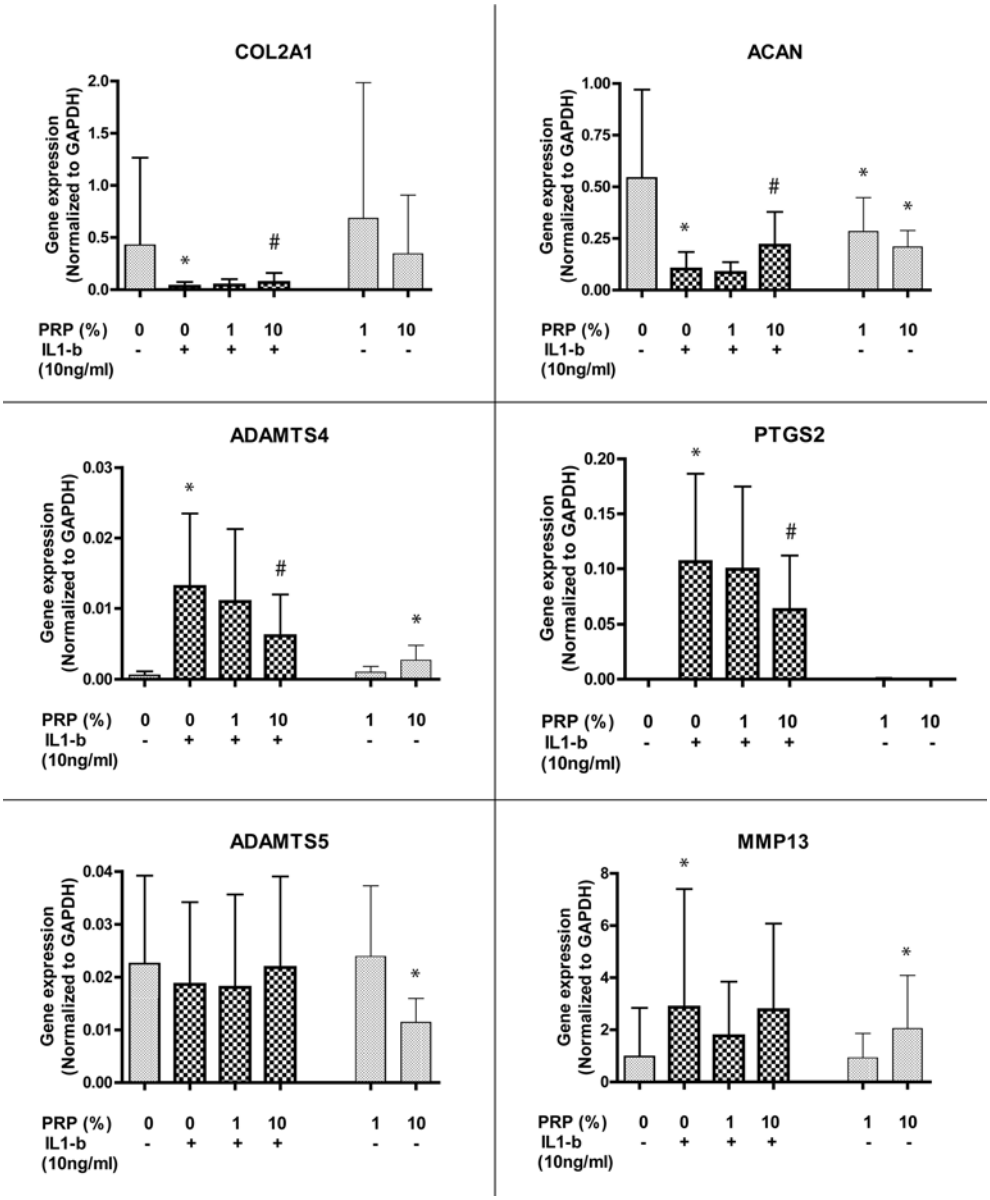

Figure 1. Effects of PRPr on OA chondrocyte gene expression.

PRPr reduced IL-1 beta effects on COL2A1, ACAN, ADAMTS4 and PTGS2 gene expression. No PRPr effects were seen on ADAMTS5 or MMP13 in the presence of IL-1 beta. Without the addition of IL-1 beta, PRPr downregulated ACAN gene expression and ADAMTS5, while ADAMTS4 and MMP13 were upregulated. PRPr did not alter COL2A1 or PTGS2 gene expression in the absence of IL-1 beta. Gene expression was normalized to GAPDH. Data are presented as means ± standard deviations for 6 experiments. * indicates $P<0.05$ compared to control without IL-1 beta, # indicates $P<0.05$ compared to control with IL-1 beta.

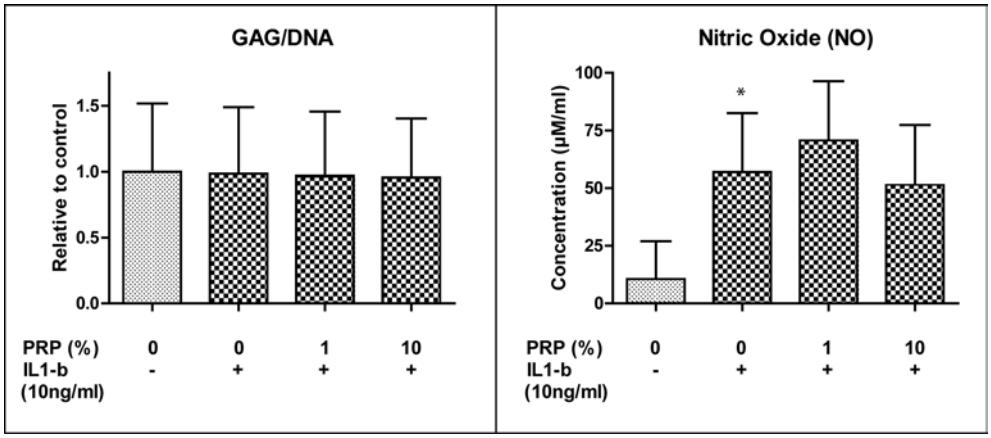

Figure 2: Influence PRPr on GAG/DNA and NO in the presence of IL1-beta.
No effects on GAG per cell were found by IL-1 beta or additional PRPr. IL-1 beta increased NO release by chondrocytes, additional PRPr did not alter this. Data are shown relative to control as means ± standard deviations for 6 experiments. * indicates P<0.05 compared to control without IL-1 beta.

PRPr reduces IL-1 beta induced NFkB activation

In order to determine a possible mechanism through which PRPr exerts its effects, we studied the effect of PRPr on NFkB activation using immunohistochemistry. This revealed NFkB activation in our IL-1 beta treated chondrocytes, which was reduced by PRPr (Fig 3A-C). Manually scoring these cells showed IL-1 beta to cause a marked increase in NFkB activated chondrocytes (Fig 3D, P=0.001). This activation was down-regulated by PRPr (P<0.001 for both doses) in a dose dependent manner. The highest concentration of PRPr (10%) reduced the amount of NFkB activated chondrocytes back to control levels.

Discussion

In this study we tested our hypothesis that PRPr has anti-inflammatory properties and influences gene expression of extracellular matrix forming and degrading proteins in an OA mimicking environment. The osteoarthritic and inflammatory environment was created through addition of IL-1 beta, one of the key players in OA pathogenesis[82,83,84,95], to chondrocytes in culture. Exposure of chondrocytes to the inflammatory cytokine IL-1 beta resulted in marked changes in the expression of genes involved in matrix formation and degradation as well as inflammation, many of which were reduced by the addition of PRPr. PRPr ameliorated the IL-1 beta induced changes on chondrocyte gene expression of COL2A1, ACAN, ADAMTS4 and PTGS2. The inhibition of NFkB activation was found to be a possible mechanism through which PRPr exerted these effects.

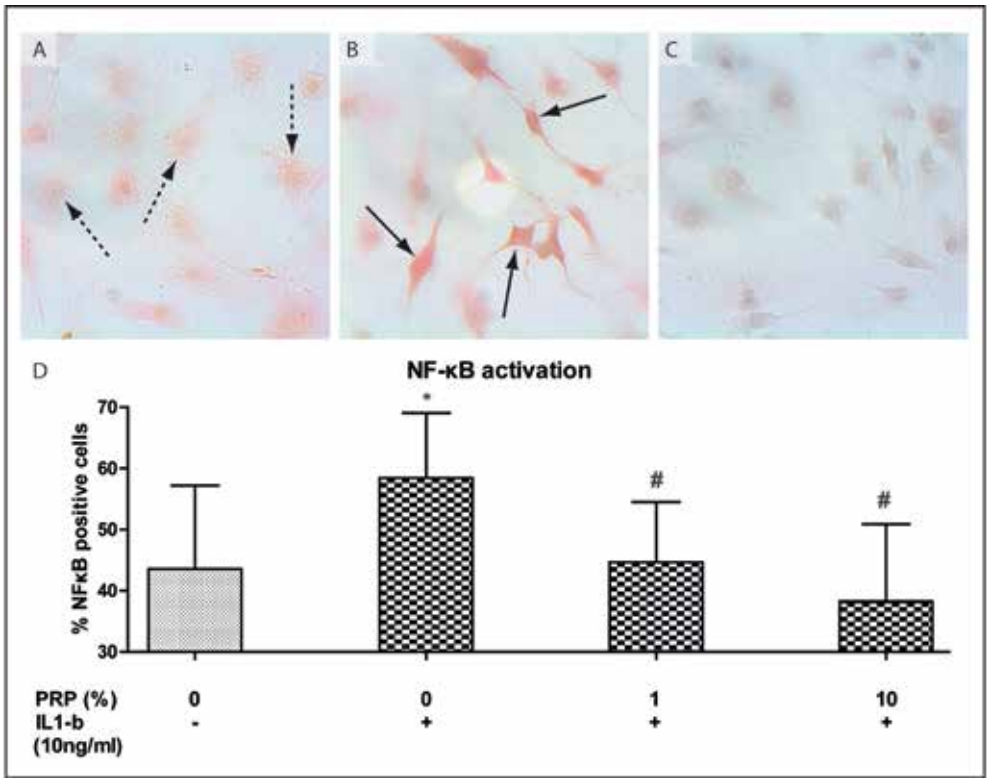

Figure 3: Impact of PRPr on NFkB activation in the presence of IL1-beta.
NFkB immunohistochemistry showing IL-1 beta treated chondrocytes in the absence of PRPr (A) and the presence of 10% PRPr (B). (C) represents IgG control. Dotted arrows in (A) resemble negative cells, whereas solid arrows in (B) indicate NFkB activated cells. IL-1 beta induced NFkB activation, which was dose-dependently reduced by PRPr (D). Data are presented as means ± standard deviations for 3 experiments. Magnification (A-C): 200 x. * indicates $P<0.05$ compared to control without IL-1 beta, # indicates $P<0.05$ compared to control with IL-1 beta.

Various research groups have studied the use of PRP mainly as a culture supplement for chondrocytes. A consistent finding in their reports is that PRP increases cell proliferation[37,38,79,80,81]. In contrast, some describe an increased gene expression or synthesis of COL2A1 and ACAN upon PRP addition[37,38], while others mention PRP to decrease these parameters[79,80,81]. We consider these contradicting reports and our own results using PRP in the absence of IL-1 beta to hinder a clear statement regarding the use of PRP as a culture supplement for chondrocytes. A major difference between these previous reports and the focus of our current study is the fact that we did not evaluate PRPr as a culture additive, but aimed at establishing the potential of PRP as a possible treatment for OA. For this application, PRP should preferably restore the anabolic-catabolic imbalance found in OA joints. By applying PRPr to OA chondrocytes in a standardized IL-1 beta mediated inflammatory and catabolic environment, our results indicated consistent anti-inflammatory effects of PRP.

PRP inhibited the translocation of NFkB to the nucleus. NFkB is present as an inactive, inhibitor-bound complex in almost all mammalian cells and gets translocated into the nucleus upon activation [96]. NFkB regulates more than 150 genes, including those involved in inflammation and other immune responses[97]. In chondrocytes, NFkB has been reported to inhibit COL2A1 gene expression and to regulate MMP-1, MMP-3, MMP13, IL-8 and MCP-1 expression[96]. Several pharmacologic agents act as NFkB inhibitors, including non-steroidal anti-inflammatory drugs, glucocorticoids and proteasome inhibitors[98,99,100]. NFkB inhibition by PRP has been recently reported in a human immortalized chondrocyte cell line[101]. We were able to confirm the latter result using primary human osteoarthritic chondrocytes, thereby providing a link towards clinical application.

NO production is increased in OA joints, and has been described to inhibit collagen and GAG synthesis, while inducing chondrocyte apoptosis and the production of metalloproteinases[102,103,104,105,106]. Vuolteenaho et al. previously reported that both TGF-beta and an NFkB inhibitor decreased IL-1 beta induced NO production by immortalized murine chondrocytes[107]. IL-1 beta induced an evident increase in NFkB activation and NO production in our experiments, but PRPr only counteracted the first effect. TGF-beta was abundantly present in our PRPr, and the amount of TGF-beta in the condition where we used 10% PRPr exceeded those used by Vuolteenaho et al. The different origin of our cells or the heterogeneity and multitude of growth factors present in PRP, affecting many regulatory pathways, could be possible explanations for the fact that we did not observe an inhibitory effect of PRPr on NO production. Although PRP could be a promising autologous treatment option with many applications, this heterogeneity and multitude of growth factors in PRP substantially limits the ability to understand and investigate the effects of PRP. This could also explain the different PRP mediated effects we observed in an environment with versus without IL-1 beta.

A limitation of our study is the use of a short term *in vitro* model to study PRP as a possible OA treatment. In our model we did not detect catabolic effects of IL-1 beta on GAG content at a protein level, although we observed clear downregulation of ACAN gene expression and upregulation of the aggrecanase gene ADAMTS4 indicating IL-1 beta did evoke inflammatory effects. Previous studies from our group did reveal an IL-1 beta effect on GAG content of constructs of immature bovine chondrocytes in alginate[108]. These bovine chondrocytes had a substantial higher GAG turnover than the human cells used in this present study. This difference could be due to the fact that we used human osteoarthritic chondrocytes from relatively aged donors. The lower overall GAG turnover of human chondrocytes in our culture model could be masking IL-1 beta and PRP effects after 48 hours. Furthermore since PRP derived growth factors may not all have their optimal effect in an *in vitro* model, further *in vivo* studies are needed to show long term beneficial PRP effects on cartilage or joints as a whole.

The use of PRP as a highly concentrated autologous source of growth factors has gained wide acceptance in various applications, such as orthopaedic surgery, otolaryngology and plastic surgery[109]. Due to the biological nature of these PRP products, inter-donor variability in the amount of platelets and growth factors is known to occur [33,71]. This was also seen in our PRP preparations, especially in the variation of VEGF concentration. Anitua et al. reported this finding before and mentioned a correlation between VEGF and platelet concentration[110], which is in line with our findings. Despite these variations, we found consistent anti-inflammatory effects of PRPr from three donors on OA chondrocytes from six donors. Next to this donor variation, multiple platelet- or growth factor concentration methods are available[111,112]. These different methods isolate diverse blood fractions with different platelet yields and with or without red and white blood cells[111,113]. All these dissimilarities complicate the comparison of results between different research groups. Therefore, caution has to be taken into account regarding safety and efficacy when considering the translation of PRP as an OA treatment into clinical studies.

We obtained PRP with an approximately 6.8 fold platelet increase above baseline. The amount of growth factors found in PRPr was comparable to those reported by others[114,115]. PRPr inhibited multiple IL-1 beta induced effects in OA chondrocytes. It beneficially affected genes involved in the formation and degradation of extracellular matrix, as well as genes related to inflammation. Additionally, PRPr diminished IL-1 beta induced NFkB activation, a mechanism known to be involved in many pathologic OA processes[96,116,117]. Revealing the relevant mechanisms and processes could improve the application of PRP in a clinical setting. Although additional studies are needed before proceeding to clinical translation, we consider these encouraging results for the further study of PRP as a conservative, autologous and low-cost treatment for OA.

Acknowledgment
We acknowledge Stefan Clockaerts for his help in scoring NFkB immunohistochemistry.

Chapter 3

Musculoskeletal stem cells

Gerben M. van Buul and Gerjo J.V.M. van Osch

Regenerative Medicine: From Protocol to Patient. 1st edition, 2011

Abstract

Probably the most striking example of musculoskeletal regeneration is the growing of an entire limb by a salamander, after it is traumatically amputated. This classic example unfortunately doesn't apply to humans, but (local) stem cells are indispensable in providing a renewable cell source for physiological tissue homeostasis and regeneration after musculoskeletal tissue injury. Stem cells have been isolated from the following musculoskeletal tissues: bone marrow, adipose tissue, periosteum, perichondrium, tendons, ligaments, muscle, cartilage, bone and synovial membrane or -fluid. We chose to refer to these cells as mesenchymal progenitor cells (MPCs). Cells from these different tissues are generally isolated by mincing the tissue followed by enzymatic digestion. Overall, the cells are positive for CD44, CD90, CD105, (CD146), CD166 and STRO-1 and negative for CD31, CD34, CD45 and CD117. On the whole, cell yields from these tissues and proliferation capacities of these cells appear to be within the same order of magnitude. Cells derived from the various musculoskeletal tissues have all been shown to have a multi-lineage differentiation potential, although they do show differentiation preferences, in general for differentiating towards the tissue they were originally derived from. Regenerative capacities of local stem cells are based on two characteristics. In the first place, they have the ability to differentiate into mature tissue cells, thereby contributing to new tissue formation. As a second quality, local stem cells secrete trophic factors that may be responsible for another mechanism of stem cell-mediated tissue repair. These trophic factors are capable of attracting (more) stem cells to the damaged area and they can play an immunomodulatory role. Musculoskeletal stem cells posses a huge capacity for application in regenerative medicine.

Introduction

Regeneration of tissues happens on a daily basis throughout our lives. For many years scientist have been trying to elucidate this process, which is delicately regulated by molecular and cellular events. The growing of an entire limb by a salamander after its traumatic amputation is one of the most remarkable examples of musculoskeletal regeneration. This process is called epimorphosis, and is characterised by cellular dedifferentiation and proliferation at the wound site [5]; Local mesenchymal cells lose their phenotype, start proliferating as blastemal cells followed by redifferentiation in order to form the tissues required for the newly formed limb [5]. This example, which is very appealing to one's imagination, regrettably doesn't relate to humans. Still, (local) stem cells are indispensable in providing a renewable cell source for physiological tissue homeostasis and regeneration after tissue injury [40]. In the 1960s, the work of Alexander Friedenstein demonstrated that mesenchymal stem cells (MSCs) are locally present in the bone marrow of adults [41]. More recently, it became clear that most specialized tissues in the body contain a local pool of stem- or progenitor cells. Local stem- or progenitor cells can be derived from all musculoskeletal tissues and show quite some resemblances to bone marrow derived MSCs (BMSCs) [118,119,120]. A multitude of nomenclature to denote these cells is being used in literature. For this chapter we will refer to them as local Mesenchymal Progenitor Cells (MPCs) that are present in the tissues of the musculoskeletal system.

Different properties and functions can be appointed to MPCs. To review them we will subsequently describe the following aspects:

- Derivation: overview of MPCs derived from various musculoskeletal tissues.
- Characteristics and properties: markers, yield and proliferation, ageing and senescence.
- Regenerative capacity: differentiation of MPCs and the secretion of trophic factors that can influence tissue regeneration.
- Potential applications: in vivo animal and clinical results regarding tissue regeneration and other applications.

Derivation

The musculoskeletal system consists of many different tissues. MPCs have been isolated from the following mesenchymal tissues: bone marrow [42], adipose tissue [121], muscle [122], cartilage [123], synovial membrane or -fluid [46,124], periosteum/perichondrium [125,126], tendons/ligaments [127,128] and bone [129] (Figure 17.1). Cells derived from these various tissues have different specific characteristics and capacities, but they also display many similarities. The main common feature of these various cell populations is that they all have the potential to differentiate into multiple mesodermal lineages. A brief description of the various tissue sources for MPCs is given below.

Bone marrow

BMSCs are the most extensively studied musculoskeletal MPCs. BMSCs are non-hematopoietic cells that reside in the bone marrow. They were first described by Friedenstein et al., as clonal, plastic adherent cells, functioning as a source of the osteoblastic, adipogenic and chondrogenic cell lineages [130].BMSCs play an important role in the bone marrow's microenvironment [131]. The main function of MSCs in the bone marrow is to create a tissue framework, serving as mechanical support for the hematopoietic cell system. For further specifics on BMSCs we refer to the previous chapter where BMSCs are extensively discussed.

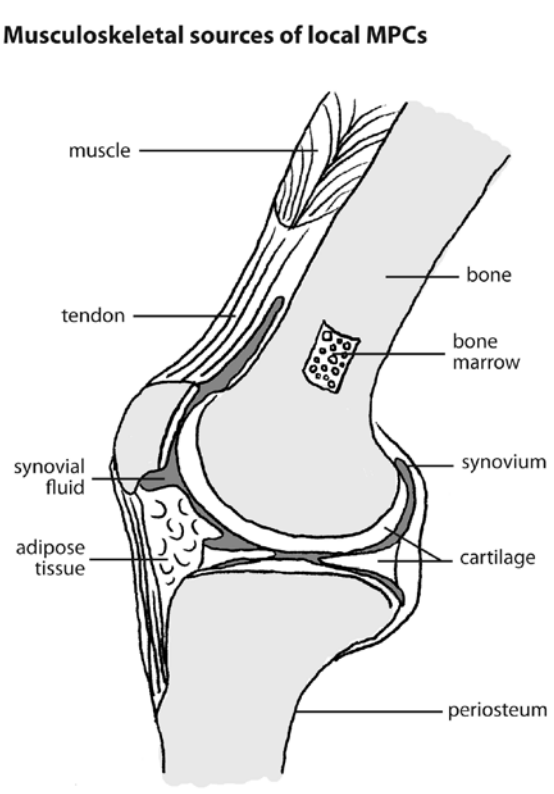

Figure 1: Local mesenchymal progenitor cells (MPCs) can be derived from all these musculoskeltal tissues. Ligaments and perichondrium are not shown in this image, but contain MPCs as well.

Adipose tissue

Adipose tissue is derived from the mesenchyme and contains a supportive stroma. Zuk et al. were the first to report the isolation of multi-potent stem cells from this stromal fraction of adipose tissue [132]. We would like to mention specifically the possibility to harvest local fat from fatpads in the joint. These stuctures have been demonstrated to contain MPCs [133,134]. A clear advantage of this cell population

is their ready accessibility and the excellent availability of large quantities of tissue that can be harvested. Also for further specifics of this cell population we refer to the previous chapter.

Muscle

Roughly two MPC groups can be found in adult muscle tissue: satellite cells (which are considered uni-potent stem cells) and multi-potent muscle-derived stem cells. Satellite cells were first described in 1963. Satellite cells comprise a heterogenous cell population that resides under the basal lamina which surrounds muscle fibres [135]. Within this heterogenous cell population a Pax7 positive sub-population can be identified which is called the "typical" satellite cell [136] Adult muscle satellite cells can give rise to transient amplifying cells (progenitors) and myoblasts. These myoblasts fuse with myofibres and play a principal role in postnatal skeletal muscle growth and regeneration [137]. The multi-potent muscle-derived stem cells (also referred to as skeletal muscle side population) have been discovered more recently. These cells comprise a more homogenous cell population that is less abundant in adult muscle [138]. These cells play a role during muscle homeostasis and regeneration. While they do posses multi-lineage differentiation potential, these skeletal muscle side population cells display a distinct preference for myogenic differentiation.

Cartilage

Articular cartilage is an avascular, aneural tissue of a stiff but compressible nature. The density of cells present in cartilage is very low, and cell mobility is limited through the surrounding matrix. The lack of vascularisation and innervation together with a low cell density and the relative immobility of the cells in cartilage, are the reasons why cartilage has a very low capacity for self-repair [123]. In general, cartilage defects caused by trauma or mechanical wear, tend to further degenerate instead of regenerate. Various studies have shown that the superficial zone of articular cartilage regulates cartilage development and growth [139,140]. The first report describing isolation of a population of stem/progenitor cells from the superficial zone dates from 2004 [141]. Recently, Koelling et al. reported on the isolation of a population of progenitor cells from repair tissue of late stage human osteoarthritic cartilage which could not be isolated from healthy cartilage [142]. These cells possessed characteristics of clonogenicity, multipotency and migratory activity. Although most studies regarding cartilage derived progenitor cells comprise articular cartilage, other cartilage types such as meniscus [118] and intervertebral discs [143,144] contain progenitor cells as well.

Synovium and synovial fluid

Cells present in the synovium produce synovial fluid that functions as a joint lubricant. The synovium is a thin (2 to 3 cell layers thick) membrane lining the non-articular joint surfaces, thereby providing a synovial fluid-filled cavity around the cartilage [124]. De Bari *et al.* were the first to report successful isolation of stem cells from synovial membrane

in 2001 [145]. Various names have been used for describing stem- or progenitor cells derived from the synovium, including synovium-derived stem cells, synovium-derived MSCs, and synovial progenitor cells. Interestingly, synovial MPCs can be generated from healthy synovium but also from rheumatoid- and osteoarthritis patients [146,147].

The number of MPC/stem cells in synovial fluid increases after trauma and these cells have been hypothesized to be important in repair of intra-articular structures after injury. For instance, the number of colony forming cells in synovial fluid was reported to be a 100-fold higher in knees several weeks after injury of the anterior cruciate ligament than in knees from healthy volunteers [46]. These cells are likely to originate from the synovial membrane, since there is a positive correlation between intra-articular synovial fragments and the number of MPCs in synovial fluid [148]. Moreover, MPCs locally present in the synovium were found to proliferate in response to damage in an animal OA model [45]. The injured tissues can attract MSC from this source to the synovial fluid by the secretion of cytokines and chemokines such as stromal cell-derived facor-1 (SDF-1), CXCR4 or VEGF [149].

Periosteum and perichondrium

These cells are amongst the first musculoskeletal tissues that have been used to regenerate bone and cartilage. The first report describing the osteogenic function of periosteum dates from 1742 [150] and Fell et al. were the first to describe culturing and isolation of periosteum cells in 1932 [151]. Periosteum as well as perichondrium contains a so-called cambium layer, which is attached to the bone and cartilage respectively. This cambium layer contains cells, capable of proliferating in order to form new tissue. The periosteum and perichondrium plays a role in cartilage or bone remodelling during the skeletal growth period and during repair after wounding.

Tendon and ligament

In their biological function, tendons and ligaments mainly transmit tensile forces. Per definition, tendons form the link between a muscle belly and a bone, while ligaments attach bone to bone [152]. Ligaments are composed of the same basic components as tendons, although differences do exist to provide the specific mechanical properties needed for their tasks. For instance, the collagen fibrils in ligaments are not uniformly parallel oriented, in order to allow for multi-axial loading patterns [152]. The primary unit of a tendon or a ligament is the collagen fiber. This extracellular matrix is produced by the tissue cells that lie between the collagen fibers [153]. An intriguing feature of tendon is its possible plasticity. Conversion of tendon into cartilage has been observed as a consequence of (non)surgical trauma [154,155]. Various tendons and ligaments, including cruciate ligaments, periodontal ligaments and patellar- and hamstring tendons, have been demonstrated to contain stem/progenitor cells. [127,156,157].

Bone

Bone and bone marrow are physically virtually co-localized. Local MPCs have been demonstrated to grow out of trabecular bone fragments, either with or without enzymatically digesting the fragments [158,159]. Since the close anatomical relationship between these cells and BMSCs, a definitive statement about whether these cells arise from a different source is difficult to make. Furthermore, MPCs derived from trabecular bone become virtually identical to bone marrow derived MSCs upon subcultivation for approximately two passages [129]. Therefore, these cells will not be discussed separately here.

Characteristics and Properties

Cell markers

Today it is unavoidable to characterize the obtained cells by fluorescence activated cell sorting (FACS) analyses. This technique is introduced from the field of haematology where research on stem and progenitor cells is further advanced. In contrast to the haematology, unique markers for mesenchymal and progenitor cells are not yet available [160]. Therefore a large set of different markers is used. Virtually all described MPC populations used in research are heterogeneous groups of cells, attributing to different characterisations by different investigators. According to the International Society for Cellular Therapy (58), a multipotent stromal cell is defined by the following criteria (a) its property of adherence to plastic; (b) its phenotype: negative for: CD14 or CD11b, CD19 or CD79a, CD34, CD45, HLA-DR, and positive for CD73, CD90, CD105; and (c) its capacity to be differentiated into three lineages, chondrocyte, osteoblast, and adipocyte. Criteria (a) and (c) are met by MPCs derived from all tissues discussed in this chapter. These topics will be further dealt with later in this section. Regarding cell phenotype, our literature review showed that MPCs derived from the previously mentioned tissues are negative for CD31, CD34, CD45 and CD117. They are reported to be positive for CD44, CD90, CD105, CD146, CD166 and STRO-1 [119,122,124,128,137,148,156,157,161,162,163,164,165,166,167,168,169]. Although this combination of positive and negative markers is quite comparable for markers generally accepted for bone marrow- and adipose tissue derived MSCs, differences between the two latter cell populations and MPCs derived from other mesenchymal tissues have been reported [118,119,120,163,170]. Most frequently reported difference between these cells is differentiation capacity, which will be discussed later in this chapter. Some groups have described a so-called “side population (SP)” of progenitor cells. These cells, originally described as hematopoietic stem cells, have a unique FACS profile after staining with Hoechst 33342 dye. They can not only be obtained from bone marrow but from other tissues as well, including synovium and muscle [171,172]. Transcriptional profiles for SP and the more differentiated non-SP cells appear to be different. Amongst the genes

upregulated in SP cells are genes that implicate the quiescent status of the cells, maintenance pluripotency and the capacity to undergo asymmetric division [173]. Some groups claim that these cells have superior properties in comparison to the remaining "main population" of cells, but this topic is too specific to include in this chapter.

Cell yield: isolation and proliferation

The common protocol for isolation of MPCs, irrespective of tissue source, is mincing the tissue followed by enzymatic digestion. The enzyme collagenase is mostly used for this purpose, sometimes in combination with other enzymes like dispase. The duration of digestion and enzyme dose is variable, but in general lower collagen content of tissues results in a shorter isolation protocol. After tissue digestion, MPCs are in general selected upon plastic adherence, which is in accordance to BMSC isolation [174]. This doesn't hold true for muscle satellite cells or cartilage derived MPCs. These cells are selected using vitrogen gel or fibronectin adherence respectively [141,175]. However, it is to be expected that these generally applied MPC isolation protocols are merely empirically based and not necessarily optimized. Changing concentration, duration and composition of enzymes might further increase cell yields as well as their viability.

Occasionally, cells are isolated by cutting the tissue in small pieces for cells to grow out. This is often done if the amount of tissue is too limited to obtain sufficient cell number after enzymatic digestion. Results of these studies demonstrate that for bone, perichondrium and tendon it is indeed possible to obtain cells with multilineage capacity this way [157,158,176].

For isolation of MPC from bone marrow aspirates a different protocol is followed. These cells are not isolated enzymatically but solely selected by adherence to tissue culture plastic after plating the biopsy. Sometimes, this is preceded by lyses of the red blood cells and/or a density gradient centrifugation to select the mononuclear fraction.

Cell yields from different tissues vary per isolation source and also depend on the original cellularity of the tissue. Sakaguchi et al. found comparable colony forming units (CFU) per 10^3 nucleated cells for adipose, synovium-, periosteum- and muscle derived MPCs [119]. Bone marrow showed less CFU per 10^3 nucleated cells, but this was compensated by a higher number of nucleated cells per tissue volume. Others report roughly comparable results with a tendency towards higher colony forming units per nucleated cells obtained from synovium compared to periosteum, muscle and cruciate ligaments [118,120]. No unambiguous differences regarding the proliferation of the isolated cell populations could be observed up to passage eight, although synovial derived cells showed a trend towards higher proliferation [119,120]. Overall, cell yields from these tissues and proliferation capacities of the isolated cells appear to be within the same order of magnitude. Therefore, the ease of harvesting sufficient amounts of tissue might become of more importance for selection of the most suitable cell source in future clinical practice.

Ageing and senescence

To evaluate the possibilities to prevent or treat musculoskeletal disorders using autologous cell-based therapies, it is important to know how the number and function of MPC in musculoskeletal tissues are affected by age and/or disease. In the ageing organism, regenerative capacities of tissues tend to decrease [177,178]. Possible explanations for this declining regenerative capacity could be an age-related change in numbers or features of MPCs (cell intrinsic factors) or modifications in the surrounding environment (cell extrinsic factors) [122]. These alterations have not been fully elucidated yet, and literature about this subject is sometimes contradictory.

In bone marrow for instance, a reduction of colony-forming efficiency was found with increasing age using donors ranging from infants to the age of 60 years [179]. Scharstuhl et al. on the other hand, found no correlation between age and the number of mononuclear cells in bone marrow, BMSC yield, cell size, proliferative capacity or cellular spectrum of the harvested cells from adult human donors [180]. Similarly, for periosteum derived MPCs opposing results have been published regarding the influence of age. Both donor age dependent [181] and independent [182] effects on osteochondrogenic potential of periosteum MPCs have been reported. Periosteal cells are reported to maintain osteochondrogenic potential up to ten population doublings. This potential eventually diminishes upon further passaging [183].

Muscle tissue is the last tissue for which inconsistencies are described regarding age-related findings. The number of progenitor cells in muscles has been reported to decrease, remain constant or increase with rising age [184]. Other cell intrinsic factors like telomere shortening and increased tendency to undergo apoptosis play a role in declining muscle regeneration upon ageing too [184]. Cell extrinsic factors that determine appropriate activation and efficient proliferation before terminal differentiation, are also proposed to play a key role in regenerating capacities of ageing muscle tissue. This is explained here with some examples. In aged muscle, progenitor cells prematurely shift from a proliferation phase to a differentiated state due to alterations of Wnt and Notch signaling [185,186]. This leads to less regeneration capability in aging organisms. Interestingly, this trend appears to be reversible. When aged satellite cells are exposed to a young systemic environment, Notch activation is re-established again [187]. Another known feature of satellite cells is the tendency to convert to fibroblasts *in vitro* upon increasing age. Exposure of aged cells to serum derived from young animals reduces this tendency, further emphasizing the role of extrinsic factors in (aged) tissue regeneration [187].

Cartilage derived chondroprogenitor cells are described to have a relative high telomerase activity [188]. Telomerase, first described by Greider et al., prevents telomere shortening during cell division [189]. By doing so, this enzyme postpones cell senescence and increases the maximum amount of cell doublings a cell can undergo. Cartilage

derived progenitors undergo a high number of initial population doublings before a plateau is reached upon approximately 50 population doublings. After approximately 25 population doublings telomerase activity appears to decrease, leading to morphological and functional cell senescence [188].

Synovium MPCs have also been described to have limited senescence and can be expanded *in vitro* to large numbers. Their proliferative capacity doesn't appear to be affected by donor age. However, despite their high proliferative capacity, synovium MPCs have undetectable telomerase activity [145]. Their multipotent capacity is not influenced by donor age, cell passages or cryopreservation [145].

In summary, the fact that regenerative capacities of ageing tissues tend to decrease is not entirely understood. However, it is clear that both cell intrinsic and cell extrinsic properties play a role in this process. In order to evaluate the applicability of cell-based therapies in musculoskeletal disorders, animal and clinical studies are a prerequisite. Results regarding this topic are mentioned in the "Potential applications for therapies" section.

Regenerative capacity

Differentiation

MPCs derived from the various described musculoskeletal tissues have all been shown to have a multilineage differentiation potential [118,119,190]. This means they all have been shown to be able to differentiate into multiple mesodermal lineages including the osteogenic, adipogenic and chondrogenic lineage. The only exception is the "typical" satellite cell, which is generally considered a unipotent myogenic stem cell [122]. Although the latter may be regarded as a myoprogenitor cell, it does have true stem cell properties including self-renewal by asymmetric division [191]. Although cells derived from these previously mentioned tissues have the capacity to differentiate into different lineages, they all show a preference, in general for differentiating towards the tissue they were originally derived from. So, cartilage derived MPCs show a tendency towards chondrogenic differentiation [192] and muscle derived MPCs towards the myogenic lineage [193]. Since no differentiation protocols are available for differentiating cells in vitro towards a tendon, synovial or periosteal lineage for instance, cells derived from these tissues commonly are differentiated into adipocytes, osteocytes and chondrocytes as well to prove multilineage differentiation potential. Periosteal MPCs have great osteogenic potential [125] whereas synovium derived MPCs have a preference for the chondrogenic lineage [194]. Nevertheless, it is very difficult to state that local progenitors from one type of tissue are more suitable to form a certain tissue than other MPCs. Due to the fact that virtually all cells discussed in this chapter comprise heterogeneous

groups of cells, it is hard to tell whether differentiation preferences arise from specific stem cell related features or are simply a consequence of differences in the presence of local (further differentiated) progenitors. Furthermore, differentiation studies *in vitro* are not directly translatable to an *in vivo* situation. MPCs are exposed to specific local (micro)environments during tissue development as well as during the mature stage. When *in vivo* applied, it seems logical that these cells can respond different to tissue specific growth factors or other stimuli. More information about the microenvironments of the various MPCs is necessary, together with knowledge about the heterogeneity of cell populations. An increasing number of clonal studies using single cells to show true multilineage differentiation and/or self-renewal of different MPCs are reported [127,137,195,196]. Direct comparison of clonal cells derived from different musculoskeletal tissues should be a focus of future research in order to form a founded opinion about optimal cell sources.

Besides differentiating into mesodermal lineages, bone marrow stromal derived cells have been shown to be able to give rise to hepatic cells (endodermal lineage) and mature astrocytes and neurons (ectodermal lineage) as well [197,198]. This process, where a stem cell differentiates into cell types from a different germ layer than the one it originally resided in, is termed transdifferentiation. Although this has not been extensively investigated for the MPCs discussed in this chapter, muscle derived MPCs for example have been demonstrated to be able to transdifferentiate into endo- and ectodermal lineages as well [199]. This might suggest that MPCs from other sources might also have transdifferentiation capacity, which has to be shown in future investigations.

Trophic factors

MPCs can contribute to tissue repair by differentiating into a mature tissue cell and forming extracellular matrix to repair damaged tissue. In addition these cells contribute to tissue repair: by the production of trophic factors. These trophic factors are capable of attracting (more) stem cells to the damaged area and they can have an immunomodulatory effect [43]. For bone marrow and adipose derived cells there is a fast increasing amount of information on trophic factors [200,201]. MPCs derived from bone marrow and adipose tissue, but also MPCs from periodontal ligament, have been shown to have a suppressive effect upon peripheral blood mononuclear cell proliferation [166]. This suppressive effect was found both in mixed lymphocyte reactions (allowing cell-cell contact) and in transwell co-cultures (avoiding direct contact). The latter emphasizes the paracrine effects of these cells.

Potential Applications for Therapies

Huge potential resides in the MPCs discussed in this chapter regarding musculoskeletal tissue regeneration, either by differentiating into more mature tissue cells or by their modulatory properties. The potential of MPCs for tissue regeneration or immune modulation is demonstrated by the huge amount of clinical trials that are already performed or are still ongoing, using bone marrow derived MSCs [131,202]. The application of these cells encompasses various fields, including cardiovascular diseases, osteogenesis imperfecta, graft versus host disease and neurological disorders like amyotrophic lateral sclerosis, etc. [160,202].

Clinical studies or applications with musculoskeletal stem cells generated from other tissues than bone marrow have been performed with muscle derived MPCs, periosteum and perichondrium. Muscle MPCs have been used in clinical trials for treating myocardial ischemia [203]. Periosteum has proven it's added value in treating bone defects [204,205] and has been used as a therapy for local cartilage defects for more than two decades [206,207]. Perichondrium has been applied as a graft to treat isolated chondral defects [208,209]. Although MPCs likely play a role in these studies, tissue transplants were used rather than isolated cells. Therefore, we cannot state that the positive results were solely based on the regenerative capacities of transplanted MPCs. Furthermore, although promising results were obtained using periosteum for treating bone defects, golden standard still is the use of autologous bone grafts. Regarding local cartilage defects, subchondral drilling and autologous chondrocyte implantation (see later) have become the main therapies. Animal experiments with MPCs of other musculoskeletal tissues show hopeful results. Muscle MPCs have been reported to contribute to up to 94% of myofibers after intramuscular injection into dystrophic mice [210]. Furthermore, satellite cells have been shown to be able to undergo approximately 14-17 cell doublings after single cell transplantation in vivo [137]. A possible drawback of muscle MPCs is their low migrating capacities requiring local delivery, although homing of these cells into damaged muscle after intravenous injection has been reported [193]. Synovial MPCs in their turn have proven in vivo to contribute to cartilage repair [211], meniscal regeneration [50], muscle repair [212] and to accelerate remodeling of tendon to bone healing in a bone tunnel model [213]. Lastly, tendon- and ligament derived MPCs can play a role in regenerating damaged tendon and ligament respectively [214,215]. Although cartilage has been shown to contain progenitor cells, applications using only these MPCs do not yet exist. In the field of cartilage repair, autologous chondrocyte implantation is a well established, cell-based and clinically applied cartilage repair technique [216]. This technique however uses predominantly differentiated chondrocytes to fill the cartilage defect, thereby not meeting the definition of stem cell therapy. The contribution of cartilage MPC to the outcome of ACI is not known and would be an interesting research question.

In summary, the applicability of MPCs of a certain tissue for an application will depend on ease of harvesting and the ability to control function. At the moment, further evidence has to come from properly organized and controlled animal as well as clinical studies.

The variability of the outcome of different studies where MPCs are used is of great concern. Part of the variation might be due to variation in MPC characteristics between donors. Better characterisation of the cells or selection of cells before use can improve outcome. The first study with cell selection (although this did not involve MPCs), for regenerative medicine of the musculoskeletal system has been performed in cartilage repair [217]. In addition to the variation in MPC characteristics, variability in outcome of clinical studies will also be caused by the differences in the host environment where the cells are introduced. Stage of disease, but also general patient characteristics like BMI, age and gender will determine MPC fate and function [179]. How these factors influence MPCs is largely unknown at the moment. It is important to take this into account when designing clinical studies.

Instead of actually applying or injecting MPCs for musculoskeletal disorders, some therapies aim at stimulating or attracting stem cells to the damaged tissue. Some examples of this approach are shockwave therapy, pulsed electromagnetic fields (PEMF) or the use of cell attracting growth factors. These strategies are being investigated as possible therapies for osteoporosis, bone non-unions, osteochondral defects and cartilage regeneration [218,219,220,221].

Tumour formation

Although formation of tumours after the use of adult MPCs have never been reported, the capacities of MPC are not yet fully investigated and understood. The role of BMSCs in carcinogenesis is a relative new feature. BMSCs have a distinct homing potential to a wide range of organs after systemic administration [222,223,224]. The ability of BMSCs to home to primary tumour sites and metastases has been demonstrated by several studies [225,226,227]. Their role at these tumour sites and their potential effect on tumour development can be bivalent. Both pro- and antiproliferative effects of BMSCs have been reported regarding this aspect (reviewed in [228]). Possible explanations for these contradictory findings might be the immunomodulatory effects of BMSCs, which can be both in favour and to the detriment of tumour development. Another factor might be the excretion of VEGF by BMSCs, which plays a role in (neo)vascularisation.

The effect or role of the MPCs in tumour formation or -growth has not been investigated so far. It is a known fact that cells can undergo karyotypic changes upon (long-term) culturing in vitro [229,230]. Cell based regenerative therapies have to be proceeded cautiously, and these aspects certainly have to be investigated before proceeding to large-scale clinical translation.

Conclusions, Discussion and Future Development in Research

It is clear that local progenitor cells can be isolated from various tissues in the musculoskeletal system and that these cells can play a role in tissue repair. Overall, cell yields from the different tissues, proliferation capacities and cell membrane markers of the isolated cells appear to be similar. Cells derived from the various musculoskeletal tissues have all been shown to have a multilineage differentiation potential. Besides differentiating into a mature tissue cell, secretion of trophic factors is assigned a possible function of stem cell that might be important in tissue repair. Musculoskeletal progenitor cells posses a huge capacity for application in regenerative medicine. Table 1 summarizes various features of MPCs derived from the tissues discussed in this chapter.

Table 1: Overview of various features of MPCs derived from different tissues.

Tissue source	**Regenerative capacity shown**	**Clonal multilineage potential shown**	**Clinical studies performed**	**Availability / accessability***
Bone marrow	Differentiation & trophic factors	Yes	Yes	+ / ±
Adipose tissue	Differentiation & trophic factors	Yes	Yes	+ / +
Muscle	Differentiation	Yes	Yes	+ / +
Cartilage	Differentiation	Yes	Performed with cartilage derived cells, not specific MPCs	- / -
Synovium and synovial fluid	Differentiation	No	No	± / ±
Periosteum and perichodrium	Differentiation	No	Performed with whole tissue, not isolated cells	- / -
Tendon and ligament	Differentiation & trophic factors	Yes	No	± / -**

* these are arbitrary measures. +, ± and - represents a good, medium and limited availability (i.e. amount of obtainable tissue) and accessability (i.e. invasiveness of procedure needed to harvest the tissue) respectively.

** in maxillofacial surgery, the periodontal ligament is readily available and accessible. However, in limbs this cell source is less abundant and accessible.

It is not always straightforward to determine whether the isolated MPCs are really local cells, especially in damaged tissues. Tissue damage has been demonstrated to attract stem cells from the circulation. These cells are recruited from the bone marrow and home in the damaged tissue to support repair [231]. These recruited cells, once isolated from damaged tissue, might therefore be mistaken for local MPCs. Furthermore, the tissues of the musculoskeletal system, with exception of cartilage and tendon, are well vascularised. These small vessels contain pericytes. A pericyte is a relatively undifferentiated cell, which serves to support small vessels. They were first described in the 19th century as cells located between the endothelial cells and the parenchymal cells in capillaries and post capillary venules. Apart from their role in blood vessel formation, pericytes were recognized to contribute to bone formation [232] and they might play an important role in tissue repair and regeneration in many musculoskeletal tissues. Pericytes have multilineage differentiation capacity too, and there could be a chance that these cells are very closely related to the local progenitors described in this chapter. However, only recently they regained a lot of interest as adult progenitor cells with multilineage capacity and methods to isolate and purify these cells are being developed [233]. Especially in well vascularised tissues, such as bone marrow, adipose tissue and muscle, the perivascular cells might be regarded as local MPC. Further research will have to elucidate the role of local stem cells as well as pericytes and systemic stem cells present in the circulation, in intrinsic tissue repair capacity as well as their use in cell therapy.

For cell therapeutic application optimal isolation and culture conditions for each cell type has to be found and tailored for every specific application. Choice for optimal cell type to regenerate a tissue might very likely depend on criteria related to ease of harvesting. In this respect, bone marrow and adipose tissue are attractive candidates for harvesting large amounts of cells in a relatively easy procedure.

The various musculoskeletal stem cells all have multilineage differentiation capacity. Although this offers interesting opportunities, it also emphasizes the importance of learning to control and direct differentiation and tissue formation by these cells to prevent undesired tissue- or tumour formation. Better characterisation of musculoskeletal stem cells and more knowledge about lineage differentiation is required to fully understand the potential of each individual source of cells for each of the different applications. In this respect, not only differentiation into mature tissue cells, but also the secretion of trophic factors deserves more study. Furthermore the fate of the cells after application in-vivo has to be studied with modern imaging techniques both in animal and in human patients [60] to be able to answer the questions: Where do they home?;How long do they stay viable?; What is their activity? Finally there is a need for more understanding of how characteristics of host microenvironment influence fate and function of the cells in order to optimize the results of cell therapy in musculoskeletal disorders.

Acknowledgements

The authors are supported by the Dutch Platform for Tissue Engineering; the Dutch Arthritis Association; the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science; the ENCITE consortium of the European Community under the 7th framework programme; and RE-MEDIC, a Network on Regenerative Medicine of the European Science Foundation. We would like to thank Monique Bernsen for critical reading the text and Ton Everaers for his assistance with creating the figure.

Chapter 4

Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture

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Osteoarthritis and Cartilage. 2012;20(10):1186-96

Abstract

Background: Mesenchymal stem cells (MSCs) are promising candidates for OA therapies, although their mechanism of action remains unclear. MSCs have recently been discovered to secrete anti-inflammatory cytokines and growth factors. We studied the paracrine effects of MSCs on OA cartilage and synovial explants in vitro.

Methods: MSC-conditioned medium was prepared by stimulating primary human MSCs with TNFα and IFNγ (50 ng/ml each). Human synovium and cartilage explants were cultured in MSC-conditioned medium or in control medium, containing the same amount of added TNFα and IFNγ but not incubated with MSCs. Explants were analyzed for gene-expression and the production of nitric oxide (NO). The presence of the inhibitor of nuclear factor kappa B alpha (IκBa) was assessed by Western blot analysis.

Results: Synovial explants exposed to MSC-conditioned medium showed decreased gene expression of interleukin-1 beta (*IL-1*β), matrix metalloproteinase (*MMP*)*1* and *MMP13*, while suppressor of cytokine signalling (*SOCS*)*1* was upregulated. In cartilage, expression of IL-1 receptor antagonist (*IL-1RA*) was upregulated, whereas a disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*)*5* and collagen type II alpha 1 (*COL2A1*) were downregulated. MSC-conditioned medium reduced NO production in cartilage explants and the presence of IκBa was increased in synoviocytes and chondrocytes treated with MSC-conditioned medium.

Conclusions: In an inflammatory environment, MSCs secrete factors which cause multiple anti-inflammatory effects and influence matrix turnover in synovium and cartilage explants. Thereby, the presented data encourage further study of MSCs as a treatment for joint diseases.

Introduction

Osteoarthritis (OA) is characterized by a catabolic and inflammatory joint environment. To this date, no drugs are available to structurally modify OA processes or prevent progression of the disease [234]. The use of mesenchymal stem cells (MSCs) as a treatment option in cartilage regenerative therapies is under extensive investigation [235]. MSCs have chondrogenic potential and are experimentally being implanted in focal cartilage defects, showing promising results [236]. In OA, more generalized cartilage lesions and joint inflammation are present, thereby limiting the usefulness of focal treatments. In order to treat the joint as a whole, MSCs have been injected intra-articularly in pre-clinical and some initial clinical studies as a treatment for OA [54,55,237]. Animal studies have shown beneficial effects of MSCs on cartilage morphology and histology in various OA models [47,54,55]. Interestingly, studies using cell tracking in cartilage repair show only limited cartilage formation by chondrogenic differentiation of the injected MSCs [54,55]. Instead, the applied cells are mostly retrieved from other articular structures, like the synovium. Apparently, the intra-articularly injected MSCs only occasionally differentiate into chondrocytes to actively produce extracellular matrix. This implies a different OA modifying mechanism, like influencing the micro-environment by paracrine actions, stimulating locally present progenitor cells to repair OA damage or by attracting circulating endogenous progenitor cells to enable repair [238]. We studied the influence of MSCs on their local micro-environment by the secretion of bioactive factors. Some of these factors, including interleukin-6 (*IL-6*), IL-10, indoleamine 2,3-dioxygenase (*IDO*), hepatocyte growth factor (*HGF*) and transforming growth factor beta (*TGF*β), have immunomodulatory properties [44,239],whereas others are involved in extracellular matrix turnover such as matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [240]. In addition, trophic effects of MSCs to stimulate chondrocyte proliferation and matrix deposition have been shown [241]. Aim of our study was to explore the protective effects of MSCs on OA related processes in a controlled and standardized environment, by investigating the paracrine effects of MSCs on OA synovium and cartilage explants *in vitro*. These paracrine effects were studied by means of MSC-conditioned medium; medium containing factors secreted by MSCs. Since MSCs increase their immunomodulatory properties in response to an inflammatory stimulus, and inflammation plays a substantial role in OA pathology, we challenged our cells with the inflammatory cytokines TNFα and IFNγ [242,243]. We measured the presence of factors in MSC-conditioned medium involved in inflammation, tissue regeneration and extracellular matrix turnover. Furthermore, we evaluated the effects of MSC-conditioned medium on osteoarthritic cartilage and synovium by analyses of the expression of genes related to inflammation and matrix turn-over, the production of nitric oxide (NO) and prostaglandin E_2 (PGE2) and activity of P38 mitogen-activated protein (MAP) kinase and nuclear factor kappa B (NFκB) pathways. These two pathways are major orchestrators in transducing inflammatory and catabolic signals in joint degeneration [96,244].

Materials & Methods

Cells and tissue preparation

Human MSCs (hMSCs) were isolated and cultured from heparinized femoral-shaft marrow aspirate of nine patients undergoing total hip arthroplasty (after written informed consent; protocol # MEC-2004-142) using previously described procedures [157]. This procedure was previously confirmed to yield MSC on the basis of morphological criteria, expression of CD105 marker and absence of CD34 marker and an adipogenic, osteogenic and chondrogenic differentiation potential [157]. Cells were seeded at a density of 2300 cells/cm^2 and cultured in DMEM containing 1 g/L glucose, 10 % fetal calf serum (FCS), 50 µg/ml gentamicin, 1.5 µg/ml fungizone, 1ng/ml fibroblast growth factor-2 and 0.1 mM l-ascorbic acid 2-phosphate (MSC culture medium). Human synovial explants (approximately 10 mm^3) and cartilage explants (approximately 70 mm^3) were obtained as surgical waste material from seven patients undergoing total knee replacement surgery. All patients implicitly consented to the use of these tissues for scientific research (protocol # MEC-2004-322). Explants were pre-cultured for 48 hours in DMEM containing 1 g/L glucose, 2 % FCS, 50 µg/ml gentamicin and 1.5 µg/ml fungizone. Synoviocytes and chondrocytes were isolated from synovium and cartilage respectively as described previously [245] by treating explants from either tissue with 0.2 % protease (Sigma-Aldrich,Zwijndrecht, Netherlands) and subsequent overnight digestion in DMEM containing 4.5 g/L glucose, 10 % FCS, 50 µg/mL gentamicine and 1.5 µg/mL fungizone, supplemented with 0.15 % collagenase B (Roche Diagnostics, Mannheim, Germany).

Conditioned medium preparation and explant culture

Subconfluent hMSC monolayer cultures (passage two) were used to obtain conditioned medium. MSC conditioned medium from four donors was prepared by incubating hMSCs for 24 hours in MSC culture medium. To stimulate the secretion of immunomodulatory factors by MSCs, TNFα and IFNγ (50 ng/ml each, PeproTech, London, UK) were added to the MSC culture medium for five other MSC donors. After 24 hours the medium was collected and centrifuged for eight minutes at 700 G to remove cellular debris. MSCs and the supernatant of the medium (MSC-conditioned medium) were harvested separately and stored at -80 °C until further use. MSC processing for gene expression analysis is described later in this section. Control (unconditioned) medium was made of plain MSC culture medium for the first four MSC donors, and MSC culture medium supplemented with TNFα/IFNγ (50 ng/ml each) for the five cytokine-stimulated MSC donors. Both control media, with or without TNFα/IFNγ, were incubated without MSCs at 37 °C for 24 hours and stored at -80 °C until further use. TNFαIFNγ. Except for the absence of MSCs during incubation, the control medium was treated identical to MSC-conditioned medium. In experiments using MSC-conditioned media from non-stimulated donors, control medium without TNFα/IFNγ was used. Stimulated MSC-conditioned media were compared to control medium with TNFα/IFNγ.

Synovium and cartilage explants were cultured in MSC-conditioned medium or in control medium for 48 hours. Explants from each synovium or cartilage donor were cultured in triplicate samples per condition in 24-well plates in a total volume of 1.0 ml, consisting of 500 µl MSC-conditioned medium and 500 µl freshly added DMEM containing 50 µg/ml gentamicin and 1.5 µg/ml fungizone. Depending on the amount of explant material that could be obtained from a given donor, explants were cultured in conditioned medium from one to four separate MSC donors. After culturing for 48 hours, explants for gene expression analyses and media were harvested and stored at -80 °C until further use. MSC conditioned medium without TNFα/IFNγ from four MSC donors was used on two synovium and cartilage donors. Conditioned medium from MSCs stimulated with TNFα/IFNγ from five MSC donors was used on five synovium and cartilage donors.

We additionally studied the effect of MSCs in a co-culture system of synovium and cartilage explants [27]. In short, synovium and cartilage explants from the same donor were cultured together, thereby preventing direct contact between cartilage and synovium by using Millicell filter inserts with a pore size of 0.4 µm (Millipore, Amsterdam, the Netherlands). These experiments were performed on synovium and cartilage from one donor in triplicates using pooled MSC-conditioned media from 5 different MSC donors.

Gene expression analysis

The frozen explants were processed using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany). RNA from explants was extracted using RNA-Bee™ (TEL-TEST, Friendswood, USA) according to manufacturer's guidelines and subsequently precipitated with chloroform 20 % (v/v). RNA from MSCs was extracted using RLT (Quiagen, Venlo, the Netherlands) and beta-mercaptoethanol 1 % (v/v). All RNA was further purified using RNeasy Micro Kit (Qiagen, Hilden, Germany) with on-column DNA digestion. Nucleic acid content was determined spectrophotometrically (NanoDrop ND1000; Isogen Life Science, IJsselstein, The Netherlands). Complementary DNA and polymerase chain reactions (PCRs) were performed as described before [89]. RT-PCR primer nucleotide sequences are listed in table 1. Data were normalized to a best keeper index (BKI) of three reference genes *GAPDH*, *UBC*, *HPRT1* [246]. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [91].

Nitric oxide and prostaglandin E2 measurements

Nitric oxide (NO) and prostaglandin E2 (PGE2) secretion by synovium and cartilage was analyzed in the cryopreserved media from the synovium and cartilage explants culture experiments. NO secretion was determined by quantifying its derived product, nitrite, in medium using a spectrophotometric method based upon the Griess reaction [94]. Briefly, 100 µl of culture medium or sodium nitrite (NaNO2) standard dilutions were mixed with 100 µl of Griess reagent (0.5 % sulphanilamide, 0.05 % naphtyl ethylenediamine dihydrochloride, 2.5 % H3PO4). The absorption was measured at 540 nm. PGE2 secretion in the media was determined using the PGE2 Assay (RnD systems, Minneapolis,

MN, USA) according to manufacturer's guidelines. NO measurements were performed on triplicate samples from five synovium and cartilage donors separately. For the PGE2 assay the triplicate samples per condition were pooled before measurement to obtain values from four synovium and four cartilage donors.

Western blotting for p38 and NFκB signaling pathway analyses

The amount of native and phosphorylated P38 MAP kinase and the amount of inhibitor of kappa B alpha (IκBa) was determined by Western blot analysis of total protein extracts from fibroblast-like synoviocytes and chondrocytes exposed to MSC-conditioned medium. Synoviocytes and chondrocytes from two donors each were used. Subconfluent monolayers of synoviocytes and chondrocytes (passage 2-4) were cultured in pooled MSC-conditioned medium (from five MSC donors) or control medium for either 10 minutes (for IκBa determination) or for 3 hours (for p38 MAP kinase signaling). The chosen incubation times were based on an experiment using different durations of IFNγ/TNFα treatment to predefine the optimal time-point to evaluate possible paracrine MSC effects (data not shown). Based on previous reports, IFNγ/TNFα dependent IκBa degradation was determined in a period ranging from five minutes to one hour and p38 MAP kinase phosphorylation in a period of 3 - 24 hours [86,247].

Total protein fractions were isolated using M-PER Protein extraction reagent (#78501, Thermo Scientific) with 1 % protease inhibitor Complete (Roche, Manheim, Germany). Protein concentrations were determined by the BCA protein assay (Pierce Chemical, Rockford, IL). For each sample, 8µg of total protein fraction was separated by 10 % SDS-PAGE gels and transferred on PVDF membranes by Western blotting. Membranes were blocked for 2h in 0.1 % TBS-T containing 5 % dry milk powder, washed three times in 0,1 % TBS-T and incubated with primary antibodies against α-Tubulin, p38 MAP kinase, phosphorylated p38 (all diluted 1:1000; respectively 2148, 9212 and 9211, Cell Signaling Technology, Leiden, the Netherlands) or IκBa (1:500; sc-371, Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4 °C following manufacturer's protocol. An anti-rabbit HPR-linked secondary antibody (1:1000; 7074, Cell Signaling) was added and incubated for 1h at room temperature. The blots were visualized by means of SuperSignal Chemiluminescent system (34077, Thermo Scientific) using manufacturer's instruction. Image analysis and quantification was performed using the National Institute of Health Image J freeware (release 1.44X; http://rsb.info.nih.gov/ij/).

Analyses of mesenchymal stem cells and conditioned media

Stimulated and non-stimulated MSCs from four donors were analysed for gene expression of *IL-6*, *TIMP2*, *HGF*, *TGFβ-1* and *IDO* as described in the gene expression analysis section. TIMP2, HGF, IL-6 and TGFβ-1 protein levels were measured in stimulated and non-stimulated MSC-conditioned media from three donors by means of ELISAs (R&D systems, Abingdon, UK) according to the protocol supplied by the manufacturer. All factors were corrected for the amounts present in standard MSC culture medium. To

determine the amount of IDO enzymatic activity in MSC-conditioned media, the level of its metabolite, kynurenine, was measured spectrophotometrically as described before [248]. In brief, 100 µl of 30 % trichloroacetic acid (Sigma-Aldrich, St. Louis, USA) was added to 200 µl of culture supernatant, which was incubated at 50 °C for 30 min, and then centrifuged at 10,000 G for 5 minutes. 75 µl of supernatant was then added to an equal volume of Ehrlich's reagent (100 mg P-dimethylbenzaldehyde and 5 ml glacial acetic acid; Sigma-Aldrich St. Louis, USA) and optical density was measured at 490 nm.

Data analyses

All data are presented as mean ± standard deviation. Statistical analyses were performed using a mixed model ANOVA (SPSS 17.0.2; SPSS Inc., Chicago, USA), which takes the within donor correlation into account. Treatment using MSC-conditioned media vs control medium was considered a fixed factor and these effect were statistically considered as independent observations. The synovium or cartilage donors were considered a random factor and measurements of the separate samples per donor were regarded correlated observations. Donor was included into the model to adjust for absolute differences in expression levels between explant donors. A log-transformation was applied to all gene expression data before statistical analyses to approach normal data distribution. A p-value < 0.05 was considered statistically significant.

Results

MSCs display immunomodulatory properties in response to inflammatory cytokine exposure.

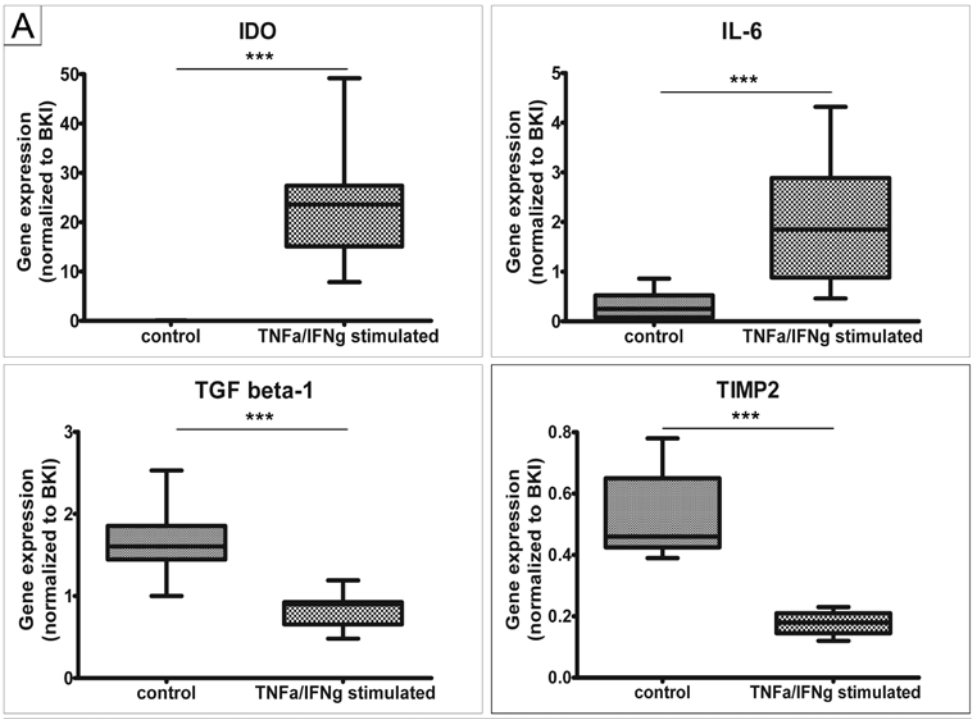
To determine which factors might be involved in the anti-inflammatory effects caused by the MSC-conditioned medium, we analyzed TNFα/IFNγ stimulated and non-stimulated MSCs for gene expression and protein secretion of various immunomodulatory factors and growth factors. Stimulation of MSC's with the inflammatory cytokines upregulated gene expression of *IDO* and *IL-6*, while *TIMP2* and *TGFβ-1* were markedly downregulated (Fig. 1a; $P < 0.001$ for all genes). *HGF*, *IL-1β* and *TIMP1* gene expression was not significantly altered by the priming procedure (data not shown).

The amount of secreted IL-6, HGF, TIMP2, TGFβ-1 and IDO enzymatic activity in conditioned medium from TNFα/IFNγ stimulated and unstimulated MSCs was measured (Fig 1b). IL-6 was significantly elevated in TNFα/IFNγ stimulated MSC-conditioned medium (approximately 15-fold, $P < 0.001$), as was IDO activity (approximately 60-fold, $P < 0.001$) compared with non-stimulated conditioned medium. HGF, TIMP2 and TGFβ-1 were found in equal amounts in both stimulated and non-stimulated conditioned medium.

Table 1. Primer nucleotide sequences of the tested genes

Gene	Primer
IL-6	Fw: TCGAGCCCACCGGGAACGAA Rv: GCAGGGAAGGCAGCAGGCAA
HGF	Fw: GGCTGGGGCTACACTGGATTG Rv: CCACCATAATCCCCCTCACAT
TGFb1	Fw: GTGACAGCAGGGATAACACACTG Rv: CATGAATGGTGGCCAGGTC
IDO	assay-on-demand (Hs00158027.m1, Applied Biosystems, Capelle a/d IJssel, the Netherlands)
IL-1beta	Fw: CCCTAAACAGATGAAGTGCTCCTT Rv: GTAGTCGGATGCCGCCAT
IL-1RA	Fw: AACAGAAAGCAGGACAAGCG Rv: CCTTCGTCAGGCATATTGGT
TNFa	Fw: GCCGCATCGCCGTCTCCTAC Rv: AGCGCTGAGTCGGTCACCCT
SOCS1	Fw: CCCTGGTTGTTGTAGCAGCTT Rv: TTGTGCAAAGATACTGGGTATATGT
SOCS3	Fw: TCGGACCAGCGCCACTT Rv: CACTGGATGCGCAGGTTCT
MMP1	Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT
MMP13	Fw: CTCAATTTCACTTCTGTTTTCTG Rv: CATCTCTGTCGGCAAATTCGT
TIMP1	Fw: TGCCGCATCGCCGAGAT Rv: ATGGTGGGTTCTCTGGTG
TIMP2	Fw: ATGGTGGGTTCTCTGGTG Rv: CGGTACCACGCACAGGA
ADAMTS4	Fw: CAAGGTCCCATGTGCAACGT Rv: CATCTGCCACCACCAGTGTCT
ADAMTS5	Fw: CAAGGTCCCATGTGCAACGT Rv: CATCTGCCACCACCAGTGTCT
COL2A1	Fw: GGCAATAGCAGGTTCACGTACA Rv: CGATAACAGTCTTGCCCCACTT
ACAN	Fw: TCGAGGACAGCGAGGCC Rv: TCGAGGGTGTAGCGTGTAGAGA

IL-6: interleukin-6, HGF: hepatocyte growth factor, TGFb1: transforming growth factor beta-1, IDO: indoleamine 2,3-dioxygenase, IL-1b: IL-1 beta, IL-1RA: IL-1 receptor antagonist, SOCS: suppressor of cytokine signalling, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of MMP, ADAMTS: a dis-integrin and metalloproteinase with thrombospondin motifs, COL2A1: collagen type II alpha 1, ACAN: aggrecan.



B

Factor	24 hour secretions by MSCs (ng/ml)	
	Non TNFa/IFNg stimulated	TNFa/IFNg stimulated
Kynurenine (IDO activity)	0.2 ± 0.4 (x10^3)	12.6 ± 1.0 (x10^3)***
IL-6	2.6 ± 3.0	40.2 ± 15.0***
TIMP2	25.2 ± 6.8	23.5 ± 7.7
TGF beta-1	0.9 ± 0.9	0.9 ± 0.9
HGF	4.6 ± 3.4	4.3 ± 3.5

Figure 1. Influence of TNFα/IFNγ stimulation on MSC gene expression (A) and MSC secretions (B). In order to stimulate the immunomodulatory capacity, MSCs were treated with TNFα and IFNγ for 24 hours. Media from TNFα/IFNγ stimulated and unstimulated MSCs were analyzed for various factors and corrected for control medium containing 10 % FCS. Gene expression data are presented as box-plots for 4 experiments performed in triplicate. Cell secretion data are presented as means ± standard deviations for 3 experiments performed in triplicate; IL-6 = interleukin 6, IDO = indoleamine 2,3-di-oxygenase, TIMP2 = tissue inhibitor of matrix metalloproteinase 2, TGF beta-1 = transforming growth factor beta-1, HGF = hepatocyte growth factor. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

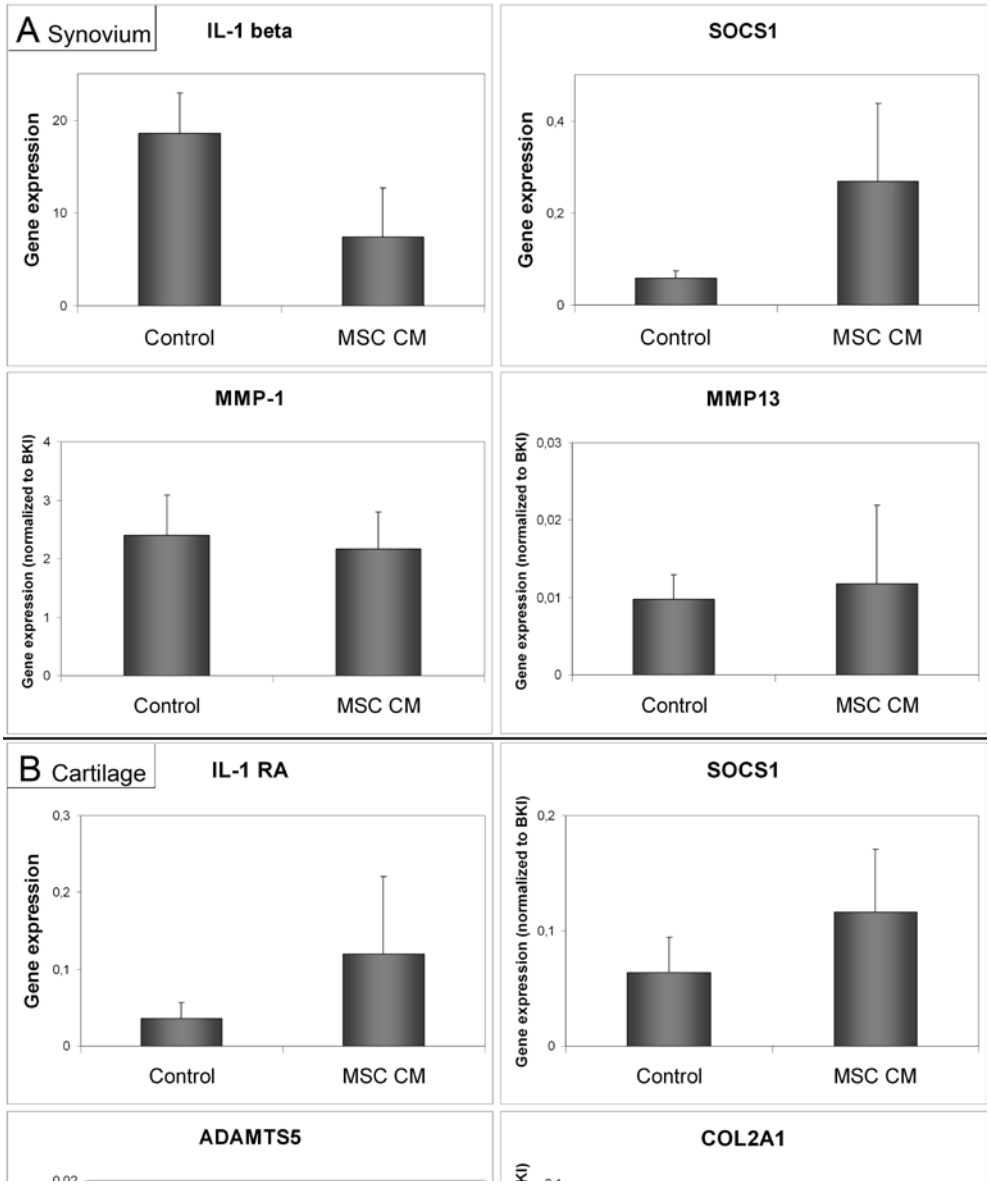


Figure S1. The paracrine effects of TNFα/IFNγ stimulated MSCs were determined in synovium and cartilage co-culture, a model resembling more closely the in vivo situation. The MSC secretome modified expression of genes related to inflammation and matrix turnover in synovium (A) and cartilage (B). This experiment was performed as confirmation of previously performed experiments using separate synovium and cartilage explants. Data are presented as means ± standard deviations for 1 experiment performed in triplicate with pooled MSC-conditioned medium of 5 donors; explants were treated for 48 hours.

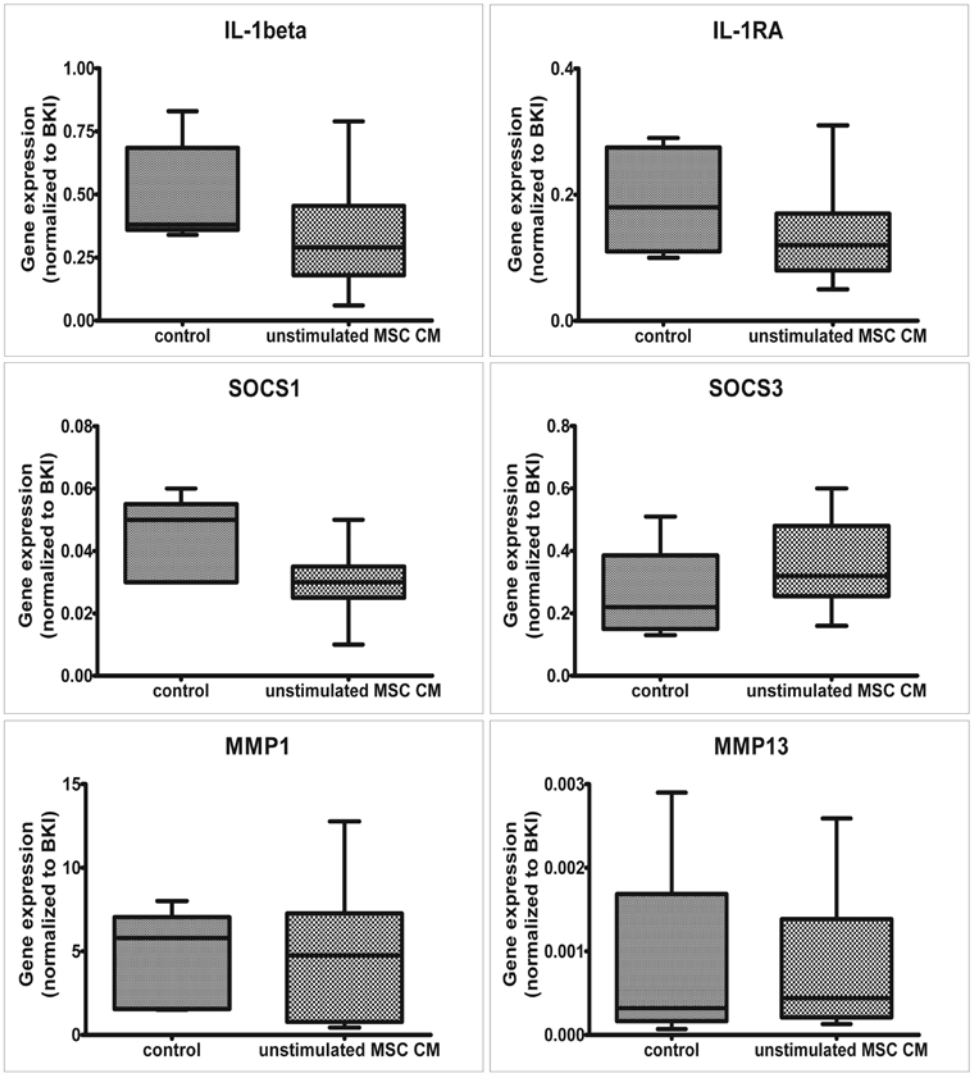


Figure 2. Effects of factors secreted by non stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic synovial explants. Data are presented as boxplots for 3 experiments, in which MSC-conditioned medium from 3 MSC donors was applied to triplicate explants from 3 OA donors; explants were treated for 48 hours. IL-1beta = interleukin-1 beta, IL-1RA = IL-1 receptor antagonist, SOCS = suppressor of cytokine signalling, MMP = matrix metalloproteinase.

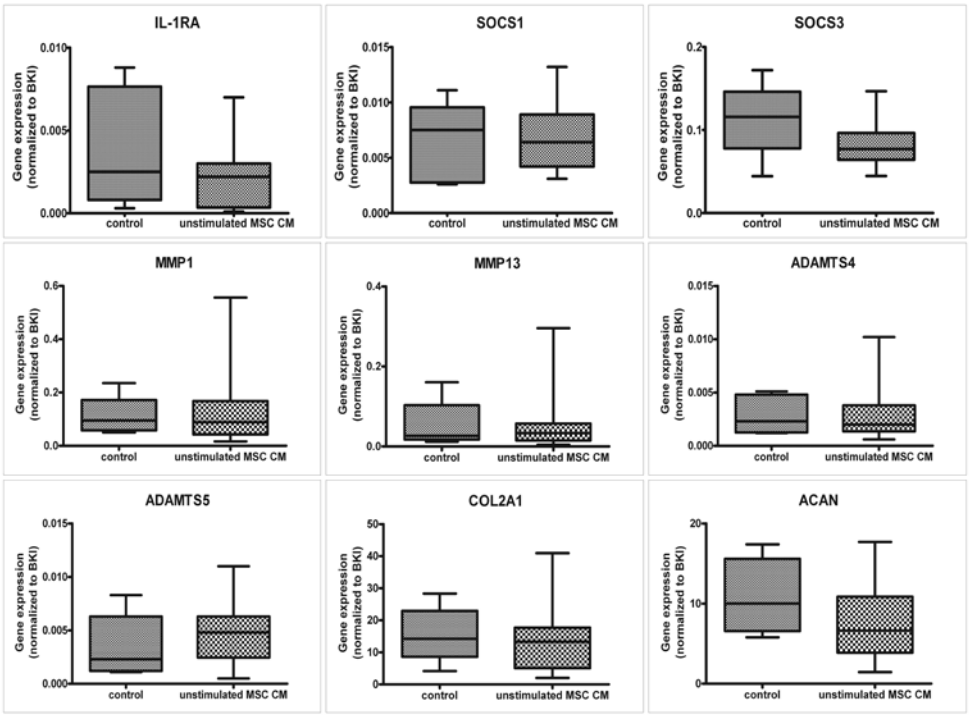


Figure 3. Effects of factors secreted by non stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic cartilage explants. Data are presented as boxplots for 3 experiments, in which MSC-conditioned medium from 3 MSC donors was applied to triplicate explants from 3 OA donors; explants were treated for 48 hours. ADAMTS = a disintegrin and metalloproteinase with thrombospondin motifs, COL2A1 = collagen type 2 alpha, ACAN = aggrecan.

Factors secreted by stimulated mesenchymal stem cells modify genes related to inflammation and matrix turnover in synovium and cartilage.

We evaluated whether factors secreted by MSCs affected inflammatory and catabolic processes in osteoarthritic synovium and cartilage. First we performed experiments in which we cultured synovium and cartilage explants in conditioned medium from MSC donors which were not stimulated with TNFα/IFNγ (unstimulated MSC-conditioned medium). None of the genes analyzed (mentioned later in this section) in either synovium or cartilage were significantly affected or affected more than two-fold by this unstimulated MSC conditioned medium (Fig. 2, 3). Therefore, we did not include this condition in our further experiments. All further presented experiments were performed using conditioned medium from TNFα/IFNγ-stimulated MSCs (designated as MSC-conditioned medium).

In synovial explants, MSC-conditioned medium downregulated *IL-1*β (P = 0.014), *MMP1* (P = 0.034) and *MMP13* (P = 0.016) gene expression, while *SOCS1* expression

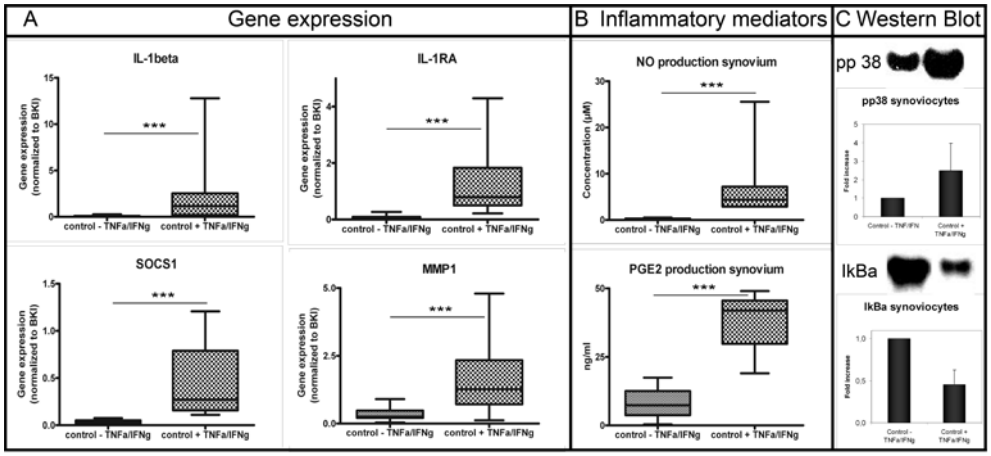


Figure S2. To evaluate whether the TNFα/IFNγ model reflected pathological OA processes, we tested the effects of TNFα/IFNγ on synovial explants from 3 donors. Data are presented as boxplots for 3 experiments performed in triplicate; explants were treated for 48 hours; ***p < 0.005.

was upregulated (P = 0.002) compared to control medium (Fig. 4). MSC-conditioned medium did not evidently affect *IL-1RA* or *SOCS3* gene expression of synovial explants. In cartilage explants, MSC-conditioned medium upregulated *IL-1RA* gene expression, while *ADAMTS5* and *COL2A1* expression were downregulated (Fig. 5; P < 0.001 for all genes). *ADAMTS4* and *SOCS3* were non significantly downregulated by 2.5-fold and 1.6 fold respectively. *SOCS1*, *MMP1*, *MMP13* and *ACAN* expression of cartilage explants were not clearly influenced by factors secreted by MSCs.

The interaction between cartilage and synovium is an important aspect in the pathogenesis of osteoarthritis. To evaluate the validity of our results in a more complex environment resembling more closely the *in vivo* situation, we exposed co-cultures of cartilage and synovium explants to MSC-conditioned medium (pooled from five MSC donors). Control experiments using pooled MSC-conditioned medium on separate cartilage and synovium explants revealed effects on gene expression level matching the effects observed using MSC-conditioned medium from individual donors (data not shown). MSC-conditioned medium affected gene expression levels in our co-culture model similar to our single explant culture model (Supp. Fig 1a,b). Synovium showed downregulation of *IL-1*β (2.7-fold) and upregulation of *SOCS1* (4.6-fold). *MMP1* and *MMP13* appeared uninfluenced by MSC-conditioned medium. In cartilage, gene expression of *IL-1RA* (3.3-fold) and *SOCS1* (1.8-fold) was upregulated, whereas *ADAMTS5* (2.0-fold) and *COL2A1* (1.9-fold) were downregulated by MSC-conditioned medium compared to control medium. These results confirm the previously described results obtained in separate cultures using synovial or cartilage tissue, indicating that MSCs can have an effect in a more complex system that better mimics in-vivo conditions.

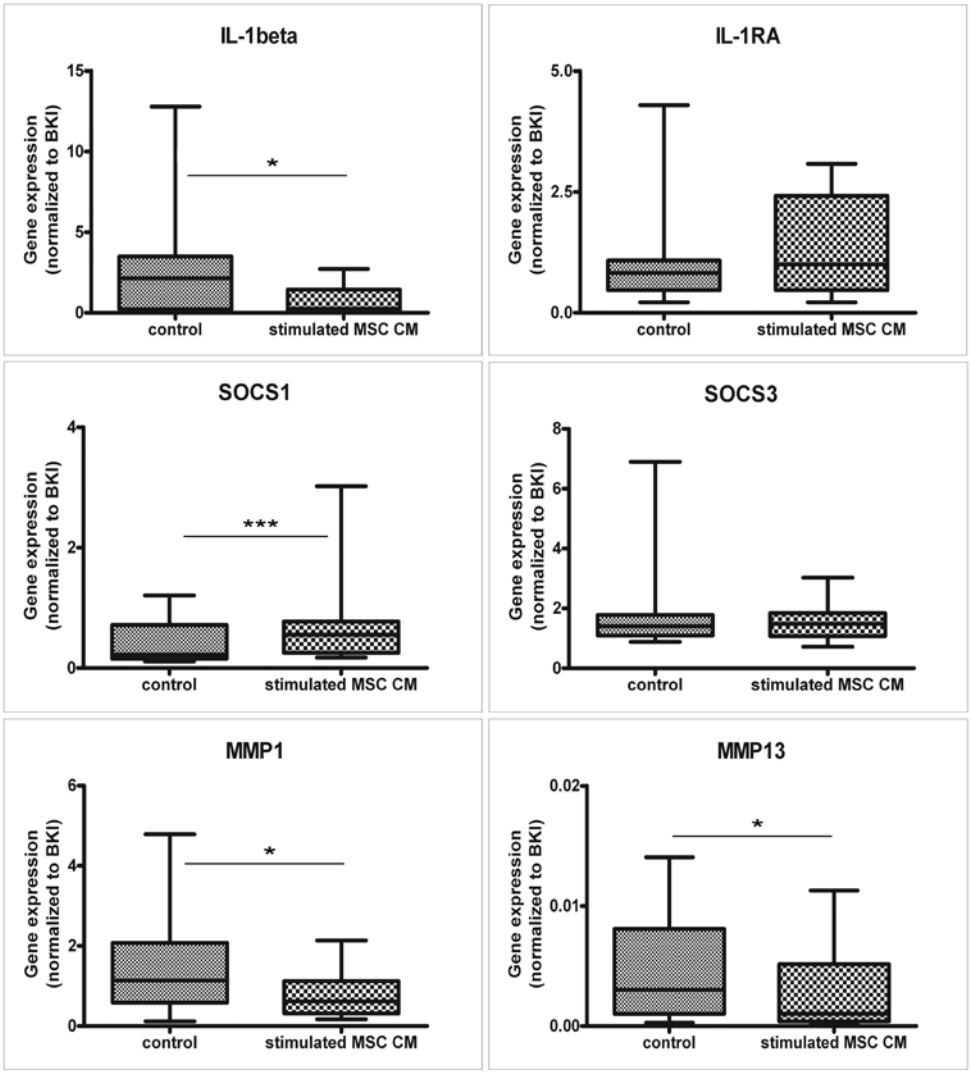


Figure 4. Effects of factors secreted by TNFα/IFNγ stimulated MSCs on expression of genes related to inflammation and matrix degradation in human osteoarthritic synovial explants. Data are presented as boxplots for 5 experiments, in which MSC-conditioned medium from 5 MSC donors was applied to triplicate explants from 5 OA donors; explants were treated for 48 hours; *p < 0.05; **p < 0.01; ***p < 0.005.

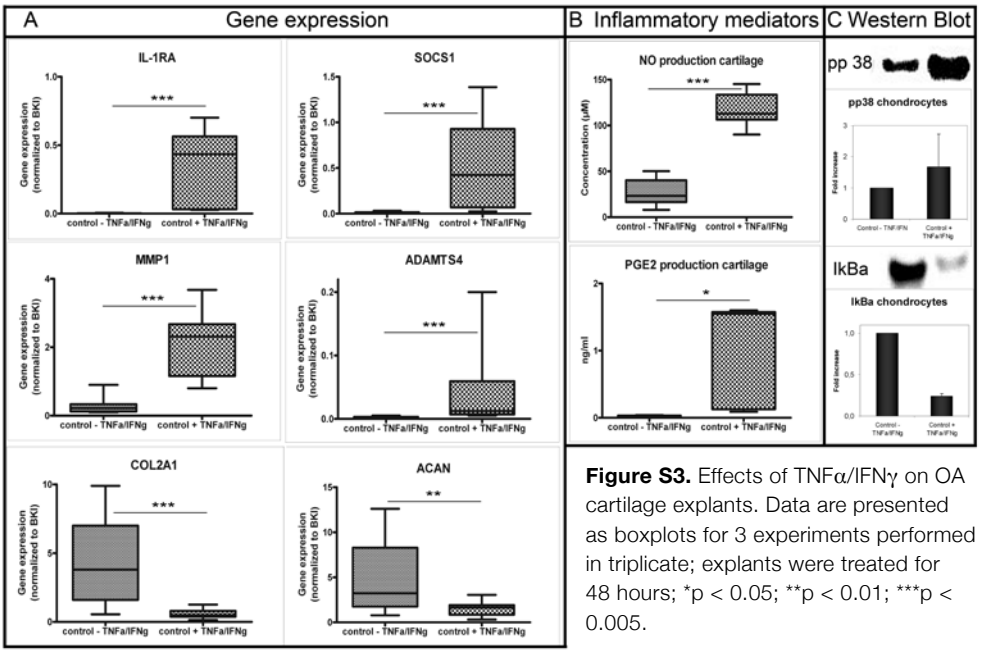


Figure S3. Effects of TNFα/IFNγ on OA cartilage explants. Data are presented as boxplots for 3 experiments performed in triplicate; explants were treated for 48 hours; *p < 0.05; **p < 0.01; ***p < 0.005.

Effects of mesenchymal stem cells on nitric oxide and prostaglandin E_2 secretion

Cartilage explants secreted more NO than synovial explants, whereas the latter produced the most PGE2 (Fig 7a-b). In cartilage explants, MSC-conditioned medium exerted a small but significant (P = 0.005) decrease in the NO secretion from 112 ± 20 µM to 93 ± 18 µM, together with a 50% decrease in PGE2 secretion (not statistically significant) from 0.98 ± 0.78 ng/ml to 0.49 ± 0.18 ng/ml. No effects were seen in synovial explants.

Effects of Mesenchymal stem cells on intracellular signalling pathways

To evaluate possible signalling pathways that are influenced by MSCs, we cultured isolated human synoviocytes and chondrocytes in MSC-conditioned medium and analyzed phosphorylated p38 MAP kinase and IκBa amounts by Western blot. MSC-conditioned medium did not influence p38 MAP kinase phosphorylation in either cell-type (Fig. 8a). Non-phosphorylated p38 MAP kinase was constitutively expressed for all conditions (data not shown), as was α-Tubulin. Treatment with MSC-conditioned medium increased the presence of IκBa (Fig 8b) in both cell-types, thereby indicating that MSCs secrete factors which inhibit NFκB activation.

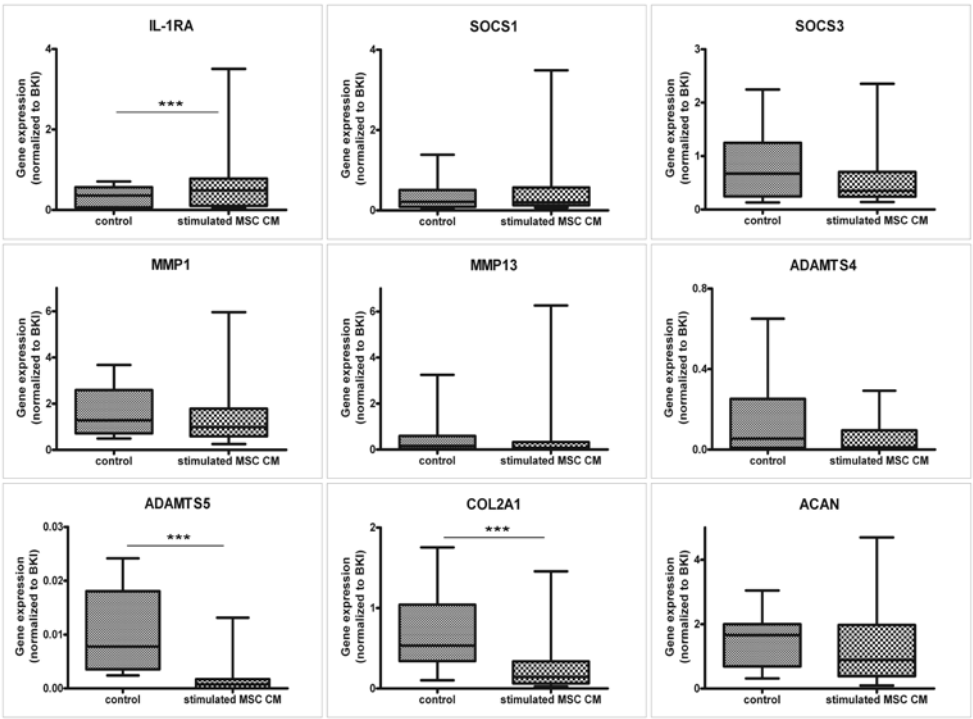


Figure 5. Effects of factors secreted by TNFα/IFNγ stimulated MSCs on expression of genes related to inflammation and matrix turnover in human osteoarthritic cartilage explants. Data are presented as boxplots for 5 experiments, in which MSC-conditioned medium from 5 MSC donors was applied to triplicate explants from 5 OA donors; explants were treated for 48 hours; ***$p < 0.005$.

Discussion

OA is a disabling disease where many catabolic and inflammatory processes play a role [8]. MSCs have chondrogenic potential, but can also play a role in immunomodulation and tissue regeneration by secretion of soluble factors [249]. In this study we showed for the first time such an effect of MSCs on OA tissues. Exposure of synovial and cartilage explants to MSC-secreted factors resulted in gene expression profiles and production of factors consistent with anti-inflammatory and anti-catabolic activity in these tissues. This included beneficial effects on the expression of genes related to inflammation (*IL-1*β, *IL-1RA*, *SOCS1*) and matrix degradation (*MMP1*, *MMP13* and *ADAMTS5*) in synovium or cartilage. These results were confirmed in a co-culture model of synovium and cartilage, a system resembling more closely the in-vivo situation. Next to the effects on gene expression, MSC-conditioned medium reduced production of the inflammatory mediator NO in cartilage explants and increased presence of IκBa in synoviocytes and chondrocytes. The phosphorylation and degradation of IκBa, which normally binds NFκB, is an essential and first step in activation of the NFκB pathway. NFκB has been

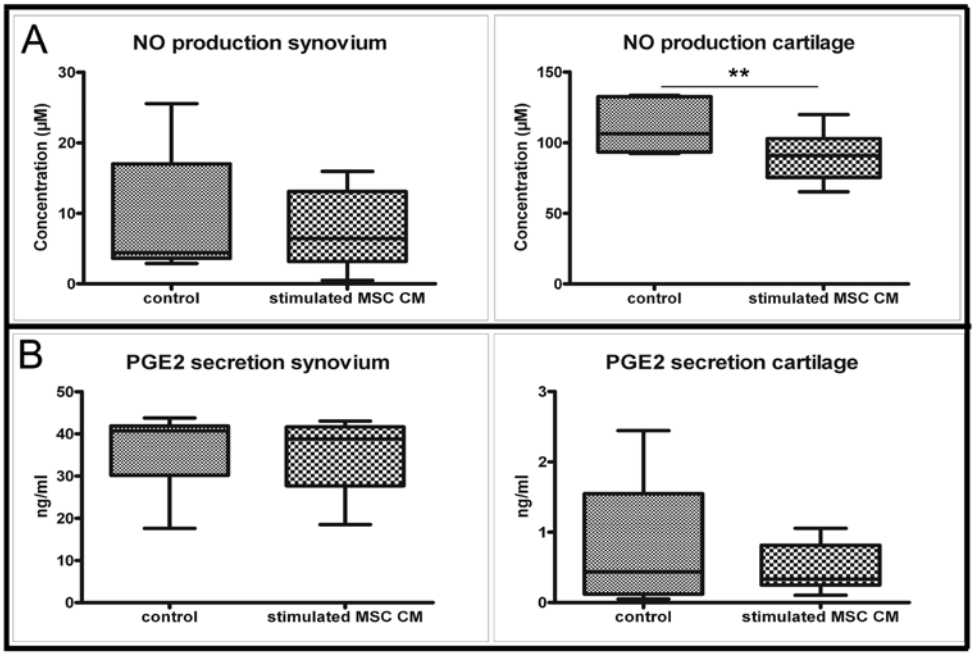


Figure 6. Influence of factors secreted by TNFα/IFNγ stimulated MSCs on NO (A, B) and PGE2 secretion (C,D) by synovium (left panels) and cartilage (right panels) explants. Data are presented as boxplots for 5 experiments (NO) or 4 experiments (PGE2), in which MSC-conditioned medium from 5 MSC donors was applied to triplicate explants from 5, respectively 4, OA donors. Regarding NO measurements, all triplicate samples were measured individually; regarding PGE2 measurements, triplicates were pooled and measured in duplicate. Explants were treated for 48 hours; NO = nitric oxide, PGE2 = prostaglandin E2; **p < 0.01.

reported to induce gene expression of, amongst others, *IL-1*β, *TNF*α*, MMP1*, *MMP3* and *MMP13*, indicating it's role as a main pathway involved in inflammation and matrix degradation [96].

We needed to stimulate MSCs to achieve secretion of sufficient amounts of immunomodulatory factors to influence OA cartilage and synovial explants. We challenged our cells with TNFα and IFNγ as described before [242,243]. TNFα is an extensively studied cytokine in OA research [8,250] and the presence of IFNγ producing T-cells in OA synovium has been indicated [251]. To study whether this model reflected pathological OA processes, we tested the effects of TNFα/IFNγ on explants from three of the five patients (Supp. Fig. 2, 3). Several inflammatory and catabolic effects were observed in both tissues at gene expression level, together with a significantly increased NO and PGE2 production as well as p38 phosphorylation and IκBa degradation. Taken together, this indicates that TNFα/IFNγ induced various processes which are relevant in OA [8,96].

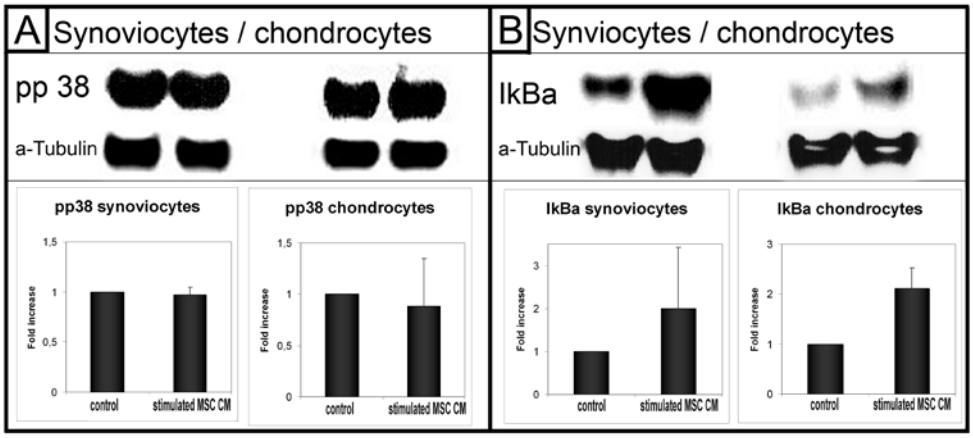


Figure 7. Influence of factors secreted by TNFα/IFNγ stimulated MSCs on P38 MAP kinase phosphorylation (A) and IκBa presence (B) in both synoviocytes and chondrocytes was measured by Western blot. Presence of IκBa was increased in both cell types by MSC-conditioned medium, thereby indicating an inhibitory effect on NFκB activation. Data are presented as means ± standard deviations for single values obtained in 2 experiments; cells were treated for 3 hours for p38 MAP kinase evaluation or 10 minutes for IκBa analysis. P38 MAP kinase = p38 mitogen-activated protein kinase, IκBa = inhibitor kappa B alpha.

The stimulated MSCs in our experiments produced IL-6, HGF, TIMP2 and TGFβ-1 and displayed a high enzymatic IDO activity. The selection of this panel of factors was based on the fact that they are known to be secreted by MSCs at high levels and their known involvement in general inflammation processes or joint metabolism [250,252]. IDO is an important factor for the inhibition of T-cell proliferation and has been reported to decrease joint inflammation [253]. The role of IL-6 in osteoarthritis, however, is controversial (reviewed in [8]). Although it is in general considered an inflammatory mediator, IL-6 deficient mice were shown to have a lower proteoglycan synthesis with a higher incidence of subchondral bone sclerosis [254], and increased cartilage damage which was reduced by IL-6 injection [255]. These results indicate possible advantageous roles of IL-6 in immune modulation. The other factors we determined in MSC-conditioned medium, HGF, TIMP2 and TGFβ-1, all play a role in tissue regeneration and cartilage matrix turnover [256,257,258].

MSC-conditioned medium upregulated *SOCS1* gene expression in synovium and caused a trend towards *SOCS3* downregulation in cartilage. *SOCS1* is a negative regulator of macrophage and dendritic cell activation, while *SOCS3* is a positive regulator of these immune cells (reviewed in [259]). Furthermore, the presence of *SOCS1* has been reported to limit joint destruction in inflammatory arthritis, whereas *SOCS3* upregulation in chondrocytes has been shown to contribute to cartilage damage [260,261].

OA synovium and cartilage are known to have a very heterogeneous gene expression

pattern between patients or between different areas within the same patient [262,263]. Due to this high variation it is challenging to obtain consistent results. Nevertheless, we found significant effects of factors secreted by MSCs on inflammatory and matrix degrading processes. Although no clear effects of stimulated MSC-conditioned medium were observed on various other genes and factors we analyzed, many trends were seen pointing towards an overall decreased inflammatory and catabolic environment. Synovial explants exhibited a lower average gene expression of *SOCS3* and a higher expression of *IL-1RA* after treatment with factors secreted by MSCs. In cartilage, *SOCS1* was higher expressed and *ADAMTS4*, *MMP1* and *MMP13* genes were lower expressed on average in MSC-conditioned medium treated samples. Next to this, in cartilage a trend towards a diminished PGE2 production was found. Even though these effects were small and not significant, partially due to a low sample size, they supported our confidence in the potential of MSCs as environmental modulators and their beneficial role in modifying OA tissues.

Wu et al. found a beneficial trophic effect of MSCs on GAG production by bovine chondrocyte pellets in a non-inflammatory environment [241]. We did not observe an effect on *ACAN* gene expression and found a downregulation of *COL2A1* by MSC-conditioned medium. It could be hypothesized that in an inflammatory environment, MSCs are mainly triggered to counteract inflammation instead of stimulating matrix formation. This is further supported by the observed increase of the immunomodulatory factors IDO and IL-6 in conditioned media from TNFα/IFNγ stimulated MSCs, although the amount of growth factors between stimulated and non-stimulated MSCs remained similar. Possible anabolic effects of secreted growth factors may become more obvious in conditions without inflammation.

We have not assessed whether our observed paracrine effects were specific for MSCs. It was recently shown that skin fibroblasts suppress inflammation in an arthritis model [264], while others found no immunosuppressive properties of skin fibroblasts in a sepsis model [265]. We did not study fibroblasts since we consider this cell-type unsuitable for intra-articular application. MSCs have the advantage of being immune privileged, and they have the capacity of chondrogenic differentiation, a mechanism of action which is likely to be at least part of their regenerative capacity for cartilage repair [266]. Next to this, MSCs are locally present in multiple joint tissues [267] and are able to react to the joint environment, as shown by the increased presence of MSCs in synovium and synovial fluid after joint injury [45,46]. This suggests that the increased intra-articular presence of MSCs is part of a natural healing process. The administration of MSCs in an osteoarthritic joint could be a therapy for OA mimicking and enhancing this healing process and thereby provide a natural and autologous treatment for OA.

To our knowledge, this is the first study indicating that factors secreted by MSCs cause multiple anti-inflammatory and anti-catabolic effects in osteoarthritic cartilage and

synovium. We performed culture experiments for 48 hours, a common time-point to evaluate processes at a gene-expression level in our group [268]. Generally, this time-point is too soon to evaluate effects at a protein level on matrix components like proteoglycans or aggrecan. Therefore we evaluated proteins which are known to respond very fast and induce other processes such as NO, PGE2, IκBa or p38 MAP kinase. Further studies are warranted to investigate the effects on structural properties of the cartilage using longer-term cultures and *in vivo* experiments.

MSC-conditioned medium undoubtedly contained many more factors than the ones we have measured, including for instance TNFα stimulated gene-6 [269]. The whole panel of bio-active factors probably works in concert to achieve the anti-osteoarthritic effects observed in our study. Since intra-articularly injected MSCs have been shown to survive in an intra-articular environment up to at least four to six weeks [47,55], they could provide the ultimate long term delivery of a cocktail of OA modifying factors.

Acknowledgements
We acknowledge Fons van de Loo for providing RT-PCR primers for *SOCS1* and *SOCS3*; Qiuwei Pan for providing the RT-PCR primers for *HGF*; Lianne van de Laar for providing the IκBa antibody and Benjamin Fernandez-Gutierrez for general support.

Conflict of interest statement
None of the authors have a conflict of interest to declare.

Declaration of funding
We acknowledge the Osteoarthritis Research Society International for providing a fellowship granted to E.V.; and the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science (SSM06004). None of the funding sources influenced the study design, data collection, data analysis, data interpretation, writing the manuscript or in the decision for submission.

Chapter 5

Effects of rat and human bone marrow derived cells on pain, inflammation and structural changes in a mono-iodoacetate rat model of osteoarthritis

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Submitted for publication

Abstract

Background: Mesenchymal stem cells (MSCs) are promising candidates for osteoarthritis (OA) therapies. We studied the effects of intra-articularly injected bone marrow derived MSCs (both rat and human), as well as freshly isolated rat bone marrow mononuclear cells (BMMNCs), on pain, structural damage and inflammation in a rat OA model *in-vivo*.

Methods: OA was induced unilaterally by injection of mono-iodoacetate (MIA). After three weeks, the 32 animals were randomly divided into four groups: 1. control, 2. rat MSCs, 3. rat BMMNCs, 4. human MSCs. Four weeks after treatment, pain was assessed with an incapitance tester, subchondral bone alterations were measured with μCT and cartilage quality and joint inflammation were analyzed with histology.

Results: All therapies were well tolerated by the animals. Animals treated with rat MSCs distributed significantly more weight to the affected limb after treatment than before treatment, although no statistically significant difference was observed compared to saline treated animals. MIA injected knees displayed significant cartilage damage, subchondral bone alterations and synovial inflammation compared to contralateral knees. No statistically significant differences between treatment groups regarding any of these outcome measures were observed.

Conclusions: This is the first study evaluating the effect of cell therapy on pain, as well as structural changes and synovial inflammation in a small animal OA model. No statistically significant effects of the cellular therapies compared to saline injection were found. Further studies should optimize cellular therapies for OA treatment.

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by inflammation and catabolic processes, leading to progressive cartilage degeneration. Cartilage has limited intrinsic repair capacity and so far no drugs are available to structurally modify OA processes [234]. Mesenchymal stem cells (MSCs) are promising candidates for cartilage regeneration and OA therapies since they have chondrogenic potential and the ability to form extracellular matrix [42]. Additionally, MSCs have immunomodulatory capacities by secreting anti-inflammatory factors and growth factors [44], which could possibly encounter inflammatory and catabolic aspects of OA. Therefore, MSCs have been injected intra-articularly in pre-clinical and some initial clinical studies as a treatment for cartilage damage and OA, showing promising results [47,50,51,53,54,55,56,237,270]. Animal studies using cell tracking after cell injection showed only limited cartilage formation by chondrogenic differentiation of these MSCs [47,50,51,54,55,56]. Instead, injected cells were mostly found in other parts of the joint, like the synovium. Furthermore, a decreased amount of inflammatory cytokines in synovial fluid was found in MSC treated OA joints [49]. These findings underline the potential dual role of MSCs as an OA modifying drug, not only able to regenerate damaged cartilage but also positively affecting the entire joint homeostasis.

The effects of MSC therapy on pain or other clinical outcome measures are difficult to assess in animal studies, and therefore they are not extensively documented. Nevertheless, pain is the main reason to proceed to joint replacement in OA patients [271]. As there appears to be limited relation to structural joint damage and pain in patients [22,271], separate evaluation of this outcome measure is essential to assess the efficacy of cell therapies. The mono-iodoacetate (MIA) model has been extensively studied as a pain model for OA in animals [272,273,274].

MSCs as an OA therapy appear promising, but several practical issues hinder broad clinical translation. Extensive culture procedures are necessary to obtain MSCs, a costly and time consuming procedure requiring special facilities. Bone marrow mononuclear cells (BMMNCs) on the other hand, can be harvested in a one-step procedure. These cells contain various progenitors [275] which could also attenuate degenerative OA processes. Another variable impeding clinical translation is the fact that many preclinical studies regarding this subject use MSCs from young and healthy animals. Conflicting results have been described regarding the influence of patient age and disease stage on the presence of MSCs and their regenerative potential [180,276,277]. OA modifying effects of MSCs from human OA patients in a pre-clinical model would facilitate clinical translation based on more realistic expectations.

In order to evaluate multiple aspects of OA pathology and to explore potential options to enhance clinical translatability, we studied the effects of intra-articularly injected cul-

tured MSCs (both rat and human), as well as rat BMMNCs, on pain structural damage and inflammation in a MIA rat OA model in vivo.

Methods

Ethical approval
All human material was obtained after written informed consent, as approved by the local medical ethical committee (protocol # MEC-2004-142). All animal experiments were performed after approval of the animal ethical committee (protocol # EMC116-10-07).

Preparation and colony forming capacity of bone marrow derived cells
Human MSCs were isolated and cultured from heparinized femoral-shaft marrow aspirate of two patients undergoing total hip arthroplasty using previously described procedures [157]. Cells were cultured in DMEM containing 10% fetal calf serum (FCS), 50 µg/ml gentamicin, 1.5 µg/ml fungizone, 1ng/ml fibroblast growth factor-2 and 0.1 mM l-ascorbic acid 2-phosphate (MSC culture medium). All media were renewed twice a week.

Rat MSCs were isolated by flushing morselized femurs and tibiae from four week old rats. The harvested cells were cultured in MSC culture medium similar to human MSCs. For both cell-types, passage 2-3 cells were used for intra-articular injection. Rat bone marrow mononuclear cells (BMMNCs) were obtained by cell separation of the harvested bone marrow using a Ficoll gradient (Ficoll-Paque™ PLUS, d = 1,077, GE Healthcare, Vienna, Austria) at 1,000 G. BMMNCs were removed from the gradient interface, washed in physiological saline and subsequently injected. To assess the presence of MSCs in BMMNCs, aliquots of these cells were seeded at a density of 12 x 10^4 cells/cm^2 and cultured in MSC culture medium. After 10 days, colonies consisting of more than 50 cells were counted to determine the percentage of colony forming cells. It was demonstrated that these isolated BMMNCs had an average of one colony forming cell per 84,000 ± 20,000 mononuclear cells, confirming previous studies describing one cell in 10,000 – 250,000 BMMNCs to have MSC characteristics [276,278].

Animal experimental design
Osteoarthritis was induced unilaterally in 32 male Wistar rats (Harlan Netherlands BV, Horst, the Netherlands) of 16 weeks old. OA induction was performed by an intra-articular injection of 300 µg mono-iodoacetate (MIA). Contralateral control knees were not injected with any substance. Rats were randomly divided into four treatment groups: 1. Control; 2. Rat MSCs; 3. Rat BMMNCs and 4. Human MSCs. Saline was used as a control and as vehicle for all other injections; all injections were applied under isoflurane anesthesia in a volume of 50 µl using a 27G needle (Sherwood-Davis & Geck, Gosport,

UK). Treatments were given three weeks after OA induction in order to allow the initial inflammatory phase after MIA injection to cease and structural damage to occur. Rat MSCs and human MSCs were given at a dose of 1 x 10^6 cells per joint, rat BMMNCs were given at a dose of 10 x 10^6 cells per joint. Viability of all cells was assessed before injection and after injection, on the remainder of the cells in the syringe, by means of trypan blue exclusion tests. Overall cell viability was 96.3 ± 3.5% before injection and 95.5 ± 1.7% (mean ± SD) for the remainder of the cells after injection. Rats where euthanized four weeks after treatment, knee joints were harvested for further analyses.

Hind limb weight distribution measurements
Hind limb weight distribution was measured using an incapacitance tester (Linton Instrumentation, Norfolk, UK) as an index of joint discomfort as described previously [273]. Animals were habituated to the apparatus starting two weeks prior to experiments. Rats were positioned on the incapitance tester with each hind limb resting on a separate force plate. The force exerted by each hind limb was measured in grams and averaged over a 3 second period. Each rat was measured for 5 subsequent times per day on 2 consecutive days by an observer blinded to the given treatment. The average of 10 obtained readings was used to calculate the weight on the affected limb as a percentage of total weight distributed by both hind limbs. Rats were measured before inducing OA, three weeks after inducing OA (before treatment) and four weeks after treatment.

µCT procedure and analysis
To evaluate cartilage damage before cell treatment a µCT arthrography (µCTa) was performed as described previously [279]. Briefly, rat knees were injected with 50 µl non-diluted ioxaglate 320 (Hexabrix, Hazelwood, MO), mixed with epinephrine 10 µg/ml (Centrafarm, Etten-Leur, the Netherlands) to induce vasoconstriction and to prevent loss of intra-articular ioxaglate. A µCTa was made using the Skyscan 1076 µCT scanner (Skyscan, Kontich, Belgium). Scan time was fifteen minutes at an isotropic voxelsize of 35 µm, voltage of 55 kV, current of 181 mA, field of view 35 mm and a 0.5 mm aluminum filter, over 198° with a 1° rotation step. All scans were reconstructed identically. Patellar cartilage volume was measured in 3D using data analysis software (CT Analyzer, Skyscan, Belgium) [280].

For subchondral bone analysis, fixated knee joints were scanned four weeks after treatment ex-vivo. Scan time was 30 minutes at an isotropic voxel size of 18 µm, voltage of 60 kV, current of 167 mA, field of view 35 mm and a 0.5mm filter over 198° with a 0.4° rotation step. Ex-vivo CT scans were segmented into binary datasets using an automated thresholding algorithm [281]. The subchondral bone part of the distal femur epiphysis was separated from other bone structures using in-house software. Subchondral plate thickness, plate volume and total pore volume was measured in the cortical bone of the femoral trochlea [20]. Subchondral plate porosity was expressed as a percentage of the subchondral plate volume.

All MIA injected knees and two contralateral control knees per treatment group were used for analyses, leading to a total of 8 knees per group.

Tissue harvest and histologic evaluations
After euthanasia, all rat knees were excised and fixed in formalin 4% (v/v) for one week. After μCT scanning, joints were decalcified with formic acid 10% (v/v) for 3 weeks and embedded in paraffin. Coronal sections were stained with Safranin O to evaluate structural cartilage damage and Thionin to visualize the amount and distribution of GAG. Thionin was used to evaluate GAG distribution due to a low sensitivity of Safranin O in the case of severe GAG loss [282]. Cartilage quality was evaluated with a score ranging from 0-6 for structure using previously described stages by Pritzker et al. [283], and a score ranging from 0-4 for GAG staining intensity. GAG staining intensity and structure grading were multiplied with a previously described staging score: 1. 0–25%; 2. 25–50%; 3. 50–75%; 4. 75–100% of cartilage surface affected [280]. This way, a maximum score of 16 for GAG staining intensity and 24 for structural damage could be obtained for each cartilage structure, where a high score represents a high amount of GAG loss or structural damage. Scoring was performed on the patella, trochlea, tibial plateau and femoral condyles at three different positions. An average score for GAG and structure was calculated for each joint compartment.

Synovial inflammation was assessed using a hematoxylin eosine staining. Synovial thickness was measured from the bone margin to the capsule in the parapatellar recesses at the location where the synovium folds from the capsule over the femoral bone, based on a previously described method [284]. Synovial thickness was determined at the medial and the lateral side at three positions and averaged to obtain a single value per knee. Measurements were performed using the NanoZoomer Digital Pathology program (Hamamatsu Photonics, Ammersee, Germany). The amount of synovial inflammation was assessed by ranking the knees in an order from minimum to maximum (score 1 - 40) based on synovial fibrillation and cellular infiltration in the subsynovial tissue.

All MIA injected knees and two contralateral control knees per treatment group were used for analysis by an observer blinded to the given treatment, leading to a total of 8 knees per group.

Statistical analysis
The effect of treatment on the difference between weight distribution on the hind limbs before and after MIA and between the cellular treatments were analyzed using a repeated measures ANOVA test for all groups, followed by a paired t-test for all groups separately. Quantitative μCT and histology data was analyzed by means of unpaired t-tests to evaluate MIA induced effects and one-way ANOVA tests for treatment effects. Semi-quantitative histology scores were compared using non-parametric Mann-Whitney tests to assess MIA induced effects and Kruskal Wallis-tests for treatment effects

(SPSS 17.0.2; SPSS Inc., Chicago, USA). For all tests, P values < 0.05 were considered statistically significant.

Results

All cell injections were well tolerated by the animals without any macroscopical sign of inflammation or rejection.

Hind limb weight distribution
As a measure of pain we determined weight distribution over the hind limbs. Rats loaded both hind limbs equally at the start of the experiments. At baseline, the limbs that received MIA bore 50.6 ± 1.6 % of the weight and the contralateral limbs 49.4 ± 1.6 % (mean ± SD). Three weeks after MIA injection, weight distributed to the affected leg was significantly reduced compared to baseline (45.8 ± 5.4 % vs 50.6 ± 1.6 %, P<0.001, Fig 1A, mean ± SD), indicating pain sensation. No differences were observed between the treatment groups at this time point (P = 0.937), pointing towards a random distribution of MIA induced pain. Four weeks after cellular treatment, the group treated with rat MSCs was the only group that had significantly more weight distributed to the affected limb after treatment than pre-treatment (51.2 ± 5.0 % vs 46.5 ± 4.1 %, P = 0.003, Fig 1B-E, mean ± SD). When comparing the treatment groups after 4 weeks, no significant differences were observed (P = 0.651, Fig. 1F).

Structural integrity: cartilage quality and subchondral bone alterations
uCT arthrography was used to assess loss of cartilage volume elicited by the MIA injection as a measure of cartilage quality before cellular treatments. MIA injected knees displayed a smaller cartilage volume than control knees (0.48 ± 0.15 mm^3 vs 0.82 ± 0.17 mm^3, P < 0.001, data not shown, mean ± SD). Cartilage volume did not differ between the MIA injected knees of the different treatment groups (P = 0.929), indicating a random distribution of cartilage loss before application of the treatment.

Cartilage quality at the end of the experiments was measured on histology with a semi-quantitive score for GAG loss and structural damage. Cartilage damage was most pronounced in the patellofemoral region and all presented data reflect this compartment. For this compartment the scores of the patella and the trochlea were accumulated, leading to a score range for GAG loss from 0 to a maximum loss of 32 and a score range for structural damage from 0 to a maximum damage of 48. MIA injected knees showed more GAG loss than non-MIA injected knees (24.0 (0.0 – 32.0) vs 1.5 (0.0 – 2.0), P < 0.001, Fig. 2A-C, range (95 % CI)). No significant between the treatment groups were observed (P = 0.393).

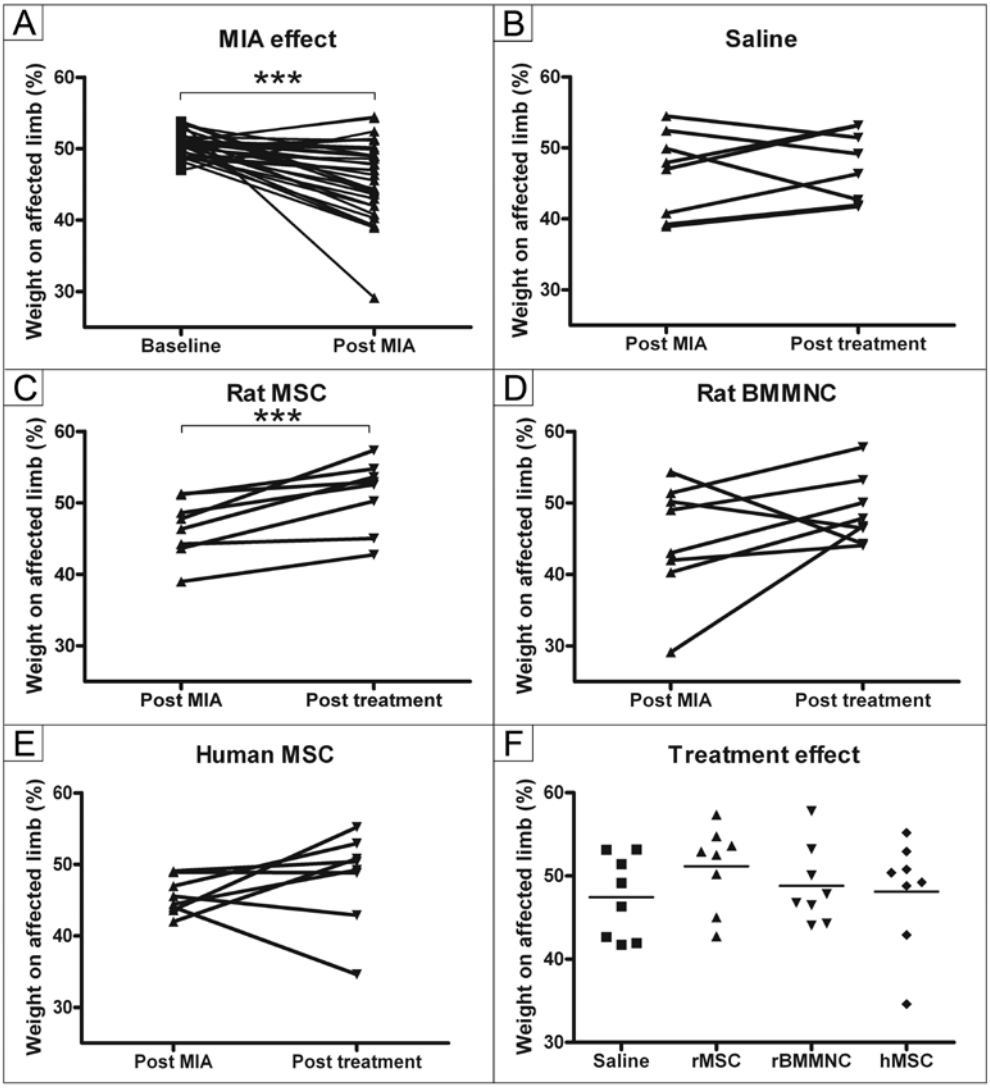

Figure 1. Assessment of hind limb weigh distribution Hind limb weight distribution was determined as an index of joint discomfort. MIA caused a reduction of weight distributed to the affected limb (A). Rat MSC injection was the only treatment that caused a significant increase in weight distributed on the OA limb (B-E), while no significant differences between treatments were observed (F). *** $P < 0.001$.

Structural cartilage damage was significantly present in MIA injected knees versus control knees (4.4 (0.0 - 8.5) vs 1.3 (0.0 - 2.2), $P < 0.001$, Fig. 2D, range (95 % CI)). Between the different treatment groups, no statistically significant differences were found ($P = 0.959$).

Ex vivo uCT was used to evaluate trochlear subchondral bone porosity and thickness after treatment. Overall, MIA injected knees had a significantly more porous (6.3 ± 6.2 % vs 2.0 ± 0.8 %, P < 0.001, mean ± SD) and thinner (221.1 ± 29.4 µm vs 253.8 ± 19.1 µm, P = 0.005, mean ± SD) subchondral plate than control knees (Fig. 3A-D), which

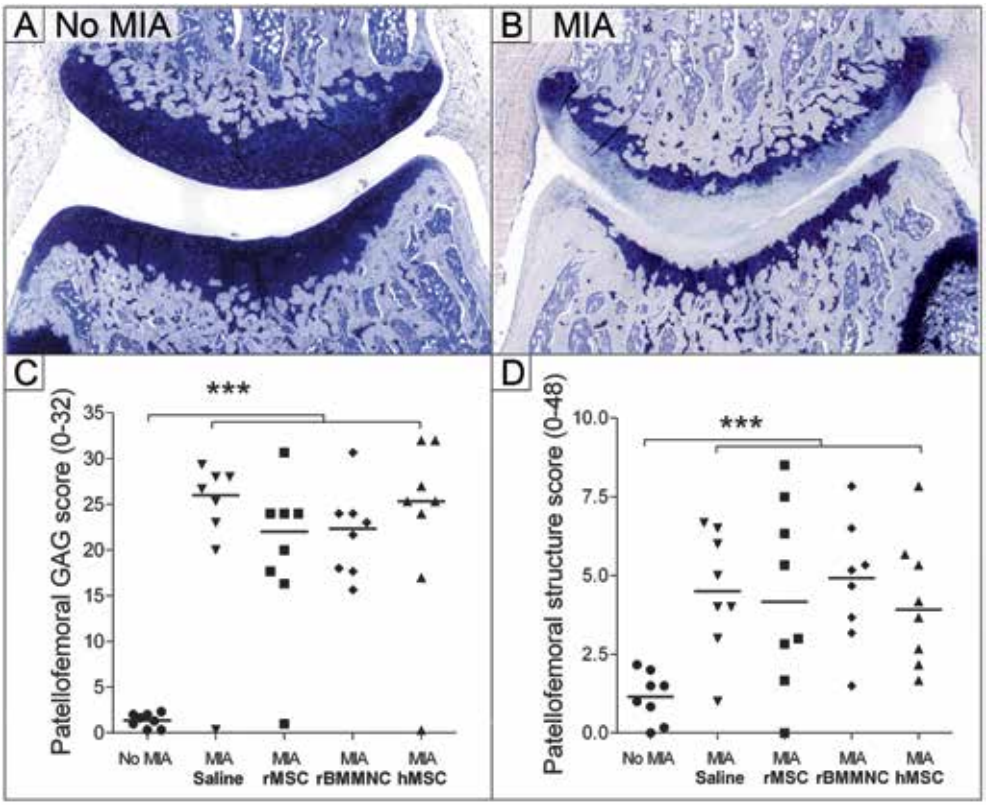

Figure 2. Cartilage quality Thionin staining demonstrated evident GAG loss and mild structural cartilage damage in MIA injected knees compared to contralateral control knees (A-D). No significant differences between treatment groups were found. Magnification 25 x, *** P < 0.001.

is in concordance with previously reported early OA related changes [20,285,286]. No statistically significant differences between treatment groups were observed regarding porosity (P = 0.208) or subchondral plate thickness (P = 0.607).

Synovial inflammation

Synovial inflammation was evaluated by measuring synovial thickness at the parapatellar recesses and ranking the samples based on synovial fibrillation and cellular infiltration (Fig. 4A,B). MIA injected knees had a thicker synovium (730.4 ± 94.8 µm vs 601.6 ± 32.6 µm, P < 0.001, mean ± SD) and inflammation was ranked higher (24.5 (5.0 – 40.0) vs 5.0 (1.0 – 12.0), P < 0.001, range (95 % CI)) than control knees without MIA injec-

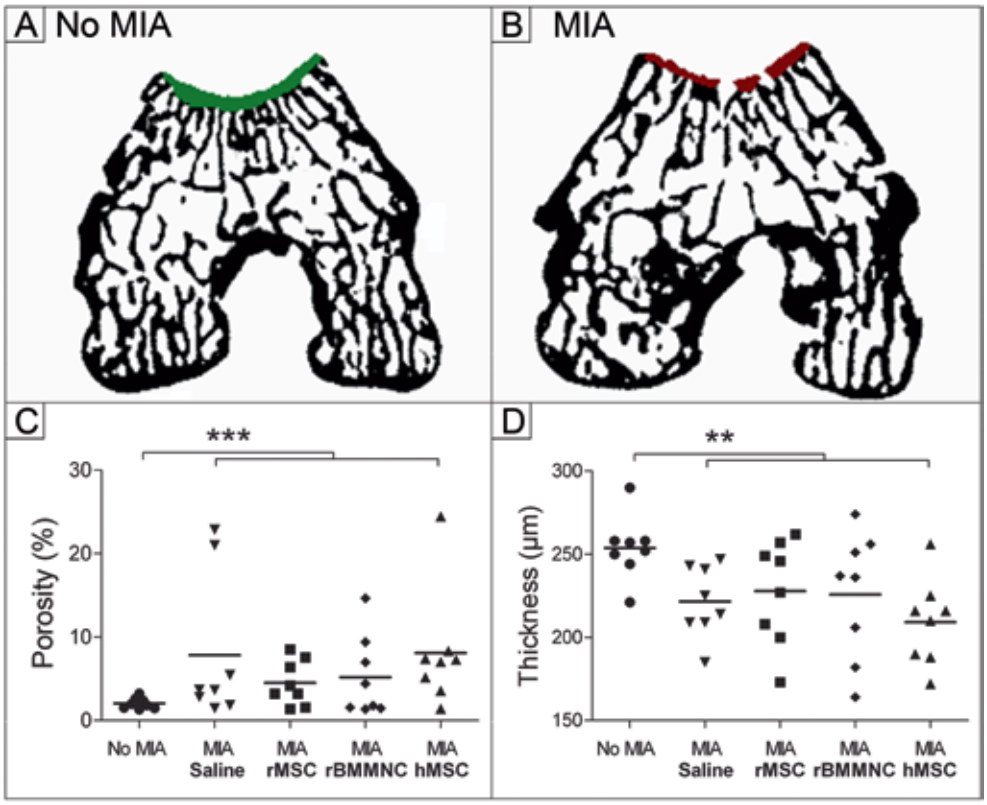

Figure 3. Subchondral bone plate evaluation µCT analyses displayed increased porosity and thinning of trochlear subchondral bone plates in MIA injected animals compared to contralateral control knees (A-D). Between de treatment groups, no significant differences were observed. ** P < 0.005, *** P < 0.001.

tion (Fig. 4C,D). No significant differences were observed between treatment groups for synovial thickness (P = 0.115) or inflammation ranking (P = 0.111).

Since the data on synovium suggested possible differences between the treatment group we performed explorative statistical analyses by comparing individual treatement groups with the saline injected control group. This revealed that synovium in the group injected with rat MSCs showed a trend to be thicker than in the saline injected group (P = 0.071) but not more inflamed (P = 0.382). Interestingly, the synovium in the group injected with human MSCs was significantly thicker (P = 0.028) and had a trend to be more inflamed (P = 0.083) than the saline injected group (P = 0.028). Injection with the mononuclear fraction of rat bone marrow did not affect synovium thickness (P = 0.733) nor inflammation (P = 0.574).

Discussion/Conclusion

OA is a disabling disease where many catabolic and inflammatory processes play a role [8]. MSCs are promising candidates for OA treatment. MSCs have chondrogenic potential, but also play a role in immunomodulation and tissue regeneration by the secretion of soluble factors [249]. We studied the effects of intra-articularly injected cultured MSCs (both rat and human), as well as rat BMMNCs, on pain in addition to inflammation and

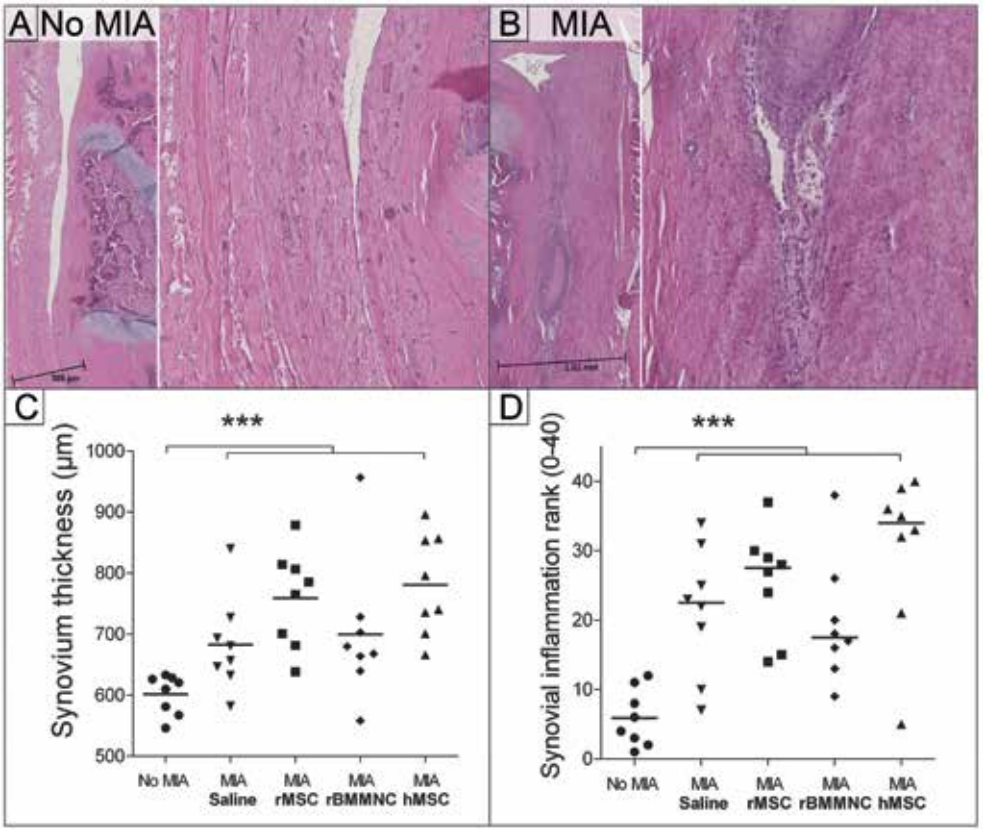

Figure 4. Synovial thickness and inflammation Hematoxylin eosine staining illustrating increased synovial thickness, fibrillation and subsynovial cellular infiltration in MIA injected knees compared to contralateral control knees. No significant differences between treatment groups were found. Magnification (A,B) left panels 25 x, right panels 100 x. *** $P < 0.001$.

structural damage in a rat OA model *in-vivo*. We used multiple techniques to evaluate these outcome measures by blinded observers, including hind limb weight distribution to assess pain, uCT analyses to assess subchondral bone changes and histological evaluation of cartilage quality and synovial inflammation.

We did not find statistically significant differences between the different treatment groups on any of the structural outcome measures. The rat MSC treated group was the only group which significantly increased loading of the affected limb after treatment, although no significant difference at the endpoint of the study was found compared to the saline injected animals.

Since knee pain in OA patients is a major reason for knee replacements and pain is known to be badly correlated to structural damage [22,271], we consider it important to include this outcome measure to evaluate the efficacy of OA modifying therapies. To date, two other studies are known describing the influence of MSCs on pain and range of motion in large animal OA models, with conflicting results. Black et al. reported an improvement in lameness, pain on manipulation and range of motion in dogs treated with MSCs for naturally developed OA [48]. Frisbie et al. on the other hand, found no improvement in pain and range of motion following MSC treatment in a horse OA model [49]. Pain can be measured using several methods and is a complicated phenomenon consisting of inflammatory and neuropathic aspects [287,288]. Further evaluation of multiple pain aspects is needed to assess the capacity of MSCs to alleviate osteoarthritic pain.

One of the goals of our study was to compare the efficacy of allogeneic rat MSCs, allogeneic rat BMMNCs and xenogeneic human MSCs in their OA modifying capacity. Due to limited overall treatment effects, the value of this comparison is fairly limited. However, it should be noted that no severe adverse effects were observed in our study after intra-articular application of allogeneic rat MSCs, allogeneic rat BMMNCs or xenogeneic human MSCs. Albeit MSCs have been previously described to be immune privileged [249,289], more recent reports show that MSCs maintain a degree of immunogenicity that may limit their longevity and attenuate their advantageous effects [290,291,292]. We found no macroscopic signs of inflammatory responses in the (sub)acute phase after cell injections, indicating that they did not induce a substantial immune response. Nevertheless, we can not rule out that the MSCs used in this study elicited a mild immunological reaction given their non-autologous origin. This could account for the histological response of the synovial membrane in the group injected with rat and, more particularly, human MSCs as indicated by our explorative statistical analyses and might also counteract possible favorable effects of these cells, resulting in mild overall treatment effects.

We used a mild OA model since we hypothesized that a fully degenerated joint would be beyond the repair capacity of cellular therapies. A disadvantage of this approach is that at the moment of cell injections certain animals had not developed evident OA-like characteristics on µCTa. After excluding animals without MIA-induced cartilage damage at the moment of treatment (four animals in total), the possible beneficial effects of rat MSC compared to saline became more pronounced for all outcome measures, but still did not reach statistical significance (data not shown).

Previous studies have shown positive effects of intra-articularly injected MSCs on cartilage quality in various animal models [47,55,57]. These studies used surgical OA models with a joint instability component. Two of these reports used cell tracking and showed homing of the cells mainly to the damaged structure and the synovium [47,55]. This indicates an indirect protective effect of the MSCs and does not point towards actual regeneration of cartilage. The fact that there was no joint instability component in our model which could have been attenuated by MSCs, could explain the modest effects of our cellular treatments. This is in accordance with Frisbie et al., who found no effect of MSCs on cartilage quality in a horse osteochondral defect OA model [49]. In addition, Matsumoto et al. found just a trend towards improved cartilage quality after injection of muscle-derived stem cells (MDSCs) in a rat MIA OA model [54]. The effects of MDSCs became only clear after they were transduced with bone morphogenetic protein 4 and sFlt-1, a vascular endothelial growth factor antagonist. We used an OA model affecting the entire joint without mechanical instability, in that way resembling the majority of clinical OA patients. The fact that in such models repeatedly very modest effects of stem cell therapies are reported and that the effects are increased after genetic cell manipulation indicates that further optimization is required before large scale clinical application.

Another factor influencing the effect of MSCs could be the time of injection. We injected our cell preparations after the inflammatory phase of the MIA model had ceased. Although MSCs are known to have immunosuppressive capacities [249], MSCs need stimulation in order to exert their immunosuppressive role [293,294]. Possibly, the amount of inflammation in our model did not elicit an immunomodulatory MSC function, thereby minimizing potential beneficial effects.

The capacity of intra-articularly injected MSCs to regenerate cartilage in defects or to limit cartilage damage in surgical OA models with a joint instability component has been demonstrated by others [47,50,51,52,53,55,56,57,295], indicating the potential of this strategy for joint diseases. The effects of MSCs applied in OA models without an instability component as found by others [49,54] and presented in the current study are slightly beneficial, but overall remain modest. The fact that we did not observe clear effects in our study could have many causes including the use of a mild OA model, the absence of apparent inflammation upon time of injection or the fact that we used non-autologous cells. Further optimization could make use of cell tracking methods allowing further insight in the function and survival of the cells [50], genetic manipulation of administered cells [54] or by selection or pre-treatment of MSCs [296] to generate subpopulations which are most suitable for modifying OA processes. Since OA is a multifactorial disease consisting of many simultaneous processes, assessment of the effect of cellular therapies on structural joint aspects, joint inflammation as well as pain in various OA models and after application in early and later stages of the disease is essential in reaching these goals.

Acknowledgements
We acknowledge Anne-Marie Zuurmond, Angela Koudijs and Koen van der Mark (TNO, Leiden, the Netherlands) for providing the incapitance tester and their help in ascertaining its use; Erik Lubberts (Erasmus MC, Rotterdam, the Netherlands) for his recommendations in assessing synovial inflammation; and Sylvia van Buul for critically reviewing the manuscript. Furthermore, the authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science (SSM06004).

Chapter 6

Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI

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Contrast Media and Molecular Imaging. 2009;4(5):230-6

Abstract

Background: The use of superparamagnetic iron oxide (SPIO) for labeling cells holds great promise for clinically applicable cell tracking using magnetic resonance imaging (MRI). For clinical application, an effectively and specifically labeled cell preparation is highly desired (i.e. a high amount of intracellular iron and a negligible amount of extracellular iron). In this article we compare ferumoxides and ferucarbotran, two previously described SPIOs for labeling human Bone Marrow Stromal Cells (hBMSCs) and chondrocytes.

Methods: hBMSCs and chondrocytes were labeled using ferumoxides-protamine sulfate complexes or ferucarbotran. Labeling protocols for either approach were based on previously reported protocols. Outcome parameters were labeling efficiency, total iron load (TIL), and intra- and extracellular iron load. TIL of labeled cells was measured using inductively coupled plasma - optical emission spectrometry. Other outcome parameters were based on light microscopy evaluation of labeled cells, which were stained using Perl's iron stain.

Results: Cell labeling using ferumoxides-protamine resulted in higher labeling efficiencies for both cell types compared to ferucarbotran (96.1 vs 89.2% for hBMSCs and 99.8 vs 97.2% for chondrocytes). Furthermore, ferumoxides-protamine showed a higher TIL compared to ferucarbotran (89.6 vs 51.3 pg/cell for hBMSCs and 75.9 vs 28.1 pg/cell for chondrocytes). For both cell-types, ferumoxides-protamine labeling resulted in a higher intracellular iron load and a lower amount of extracellular iron aggregates compared to ferucarbotran.

Conclusions: Cell labeling using ferumoxides-protamine complexes and ferucarbotran resulted in histologically clearly visible labeling of both hBMSCs and chondrocytes. Ferumoxides-protamine complexes resulted in a superior labeling efficiency and TIL relative to labeling with ferucarbotran. Furthermore, ferumoxides-protamine complexes resulted in a higher intracellular iron load and a lower amount of extracellular iron. We consider ferumoxides-protamine to be a more effective and specific labeling method over ferucarbotran for SPIO labeling both hBMSCs and chondrocytes.

Introduction

Cell tracking is a necessary tool for determining the efficacy and safety of cell based regenerative therapies. Cell labeling using superparamagnetic iron oxide (SPIO) particles allows subsequent in vivo cell tracking using magnetic resonance imaging (MRI) and has already entered the clinical arena [62,63,64]. Ferumoxides and ferucarbotran are two SPIOs frequently described for the purpose of cell labeling and -tracking using MRI. Both SPIOs have comparable particle sizes (60-150 nm), but whereas ferumoxides particles have a dextran coating, ferucarbotran particles are carboxydextran coated [297]. These additional carboxyl groups associated with ferucarbotran lead to a higher affinity to the cell membrane [298]. This difference in coating appears to be the main reason why efficient labeling of non-phagocytic cells with ferumoxides requires additional use of a transfection agent, while adequate labeling of non-phagocytic cells with ferucarbotran can be accomplished without the need of an additional agent [297,298]. Several transfection agents are being used to facilitate cellular incorporation of ferumoxides [298,299]. Amongst them, protamine sulfate is the most interesting from a clinical point of view since it is already used for, and has FDA approval as, a heparin antidote [300]. When applying SPIO labeled cells in vivo or clinically, an effectively and specifically labeled preparation of cells is highly desired (i.e. a high intracellular iron load and a negligible amount of extracellular iron). Effective labeling is essential since MRI sensitivity is reported to be influenced by total iron load (TIL) per cell [301]. Moreover, the endocytosed SPIO particles are known to dilute upon 5-8 cell divisions [302]. A high intracellular amount of iron is likely to positively influence the duration of MRI traceability. Extracellular iron on the other hand, could potentially be endocytosed by host cells, or generate MRI signal voids on its own. Effective cell labeling of different cell types, including human bone marrow stromal cells (hBMSCs), using ferumoxides-protamine or ferucarbotran has been described in numerous publications [297,298,300,303,304,305,306]. Although for both approaches comparable labeling efficiencies up to approximately 100% have been reported regarding hBMSC labeling, both methods are hampered by the occurrence of extracellular iron nanoparticle aggregates [298,300,304,305,306,307]. No publications are known comparing ferumoxides-protamine sulfate to ferucarbotran labeling directly on the same cell-types. This study aims to compare both approaches based on accepted protocols found in current literature. Outcome measures were labeling efficiency, TIL, intra- and extracellular iron load. We performed our experiments on the two cell types most commonly used in cell based cartilage repair: chondrocytes and hBMSCs.

Methods

Cell culture
hBMSCs and human chondrocytes were isolated and cultured using previously described procedures [157,313] (after informed consent; MEC-2004-142)(after approval by the local ethical committee; MEC-2004-322).. .

Fresh medium was applied to all cells every 3 to 4 days. All media consisted of DMEM containing 10% FCS, 50 µg/ml gentamicin and 1.5 µg/ml fungizone. Cells were trypsinized at subconfluency and subsequently passaged. Cells from the third to the sixth passage, both freshly collected and cryopreserved, were used for the labeling experiments.

Cell labeling
Cells were labeled using ferumoxides (Endorem®, Guerbet S.A., Paris, France) -protamine sulphate (LEO Pharma N.V., Wilrijk, The Netherlands) complexes or ferucarbotran (Resovist®, Bayer Schering Pharma AG, Berlin, Germany). Labeling protocols used for both SPIOs closesely resembled labeling protocols found in literature [297,298,300,303,304,305,306].

Ferumoxides- protamine sulfate
Both hBMSCs and chondrocytes were grown till 80-90% confluency in six-well plates. At that time, existing medium was removed and 1 ml of fresh DMEM containing 10% FCS was added per well. Protamine sulfate was prepared as a fresh stock solution of 1 mg/ml in distilled water. Ferumoxides was diluted in serum-free DMEM to a final concentration of 100 µg/ml. Protamine sulfate was added to the ferumoxides solution to a final concentration of 5 µg/ml. After 3 to 5 minutes of intermittent shaking by hand at room temperature, the ferumoxides-protamine solution was added to the freshly applied medium on the cells in a 1:1 v/v ratio (final dose 100 µg of iron per well of a six-well plate).

Ferucarbotran
Both hBMSCs and chondrocytes were seeded in six-well plates at a density of 50,000 cells per cm^2 on day one. After 24 hours, existing medium was discarded. Subsequently, 1 ml of fresh medium was applied and ferucarbotran was added to the media at a final concentration of 100 µg/ml (final dose 100 µg iron per well of a six-well plate).

Labeling efficiency
Medium was removed after 24 hours of incubation with either ferumoxides-protamine complexes or ferucarbotran. Cells were washed twice using PBS, trypsinized and replated in six-well plates (seeding density 10,000 cells/cm^2). Replated cells were fixed within one day and stained using Perl's iron stain (Klinipath BVBA, Duiven, The Netherlands) according to the manufacturers protocol. All samples were evaluated using light microscopy. Labeling efficiency was based on manually counting stained cells. Cells

were considered positive if blue granules were present within cell boundaries. A minimum of 100 randomly selected cells was counted per sample at 400X magnification.

Total iron load per cell

TIL was measured in samples containing trypsinized cells using inductively coupled plasma - optical emission spectrometry (ICP-OES). Cell pellets of unlabeled and labeled cells were dried for 72 h at 60°C. Then they were digested in 40µl of a 3:1 v/v mixture of ultra-pure perchloric acid (EM Science, Gibbstown, NJ, USA) and ultra-pure nitric acid (JT Baker, Deventer, The Netherlands) at 60 ºC for 24 hours. To the digested substance 4 ml MiliQ was added and emission was measured at 259nm with a Perkin Elmer Optical Emission Optima 4300 DV Spectrometer. The amount of iron per sample was determined by calibration to a standard curve, which was generated using a commercially available ICP-OES standard iron solution (Merck, Schiphol, The Netherlands) in a range of 0 to 50 µg/ml.

Evaluation of intra- and extra-cellular iron

To further discriminate between intra- and extracellular iron load, a grading score was developed. In this score, quantity of both intra- and extracellular iron was graded on a four-point scale (table 1). All samples were stained using Perl's iron stain and evaluated using light microscopy. Amount of intracellular iron was based on number of intracellular blue granules. A minimum of 100 randomly selected cells per sample was manually evaluated for intracellular iron according to this score at 400X magnification. Extracellular iron was quantified based on number and size of extracellular SPIO aggregates. Analysis of extracellular iron was based on 40 randomly selected fields of view at 100X magnification in order to get a representative evaluation of the total well. Iron particles were considered extracellular if they were not localized within cell boundaries. Iron within the boundaries of dead cells was not considered extracellular. All samples were evaluated independently by two blinded observers.

Data analysis

All experiments have been performed with triplicate samples for each of the three different hBMSC and chondrocyte donors except for TIL measurements, which were performed with triplicate samples for one hBMSC and one chondrocyte donor. Independent scores of both observers were compared and proved to be similar. For further calculations all data from both observers regarding intra- and extracellular iron was accumulated per sample. Data is shown in terms of percentage distribution of different grades of intra- and extracellular iron of all evaluated samples. Labeling efficiencies and percentages of different grades were compared using a mixed model ANOVA, in which treatment was considered a fixed factor and the three different donors a random factor. A P value < 0.05 was considered statistically significant.

Results

Labeling efficiency and total iron load per cell

SPIO labeling using ferumoxides-protamine or ferucarbotran resulted in histologically clearly visible label uptake in both cell types (Figure 1). Blue stained SPIO particles were seen in the cytoplasm of cells, mostly around the nuclei. No apparent effects of either labeling procedures were seen in terms of changes in cell morphology or viability. Ferumoxides-protamine showed a higher labeling efficiency compared to ferucarbotran for hBMSCs (96.1 ± 4.5% vs 89.2 ± 8.0%; P = 0.008) and chondrocytes (99.8 ± 0.3% vs 97.2 ± 1.8%; P < 0.000). ICP-OES measurements of ferumoxides-protamine labeled cells showed a higher TIL for hBMSCs (of 89.6 ± 14.9 vs 51.3 ± 4.35 pg/cell) and chondrocytes (75.9 ± 9.53 vs 28.1 ± 3.04 pg/cell) compared to ferucarbotran.

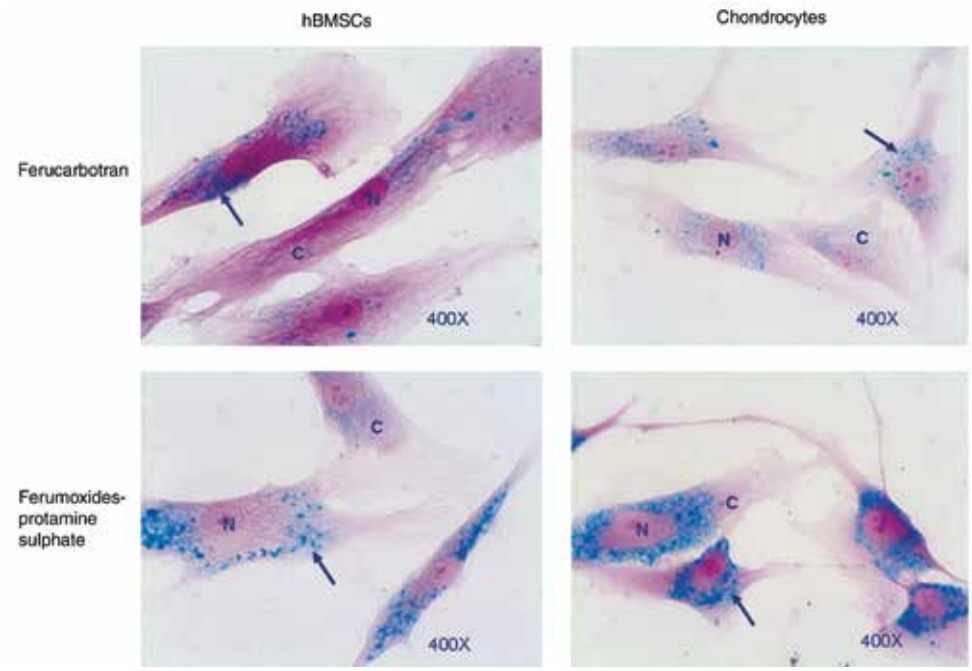

Figure 1: Perl's iron stain of SPIO labeled hBMSCs (left) and chondrocytes (right). Blue stained SPIO particles (arrows) are present in the cytoplasm (C) around the nuclei (N) in all conditions.

Evaluation of intra- and extracellular iron

For further evaluation of the distribution of TIL between the intra- and extracellular compartment we scored all samples using a four-point grading system (table 1). Figure 2A shows representative examples of all different grades of intracellular iron. Ferumoxides-protamine complexes resulted in a markedly higher intracellular iron load compared to

ferucarbotran for both cell types (Figure 2B). In ferumoxides-protamine labeled hBMSCs 64.2 ± 26.1% of cells had an intermediate to high intracellular iron load compared to 21.9 ± 22.0% of ferucarbotran labeled hBMSCs ($P < 0.000$). In ferumoxides-protamine labeled chondrocytes 96.6 ± 2.2% of cells showed an intermediate to high amount of intracellular iron versus 45.6 ± 8.4% in the ferucarbotran labeled cells ($P < 0.000$).

Table 1: Description of four-point grading score used for evaluation of intra- and extracellular iron

Intracellular iron load	**Appearance intracellular compartment**
Absent	No blue granules
Low	1-20 blue granules
Intermediate	21-100 blue granules
High	>100 blue granules or granules no longer separately distinguishable
Extracellular iron load	**Appearance extracellular compartment**
Absent	No aggregates
Low	1-5 aggregates < 1 cell size
Intermediate	>5 aggregates < 1 cell size or 1-5 aggregates 1-10 cell sizes
High	>5 aggregates 1-10 cell sizes or aggregate(s) > 10 cell sizes

The amount of extracellular iron was evaluated using a comparable four-point grading system (table 1). Extracellular iron presented as aggregates varying in size from barely visible to large aggregates up to the equivalent of fifty cell diameters. Aggregates in all samples were seen both sticking to cell membranes and as loose aggregates. Figure 2C shows representative examples of all different grades of extracellular iron. Ferumoxides-protamine labeling of cells resulted in less extracellular iron compared to ferucarbotran in both cell-types (Figure 2D). In ferumoxides-protamine labeled hBMSCs 19.3 ± 13.7% of fields of view showed an intermediate to high amount of extracellular iron, compared to 47.6 ± 19.1% in the ferucarbotran labeled conditions ($P = 0.001$). When labeling chondrocytes a comparable difference was observed. In 20.2 ± 11.1% of fields of view an intermediate to high amount of extracellular iron was observed in the ferumoxides-protamine labeled condition, compared to 57.2 ± 10.2% in the ferucarbotran labeled cells ($P < 0.000$).

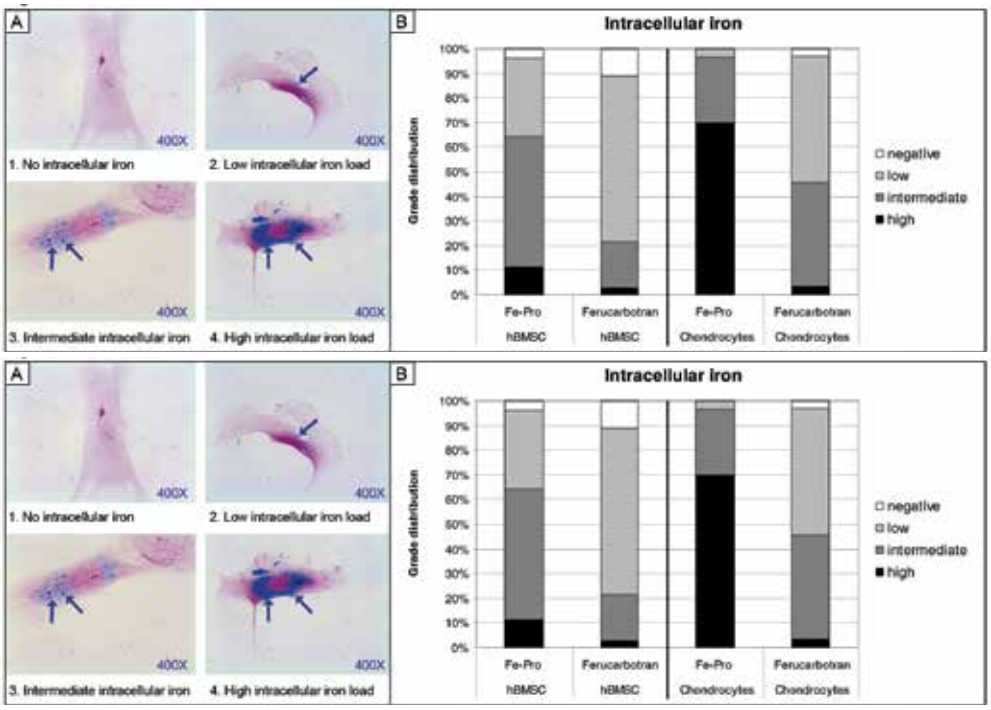

Figure 2: Perl's iron stain of representative examples of all four different intra- and extracellular iron grades (A, C) and intra- and extracellular iron grade distribution (B, D) of hBMSCs and chondrocytes labeled using ferumoxides-protamine (Fe-Pro) and ferucarbotran. Ferumoxides-protamine resulted in a higher percentage of cells showing a high to intermediate amount of intracellular iron particles (B) and a lower percentage of fields of view showing a high to intermediate amount of extracellular iron aggregates (D). Arrows: intra- and extracellular blue stained SPIO particles.

Discussion

In this study we performed a direct comparison on two primary cell types (hBMSCs and chondrocytes) of two SPIO labeling approaches using ferumoxides-protamine complexes and ferucarabotran. Both strategies resulted in effective labeling of both cell types. However, we did find significant differences in the quality of cell labeling. For both cell types ferumoxides-protamine resulted in a higher percentage of labeled cells (96.1-99.8% vs 89.2-97.2%) and a higher TIL (75.9-89.6 pg/cell vs 28.1-51.3 pg/cell) compared to ferucarbotran. In addition, ferumoxides-protamine complexes also resulted in a higher amount of intracellular iron together with a lower amount of extracellular iron aggregates in both cell-types.

Both ferumoxides and ferucarbotran contain particles ranging from 60-150 nm [297]. When complexing ferumoxides to protamine sulfate however, much larger particles are formed up to approximately 2,000-2,500 nm [308]. Several articles reported the fact

that cellular uptake in transfection experiments is positively influenced by increasing particle size of transfection complexes [309,310]. These articles describe the fact that larger particles can sediment faster onto cells and that larger particles have a bigger payload. The latter results in the delivery of more transfection material at a similar cell-surface occupation.

Table 2. Overview of literature reporting hBMSC labeling using ferumoxides-protamine sulfate or ferucarbotran.

Reference	SPIO-TA + concentration SPIO	Labeling efficiency	Cellular iron load (pg/cell)	Extracellular SPIO aggregates
[303] Arbab et al. (2004)	Ferumoxides-protamine 50µg/ml	± 100%	10.94 ± 1.86	NM
[304] Arbab et al. (2005)	Ferumoxides-protamine 50µg/ml	± 100%	NM	Describes heparin wash to remove extracellular aggregates
[300] Pawelczyk et al. (2006)	Ferumoxides-protamine 50µg/ml	± 100%	44.7 ± 0.34.	Describes heparin wash to remove extracellular aggregates
[305] Omidkhoda et al. (2007)	Ferumoxides-protamine 25-125µg/ml	71-87%	Absolute value NM	Minimal extracellular aggregates observed
[306] Pawelczyk et al. (2008)	Ferumoxides-protamine 50µg/ml	± 100%	34.75 ± 0.32	Describes heparin wash to remove extracellular aggregates
[297] Hsiao et al. (2007)	Ferucarbotran 100 µg/ml	± 100%	23.4	NM
[298] Mailander et al. (2008)	Ferucarbotran 250 µg/ml	NM	± 43. After FACS for live cell fraction ± 9	Extracellular aggregates indirectly quantified

Comparable labeling efficiencies and cellular iron loads have been reported for hBMSCs labeling using ferumoxides-protamine sulfate and ferucarbotran. Majority of articles mention the occurrence of extracellular iron aggregates. Mailander et al. indirectly quantified this amount of extracellular iron. TA: transfection agent. NM: not mentioned.

Several studies describe the use of ferumoxides-protamine complexes or ferucarbotran for labeling of hBMSCs [297,298,300,303,304,305,306]. In general, these reports show comparable labeling efficiencies and cellular iron loads for both techniques (table 2). Labeling efficiencies of 71-100% have been described, dependent on the protocol used. Iron load per cell ranged from 10.9 - 44.7 pg/cell. Unfortunately none of the published articles directly compared both labeling methods. In our study the observed labeling efficiencies and TIL per cell for hBMSCs labeling using both ferumoxides-protamine and ferucarbotran were similar to those previously reported, making it possible to relate our results to the articles listed in table 2. In various studies modifications of labeling protocols have been mentioned in order to increase cellular uptake or decrease the occurrence of extracellular iron deposits. Several publications describe the use of heparin washes (10 U/ml) to dissolve extracellular ferumoxides-protamine sulfate complexes [300,304,306]. Moreover, Pawelczyk et al. report in their publications the approach of allowing cells to take up additional iron complexes for two days after the labeling procedure [300,306]. We did not include heparin washes or additional time for ferumoxides-protamine complex endocytosis in our main experiments, in order to better compare both techniques. In additional experiments, where we included heparin washes (10 U/ml) and additional time to endocytose ferumoxides-protamine complexes, the amount of extracellular iron appeared to decrease during microscopic evaluation (data not shown). This observation was made for both cell types, making ferumoxides-protamine even more favourable over ferucarbotran.

Ferucarbotran labeled hBMSCs showed rather poor intracellular labeling with approximately 80% of cells showing no or low amounts of intracellular iron. Although not the primary scope of this report, we adjusted the labeling protocol regarding ferucarbotran in order to increase the quantity of intracellular iron. We were able to greatly improve cellular SPIO uptake of hBMSCs labeled with ferucarbotran, by labeling 5 hours after cells were seeded instead of 24 hours and by labeling in a serumfree environment (data not shown). Unfortunately this simultaneously led to an increase in amount of extracellular iron observed, making this an unfavourable option.

In our study we did not perform differentiation, viability or cytotoxicity assays. Cell labeling using both SPIOs has been shown not to influence several cell behaviour characteristics [297,298,300,303,304,305]. [311]. We did not observe increased cell death or changes in morphology between labeled cells versus unlabeled controls for both cell-types. Labeling chondrocytes using ferumoxides-protamine did result in a distinct higher amount of intracellular iron compared to hBMSC labeling. Differences between labeling different cell-types using SPIOs have been reported previously [302,312]. The high amount of intracellular iron might have an effect on chondrocyte metabolism. This needs to be further investigated before proceeding to any in vivo or clinical experiments.

Conclusions

We have shown ferumoxides-protamine sulfate to be a more effective and specific way of SPIO labeling compared to ferucarbotran for both primary hBMSCs and human chondrocytes.

Acknowledgements
The authors thank Erwin Waarsing (Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands) for his assistance with statistical analyses and Ulla Woronieck and Bert Wolterbeek (Technical University Delft, Delft, The Netherlands) for their assistance with the ICP-OES measurements. Furthermore, the authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science; and the ENCITE consortium of the European Community under the 7th framework programme

Chapter 7

Clinically translatable cell tracking and quantification by MRI in cartilage repair using Superparamagnetic Iron Oxides

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PLoS One. 201;6(2):e17001

Abstract

Background: Articular cartilage has very limited intrinsic regenerative capacity, making cell-based therapy a tempting approach for cartilage repair. Cell tracking can be a major step towards unraveling and improving the repair process of these therapies. We studied superparamagnetic iron oxides (SPIO) for labeling human bone marrow-derived mesenchymal stem cells (hBMSCs) regarding effectivity, cell viability, long term metabolic cell activity, chondrogenic differentiation and hBMSC secretion profile. We additionally examined the capacity of synovial cells to endocytose SPIO from dead, labeled cells, together with the use of magnetic resonance imaging (MRI) for intra-articular visualization and quantification of SPIO labeled cells.

Methods: Efficacy and various safety aspects of SPIO cell labeling, were determined using appropriate assays. Synovial SPIO re-uptake was investigated *in vitro* by co-labeling cells with SPIO and green fluorescent protein (GFP). MRI experiments were performed on a clinical 3.0T MRI scanner. Two cell-based cartilage repair techniques were mimicked for evaluating MRI traceability of labeled cells: intra-articular cell injection and cell implantation in cartilage defects. Cells were applied *ex vivo* or *in vitro* in an intra-articular environment and immediately scanned.

Results: SPIO labeling was effective and did not impair any of the studied safety aspects, including hBMSC secretion profile. SPIO from dead, labeled cells could be taken up by synovial cells. Both injected and implanted SPIO-labeled cells could accurately be visualized by MRI in a clinically relevant sized joint model using clinically applied cell doses. Finally, we quantified the amount of labeled cells seeded in cartilage defects using MR-based relaxometry.

Conclusions: SPIO labeling appears to be safe without influencing cell behavior. SPIO labeled cells can be visualized in an intra-articular environment and quantified when seeded in cartilage defects.

Introduction

Articular cartilage provides low friction and allows for efficient load bearing and distribution in synovial joints. Cartilage is avascular, aneural and lacks lymphatic drainage. Consequently, natural repair mechanisms are underprovided, giving cartilage a very limited intrinsic regenerative capacity [13]. Current cell-based cartilage repair techniques aim at restoration of the functional properties of damaged cartilage. Human bone marrow-derived mesenchymal stem cells (hBMSCs) and chondrocytes are two cell-types currently used for these approaches

[216,237,314,315]. hBMSCs can be either implanted in focal defects, or injected intra-articularly to repair more generalized cartilage lesions like osteoarthritis [237,315]. Chondrocytes are mainly being used for implantation in focal cartilage defects, a procedure known as autologous chondrocyte implantation (ACI) [216,314]. Despite the improvements made in cell-based cartilage regeneration in the past decades, repaired cartilage does not entirely resemble native cartilage and can not guarantee a permanent solution at this moment [217].

To elucidate the relevant repair mechanisms and for optimization of cell-based therapies, it is necessary to determine the fate of implanted cells. Superparamagnetic iron oxides (SPIOs), such as ferumoxides, have already been used clinically for cell labeling and *in vivo* cell tracking in dermal oncology, neural regeneration and pancreatic islet transplantation [316]. No negative effects of the labeling procedure have been reported in these initial clinical studies. Before implementing this approach to an orthopedic application, any possible negative influence of SPIO labeling on cell behavior must be ruled out. Besides repopulation of damaged tissue through proliferation and differentiation of hBMSCs, secretion of certain trophic factors by transplanted hBMSCs has more recently been suggested as a possible mechanism by which hBMSCs promote tissue regeneration [4]. These bioactive molecules could provide a regenerative microenvironment to initiate a self-regulated regenerative response. To our knowledge, the effects of SPIO labeling on factors secreted by hBMSCs have not been investigated before. Another point of interest is the feasibility of tracking SPIO-labeled cells within the joint in relation to other anatomical structures, using a clinical magnetic resonance imaging (MRI) protocol, in a model of clinically relevant size. For these purposes, it must be checked how long cells retain the SPIO label and it should be validated if generated MRI signals arise from originally labeled cells. Next to cell visualization, quantification of labeled cells implanted in cartilage defects by MRI could provide an exquisite opportunity to longitudinally map the role and contribution of implanted cells to cartilage repair. The aim of this study was to label hBMSCs using SPIOs and evaluate the effects of iron incorporation on cell viability, long term metabolic cell activity, differentiation and secretion profile. Furthermore we examined the SPIO retention of labeled cells and the capacity of synovial cells to endocytose SPIO from dead, labeled cells. Finally, we studied the use of MRI for accurate intra-articular visualization and quantification of SPIO labeled cells.

Materials and Methods

Ethics Statement

All human samples were obtained after approval by the Erasmus MC medical ethical committee. hBMSCs were isolated from heparinized femoral-shaft marrow aspirate of patients undergoing total hip arthroplasty (after written informed consent; protocol # MEC-2004-142). Articular cartilage and synovial explants were obtained as redundant material from patients undergoing total knee replacement surgery. All patients implicitly consented to the use of these tissues for scientific research (protocol # MEC-2004-322).

Cell isolations and cultures

hBMSCs and human chondrocytes were isolated and cultured using previously described procedures [157,245]. All isolated cells or explants were cultured in DMEM containing 10% FCS, 50 µg/ml gentamicin and 1.5 µg/ml fungizone (defined as standard medium) unless stated otherwise. hBMSCs were cultured using additional 1ng/ml FGF2 and $1x10^{-4}$ M vitamin C. All hBMSCs and chondrocytes were used at passage 2-5 in labeling experiments.

SPIO labeling

SPIO labeling was performed using ferumoxides (Endorem®, Guerbet S.A., Paris, France) complexed to protamine sulfate (LEO Pharma N.V., Wilrijk, Belgium) as described earlier [331]. For removal of extracellular iron, cells were washed with PBS containing heparin 10 U/ml (LEO Pharma B.V., Breda, the Netherlands). SPIO labeling mix was made at a constant concentration of 100 µg/ml ferumoxides with 5 µg/ml protamine to ensure identical particle formation [308]. Cells were labeled with doses ranging from 2.5–25 µg/cm^2 of ferumoxides.

Labeling efficiency, chondrogenic differentiation and functional assays

Histological iron detection was performed using a Perl's iron stain (Klinipath BVBA, Duiven, the Netherlands) according to the manufacturer's protocol. Labeling efficiency was determined in triplicate samples for three donors by manually counting labeled cells stained with Perl's iron [331]. Total iron load was measured in triplicate samples from three donors by inductively coupled plasma - optical emission spectroscopy [331]. Cell viability was evaluated by trypan blue exclusion. After labeling, cells were seeded in 96-well plates at a density of 10,000 cells per well. AlamarBlue® (Invitrogen, Breda, the Netherlands) was used according to manufacturer's protocol for longitudinal measurement of metabolic cell activity at day 0, 3 and 7.

Chondrogenic differentiation was performed in triplicate samples on hBMSCs pellets from two donors according to previously reported protocols [334]. Chondrogenically differentiated hBMSCs were stained using a collagen II monoclonal antibody (II-II6B3;

Developmental Studies Hybridoma Bank, Iowa City, IA) and thionine stain [334,335]. hBMSCs used for gene expression analysis and glycosaminoglycan (GAG) measurements were chondrogenically differentiated in alginate beads [87] using an otherwise identical differentiation protocol. RNA extraction, cDNA synthesis, real-time RT-PCR and collagen II primers for gene expression analysis were described earlier [90]. GAG measurements were performed using a dimethylmethylene blue (DMB) assay [336].

To investigate the influence of SPIO labeling on cytokines secreted by hBMSCs, labeled and unlabeled cells from five donors were cultured for 24 hours in serumfree standard medium supplemented with ITS+1 (serum replacement; BD Bioscience, Bedford, MA) in a 1:100 v/v ratio. Secretion of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-6, IL-10, vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) were analyzed in duplicate samples using a human Cytokine Bead Array flexsets (BD Bioscience, Breda, the Netherlands) according to the manufacturer's protocol. Flow cytometric measurements were performed on a FACSCanto II (BD, San Jose, CA, USA) and analyzed using FACSDiva software version 6.1.2 (BD Biosciences).

Intra-articular cell injections and cell implantations

Due to the *ex vivo* nature of our MRI experiments, all cells were fixed in 4% formalin prior to injection or implantation. SPIO-labeled hBMSCs were injected *ex vivo* into porcine knees (Yorkshire x Landrace, 35 kilograms, age 4-5 months) in a dose range of $0.1x10^6$ to $5x10^6$ labeled cells (injected in 100 µl physiological saline). For cell implantation, osteochondral plugs with a diameter of 8 mm were harvested from porcine femoral condyles *ex vivo*. Circular full-cartilage defects of 6 mm diameter (approximate volume of 75 µl) were created in those plugs. Cells from two donors were suspended in 1% Agar (Fluka BioChemika, Sigma-Aldrich, Buchs, Switzerland) in saline before implantation. A total of $1x10^6$ cells were seeded per defect *in vitro* (cell concentration of $13x10^6$ cells/ml), containing 0.3 – 100% labelled cells (i.e. $0.04x10^6$–$13x10^6$ labeled cells/ml). Cell implantation was also performed *ex vivo* in an intact porcine knee according to previously described autologous chondrocyte implantation (ACI) procedures [216]. The knee joint was opened and a circular cartilage defect was created (diameter 6 mm; volume 75 µl). A periosteal flap was sutured on top of the defect using PDS 6-0 sutures (Johnson & Johnson, New Brunswick, New Jersey) and fibrin glue (Tissucol® Duo 500, Baxter, Uden, the Netherlands) was applied at 90% of the edges of the flap as a sealant. $1x10^6$ SPIO-labeled cells suspended in 1% Agar, were seeded into the defect and fibrin glue was used to seal the final 10% of the periostal flap margin. The joint was closed in layers afterwards. All surgical procedures - except the cell seeding and fibrin glue application - were performed under water in order to prevent air artifacts on MRI.

Validation SPIO-generated signal

To validate if SPIO-generated signal was related to originally labeled cells, or that SPIO particles were taken up by host cells, human green fluorescent protein (GFP) trans-

fected chondrocytes were co-labeled with SPIO. Cells were lentivirally transduced and constitutively expressed GFP under the control of a CMV promoter (SHC003 vector, Sigma-Aldrich Mission®, shRNA library). To investigate the fate of SPIO after cell death, we considered the cell type used for labeling less relevant and chose chondrocytes over hBMSCs due to their substantially higher transduction efficiency. 25 x 10^4 GFP^+-$SPIO^+$ cells were seeded in duplicates on synovial explants from two human donors. Cells were alive or killed (by repeated free-thaw cycles) before seeding. Cells and synovial explants were co-cultured for five days. Fluorescent images were made from frozen sections using an Axiovert s100 microscope (Carl Zeiss B.V., Sliedrecht, the Netherlands). Subsequently, samples were stained using Perl's iron stain and corresponding fields of view were evaluated by light microscopy. GFP and SPIO containing cells were determined by manually counting a minimum of 100 cells per sample.

Magnetic Resonance Imaging

Scanning was performed on a clinical 3.0T MRI scanner (General Electric Healthcare, Milwaukee, Wisconsin). A 3D SPGR sequence was used to scan labeled hBMSCs *in vitro* (TR 87.7 ms, TE 15.1 ms, FOV 17 mm, matrix 512x512, scanning time 11 min. 35 sec.) in combination with a custom made receive-only surface coil with a diameter of 1 cm. Scanning of injected or implanted hBMSCs in intact porcine knees was performed using a FIESTA-3D sequence (TR 7.9 ms, TE 2.2 ms, FOV 140 mm, matrix 512x256, scanning time 28 min. 46 sec.) and a clinical phased-array head coil. Images of hBMSCs implanted in defects *in vitro* were obtained by a Spin Echo sequence (TR 1500 ms, TE 40 ms, FOV 40 mm, matrix 512x512, scanning time 14 min. 42 sec.) and a custom made receive-only surface coil with a diameter of 3 cm. R2*-mapping was performed in duplicate samples for two hBMSC donors by a SPGR sequence (TR 45ms, TE 4 – 120 ms, FOV 40 mm, matrix 256x256, scanning time 21 min. 21 sec.). R2* values were calculated and validated by mono-exponential curve fitting (MATLAB 7.0.1, MathWorks, MA). Voxels with an r^2 <0.95 were excluded from the measurements.

Statistical analysis

Statistical differences between different treatment groups were analysed by the paired Student t-test (normality test $p>0.05$). P-values of ≤ 0.05 were considered statistically significant. All numerical data are presented as mean ± standard deviation.

Results

Labeling effectivity and cell behavior.

hBMSCs were efficiently labeled with ferumoxides-protamine sulfate complexes (Figure 1A). We observed a labeling efficiency ranging from 41.2 ± 33.5 % at an SPIO dose of 2.5 µg/cm2 to 94.5 ± 7.8 % at an SPIO dose of 25 µg/cm2 (Figure 1B) with resulting average total iron loads (TILs) of cells ranging from 4.0 ± 2.9 pg/cell to 19.5 ± 6.1 pg/cell (Figure 1C). Both labeling efficiency and TIL increased in relation to SPIO labeling dose up to a dose of 10 µg/cm^2. The higher SPIO dose of 25 µg/cm^2 did not significantly increase labeling efficiency (*P = 0.42)* or average TIL (*P = 0.50)*. hBMSC viability was not influenced by SPIO labeling for dosages up to 25 µg/cm^2 (Figure 1D). Additionally, SPIO labeling did not inhibit cell metabolic activity compared to unlabeled controls for at least seven days (Figure 1E). Based on these results an SPIO dose of 10 µg/cm^2, corresponding to a final labeling concentration of 50 µg/ml SPIO, was considered optimal and used in all further experiments.

Chondrogenic differentiation was studied in cell pellets consisting of labeled and unlabeled cells at different ratios (0, 10, 50 and 100% of labeled cells). Differentiation was unaffected in all pellets containing labeled hBMSCs (10 - 100%) compared to control pellets consisting of unlabeled cells only. All pellets displayed a cartilaginous extracellular matrix rich in GAG and collagen type II (Figure 2A-H). Moreover, quantitative measurements revealed SPIO-labeled hBMSCs to produce comparable amounts of GAG

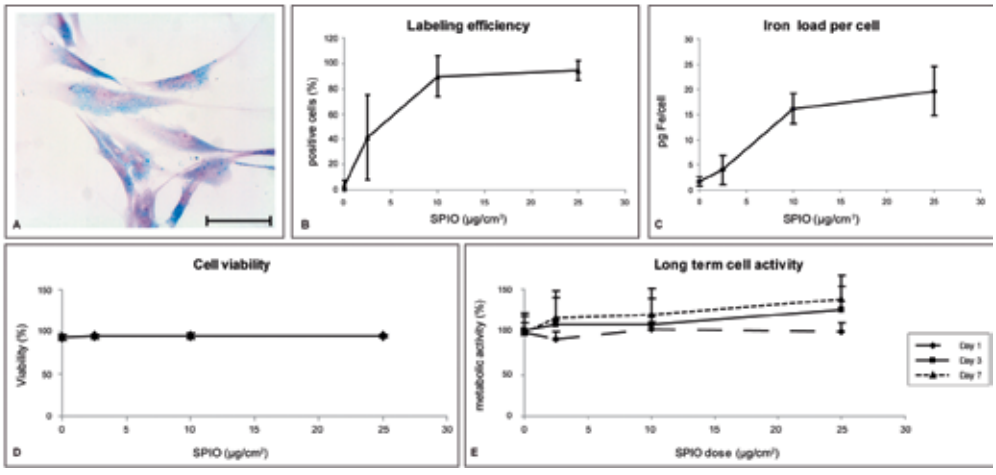

Figure 1. Labeling human Bone Marrow stroma-derived mesenchymal Stem Cells (hBMSCs) using Superparamagnetic Iron Oxides (SPIO). Perl's iron stain showing effective endocytosis of SPIO (A), leading to labeling efficiencies of approximately 95% (B) and a total iron load of approximately 20 pg/cell (C). SPIO labeling did not impair cell viability (D) or subsequent metabolic cell activity (E) at any dose used. Results shown for triplicate samples from three hBMSC donors.

(Figure 2I). In addition, gene expression of collagen type II was unaltered in SPIO-labeled hBMSCs after chondrogenic differentiation (Figure 2J).

To evaluate the influence of iron incorporation on cell secretions, we tested six cytokines containing anti- and pro-inflammatory cytokines as well as growth factors. No effect of SPIO-labeling on the secretion profiles was found (Table 1). SPIO-labeled hBMSCs produced comparable quantities of the anti- and pro-inflammatory cytokine IL-6 (P = 0.39) and the growth factor VEGF (P = 0.31) compared to unlabeled control cells. Further-

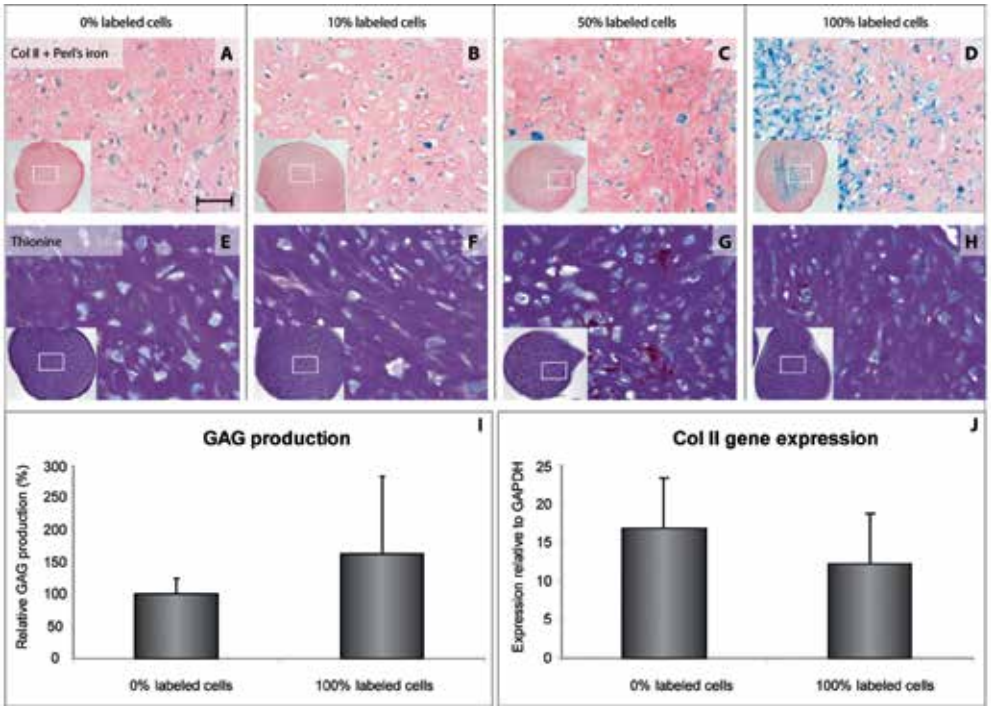

Figure 2. Chondrogenic differentiation of hBMSCs is not affected by SPIO labeling. Collagen II immunohistochemistry and Perl's iron stain demonstrated SPIO labeling to have no effect on pellet size or collagen II production, while iron remained present in differentiated pellets (A-D). Glycosaminoglycan deposition was also unaffected, as can be seen by thionin stain (E-H) and DMB assay (I). Gene expression of collagen II was unaltered by SPIO labeling (J). All analyses were performed after 35 days of differentiation. Insets display low magnification overviews of the entire pellets. Results shown for triplicate samples from two hBMSC donors.

more, SPIO labeling did not induce the production of the pro-inflammatory cytokines TNFα and IFNγ, the anti-inflammatory cytokine IL-10 or the growth factor FGF2. These four factors were not detectably secreted by control or SPIO-labeled hBMSCs.

Cell visualization by MRI.

All cell doses injected intra-articularly could be visualized by MRI. The number of injected cells was related to the number and extent of areas with signal loss detected by

Table 1: Cytokine secretion of hBMSCs

Cytokine (all values in pg/ml)	Control cells	SPIO labeled cells
TNFα	≤ d.l.	≤ d.l.
IFNγ	≤ d.l.	≤ d.l.
IL-6	489 ± 613	330 ± 242
IL-10	≤ d.l.	≤ d.l.
VEGF	193 ± 103	265 ± 102
FGF2	≤ d.l.	≤ d.l.

hBMSC secretion profile of four cytokines and two growth factors was not influenced by SPIO labeling. Cytokines were measured in serum free 24 hour supernatant of labeled and unlabeled cells. SPIO did not induce the secretion of pro-inflammatory cytokines (TNFα, IFNγ), nor did it alter the secretion of the anti- and pro-inflammatory cytokine IL-6 or the growth factor VEGF. The anti-inflammatory factor IL-10 and growth factor FGF2 were not secreted at a detectable level by control or SPIO-labeled cells. TNF-α: tumor necrosis factor-alpha, IFN-γ: interferon-gamma, IL: interleukin, VEGF: vascular endothelial growth factor, FGF: fibroblast growth factor, ≤ d.l.: below detection level (10pg/ml). Results shown for duplicate samples from five hBMSC donors.

MRI (Figure 3A-D, arrows). Injected hBMSCs were found scattered throughout the joint, clearly distinguishable from anatomical structures in all conditions. In addition, presence of labeled cells did not interfere with regular evaluation of knee anatomy.

For cell implantation experiments we created cartilage defects of 75 µl in porcine osteochondral plugs *in vitro* (Fig. S1A). Labeled hBMSCs implanted in cartilage defects were homogenously distributed and could be clearly distinguished from surrounding bone and cartilage by MRI. The extent of signal loss in the seeded plugs was dependent on the labeled cell dose used (Figure 3E-I). The minimal labeled cell concentration we could visualize was 0.4 x 10^6/ml. To show the potential applicability of this technique in a clinical ACI-like procedure, we subsequently implanted labeled hBMSCs in a cartilage defect in an intact porcine knee. In this setting the SPIO-labeled cells were also clearly distinguishable from the surrounding tissues (Figure 3J). Post MRI, Perl's iron stain confirmed SPIO positive cells to be present inside the defect (Fig. S1B).

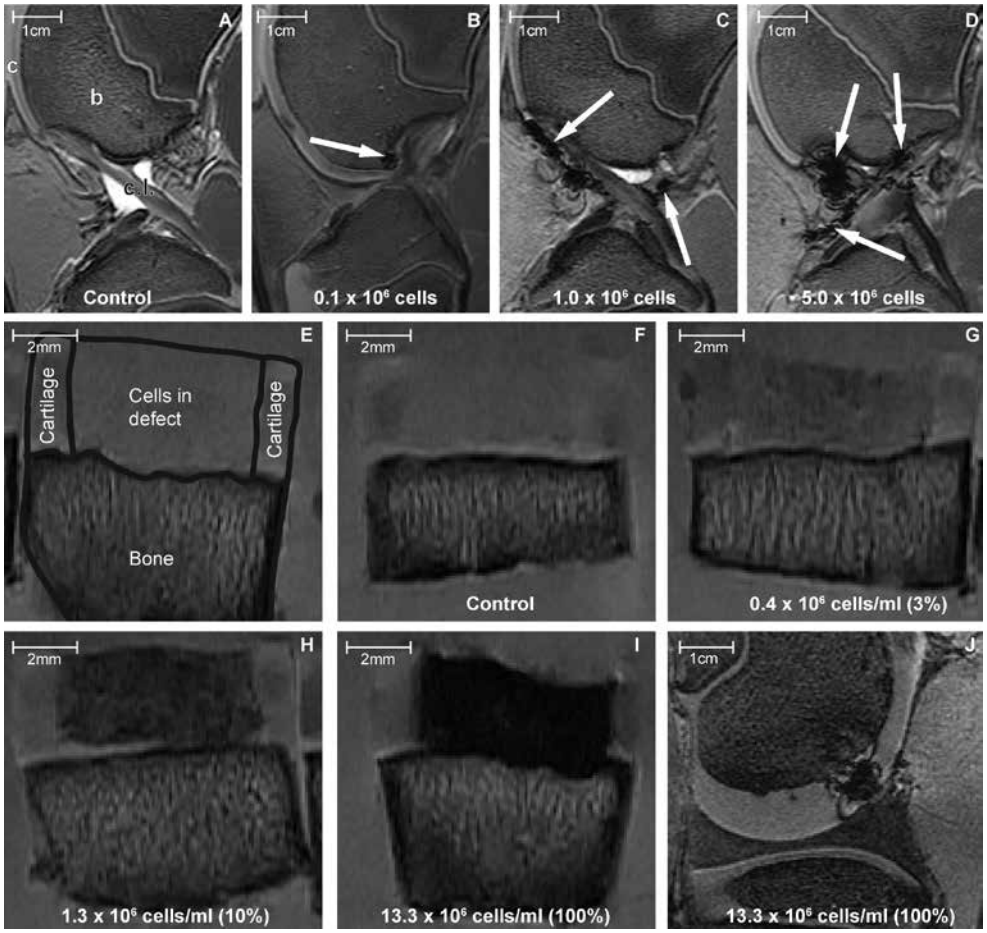

Figure 3. MRI visualization of SPIO-labeled hBMSCs in an intra-articular environment. Injected cells were visualized in a dose dependent manner (A-D, in-plane resolution 273 x 273 µm; slice thickness 600 µm). Cells were found scattered throughout the joint (arrows) and could be discerned from intra-articular structures. Labeled cells implanted in cartilage defects in vitro were homogeneously distributed and imaged in a comparable way; dose dependent and clearly distinguishable from structures like cartilage and bone (E-I; in-plane resolution 78 x 78 µm; slice thickness 1500 µm). After implanting cells in a cartilage defect in an intact porcine knee, MRI showed the cells inside the defect (J; in-plane resolution 273 x 273 µm; slice thickness 400 µm). In (A); b: bone; c: cartilage; c.l.: cruciate ligament. Intra-articular cells in porcine knees were imaged as a single sample for one donor. hBMSCs implanted in cartilage defects in vitro were imaged in duplicate samples from two hBMSC donors.

A point of interest is whether observed areas with signal loss are generated by originally labeled cells, since SPIO remains in the joint after labeled cells die. The joint cavity is lined by an inner layer of synovial tissue that contains macrophages. We investigated the capacity of synovial cells to endocytose SPIO from labeled cells, by seeding dead

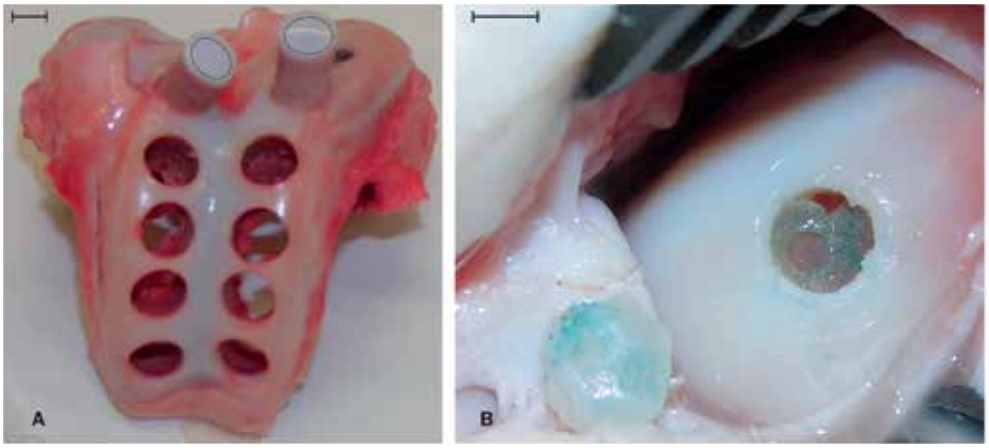

Figure S1. *In vitro* and *ex vivo* cell implantation procedures. Osteochondral plugs with a diameter of 8mm were created from the femoral part of porcine knees (A). *Ex vivo*, post-MRI Perl's iron stain shows macroscopically the presence of blue, iron containing cells in the defect (B). Dotted line in (A) represents created defect. Intra-articular cell implantation in a porcine knee was performed for one donor.

or living GFP-SPIO co-labeled chondrocytes on synovial tissue explants. In the synovium samples seeded with living GFP-SPIO co-labeled cells, GFP^+ cells were observed immediately after seeding (Fig. S2A). Moreover, GFP^+-$SPIO^+$ cells were found after the co-culture period of five days, suggesting these cells to be originally labeled chondrocytes (Figure 4A and 4B). When seeded with dead GFP-SPIO co-labeled cells, fluorescence microscopy confirmed the vast majority of cells to have died directly following

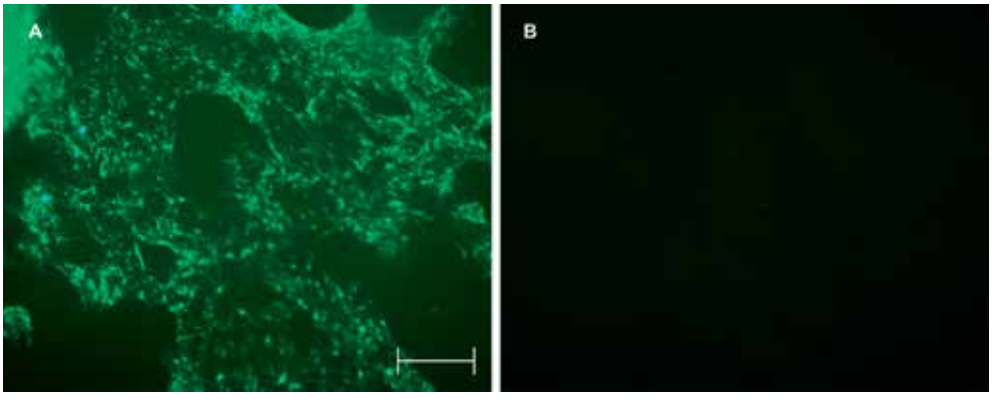

Figure S2. Cell seeding on synovial explants. Fluorescent images directly after seeding GFP^+-$SPIO^+$ cells on synovial explants showing abundant GFP signal in samples seeded with living cells (A), while GFP signal is absent in samples seeded with dead cells (B). This confirmed that the vast majority of killed cells had not survived the multiple freeze-thaw procedures. Representative images are shown for triplicate samples from two synovium donors.

the freeze-thaw procedure (Fig S2B). After five days of co-culture, 88.4 ± 11.4 % of the SPIO$^+$ cells were GFP$^-$ in these samples, indicative of iron re-uptake by synovial cells (Figure 4C-E). Next to this, extracellular SPIO aggregates were found attaching to the synovial membrane (Figure 4D inset). In samples seeded with living chondrocytes, 25.5 ± 10.5 % of SPIO positive cells showed no GFP signal either. Whether this was due to extinguishing GFP signal from originally labeled chondrocytes, synovial re-uptake of SPIO from living cells, or caused by iron re-uptake from seeded cells that died during the co-culture period, could not be determined.

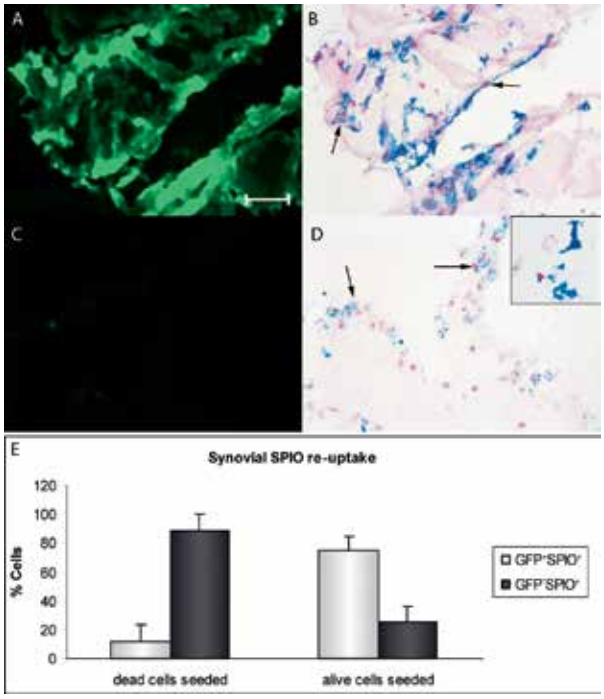

Figure 4. SPIO re-uptake by human synovial explants. Fluorescent images and Perl's iron stain of GFP-SPIO co-labeled chondrocytes seeded on synovial explants. GFP$^+$-SPIO$^+$ cells, indicating originally seeded chondrocytes, were seen in samples seeded with living cells (A,B). In samples seeded with dead cells, GFP$^-$-SPIO$^+$ cells were found, indicating SPIO re-uptake by synovial cells (C,D). Furthermore, extracellular SPIO aggregates were observed attached to the synovial explants (D, inset). Approximately 90% of SPIO$^+$ cells in samples seeded with dead chondrocytes were not originally labeled cells, compared with 25% of SPIO$^+$ cells in samples seeded with living cells (E). Arrows indicate SPIO containing cells. Results shown for triplicate samples from two synovium donors.

Cell quantification by MR-based relaxometry.

In addition to visualization of seeded cells by MRI, we were able to quantify the concentration of hBMSCs seeded in defects *in vitro* by applying a R2* mapping technique, in which R2* = 1/T2* (Figure 5A-E). A linear relationship between R2* values and labeled cell concentrations of 0.4 x 10^6 – 2.6 x 10^6 cells/ml was found (Figure 5F). R2* values ranged from 45 ± 22 - 460 ± 120 ms^{-1} for these labeled cell concentrations. Below

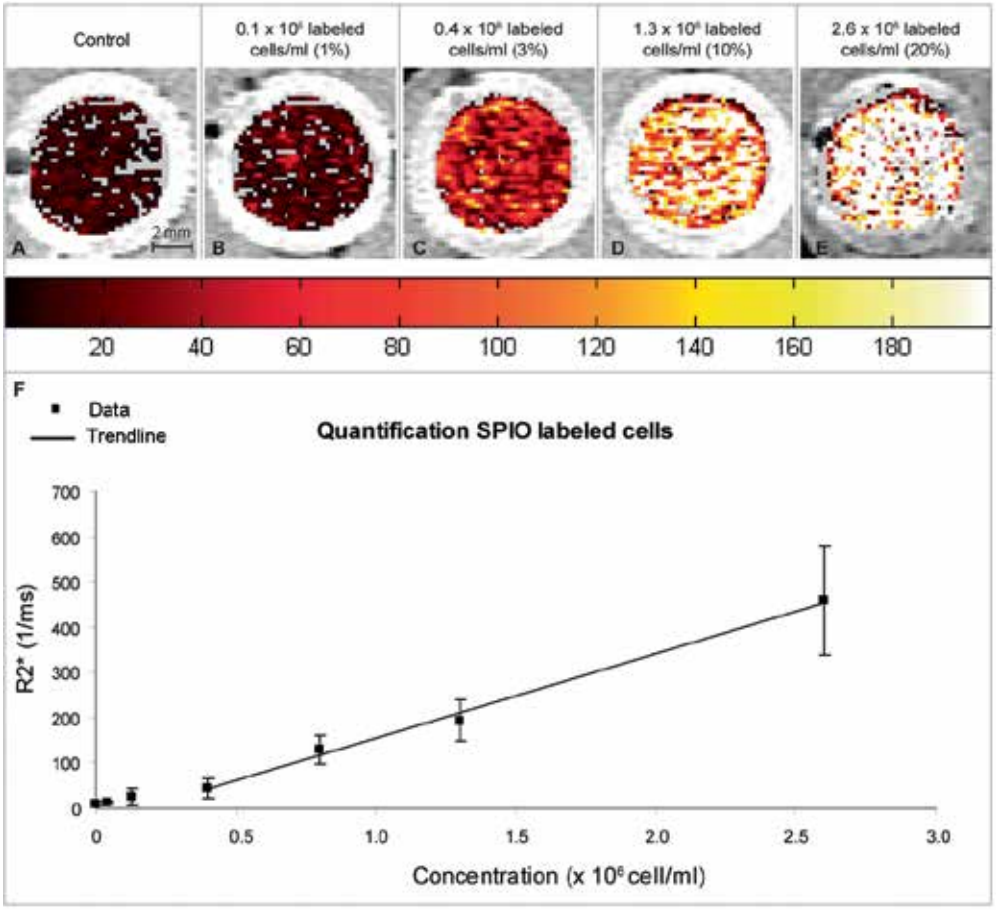

Figure 5. Quantification of SPIO-labeled cells implanted in cartilage defects *in vitro*. Transverse R2* maps from identical osteochondral plugs as shown in figure 3, visualize the differences in labeled cell concentrations (A-E; in-plane resolution 156 x 156 µm, slice thickness 700 µm). A linear relationship between labeled cell concentration and R2* value was found in a range of 0.4 x 10^6 – 2.6 x 10^6 cells/ml (F). Grey voxels inside the defects represent positions where R2* values could not be obtained due to a too low (mainly in A,B) or too high (mainly in E) local concentration of SPIO. Results shown for duplicate samples from two hBMSC donor for samples containing 0; 1.3 x 10^5; 4.0 x 10^5 and 1.3 x 10^6 cells/ml. Samples containing 4.0 x 10^4; 8.0 x 10^5 and 2.6 x 10^6 cells per ml were scanned in duplicates for one hBMSC donor.

$0.4x10^6$ cells/ml, SPIO-generated R2* signal was insufficient for reliable differentiation from defects seeded with unlabeled hBMSCs. At the very high labeled cell concentration of $13x10^6$ labeled cells/ml R2* values could no longer be determined, since the values exceeded those measurable with our clinical acquisition parameter range. At this high SPIO concentration a signal-to-noise ratio of approximately 1.6 was obtained, which signifies that the signal loss was not reliably discernable from background noise.

Discussion

Cell-based cartilage repair techniques are developing rapidly, and the use of hBMSCs as a treatment for focal cartilage defects and osteoarthritis shows promising results [315,317]. Clinically translatable cell tracking represents a vital tool to aid in the elucidation of the repair mechanisms of these therapies, and in further improving them. In this study we have shown that SPIO labeling of hBMSCs is effective and does not impair cell viability, long term metabolic cell activity, chondrogenic differentiation, or the secretion of a panel of six cytokines involved in inflammation and tissue regeneration. We were able to accurately visualize intra-articular SPIO-labeled hBMSCs and quantify cells seeded in localized cartilage defects by means of an R2* mapping MRI technique.

Regarding the influence of SPIO labeling on chondrogenic capacity of BMSCs, some discrepancies can be found in recent reports. Some groups mention no influence of SPIO labeling on chondrogenic differentiation of BMSCs [300,318], whereas others do report a negative effect [319,320]. These conflicting data could be due to the wide variety in protocols for SPIO labeling and chondrogenic differentiation used. Furthermore, the influence of cellular iron load on chondrogenic differentiation has been described [320,321]. As reported in this paper we found no SPIO-related negative effects in our

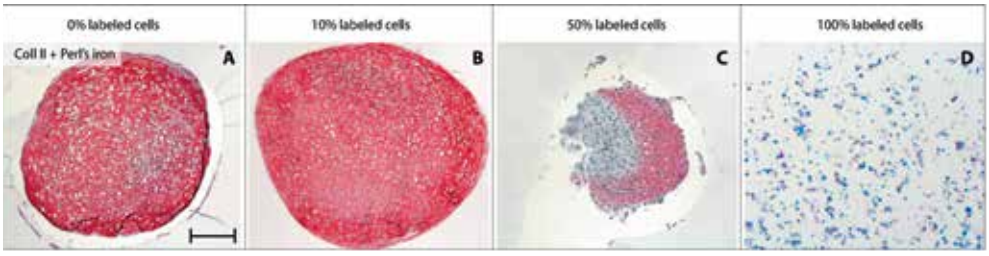

Figure S3. Chondrogenic differentiation of cryopreserved SPIO-labeled hBMSCs. Perl's iron stain (A-D) and collagen II immunohistochemistry (A-C) of chondrogenically differentiated cryopreserved hBMSCs. SPIO labeling of 10% of the cells did not influence pellet size or collagen II deposition compared to control cells (A and B). Using 50% of SPIO-labeled cells did negatively influence these outcome measures (C). Pellets consisting of 100% of labeled cells disintegrated within 7 days, showing viable and iron containing cells on a cytospin (D). Results shown for triplicate samples from two hBMSC donors.

chondrogenic assays with freshly isolated cells. However, we have observed inhibitory effects if we used cryopreserved cells in pellets containing ≥50% labeled cells, although pellets with 10% labeled cells appeared unaltered (Fig. S3A-D). Therefore we recommend further investigation regarding this subject when using cryopreserved hBMSCs.

Besides cell proliferation and differentiation, other hBMSC capacities are relevant in regenerative medicine as well. Recently, secretion of trophic factors was postulated as a possible role for hBMSCs in cartilage regeneration [4]. We demonstrated that the hBMSC secretion profile of six important factors involved in inflammation and cell growth was not affected by SPIO. Previous research has shown that SPIO labeling does not impair hBMSC immunomodulatory capacities in a mixed lymphocyte reaction [322]. Furthermore, it has been described that gene expression of neural stem cells, with the exception of iron homeostasis related genes, remains largely unaltered after SPIO labeling [323]. We report, to our knowledge, for the first time the influence of SPIO labeling on cell secretions at a protein level. These findings could be relevant for other regenerative medicine fields using hBMSCs, like cardiology, neurology and organ transplantation [324]. In summary we found no deleterious effects of our labeling procedure on cell functionality. When labeling only part of the applied cells, which in our experiments sufficed for accurate intra-articular MRI traceability, the risk of possible negative effects will even be further diminished.

In order to translate our labeling method to a cell tracking approach in an intra-articular environment, we investigated the MRI traceability of clinically used amounts of SPIO-labeled cells in a joint model of a clinically relevant size. Jing et al. previously visualized cells labeled with SPIO in rabbit knee joints by MRI [51]. Through the use of a clinical phased-array head coil and specific MRI parameters we were able to increase MRI resolution (in plane resolution 273 x 273 µm), which is higher than in other studies performing intra-articular cell tracking or general clinical anatomical intra-articular imaging by MRI [325,326]. This enabled dose-dependent visualization of labeled cells and discrimination from intra-articular structures like cruciate ligaments, cartilage and subchondral bone. Following our *in vitro* cell implantation experiments, we visualized SPIO-labeled cells implanted in an ACI-like procedure in an intact porcine knee. Air artifacts show up as hypointensities on MRI, which interferes with the signal loss generated by labeled cells. To avoid these artifacts, we were obliged to perform this operation under water. This is impracticable in a clinical setting indicating that immediately post-operative this technique can be used to visualize cells that remained in the defect, but that the technique is unsuitable to discern labeled cells that might have leaked in the intra-articular space. These leaked-out cells could be identified after intra-articular air has resolved, which in our personal clinical experience occurs within a day.

SPIO for cell-labeling has great clinical potential, but unfortunately the label will dilute upon cell division. This could limit the duration that cells can be accurately detected by MRI. Previous results from our group and others show an approximate 75% decrease in SPIO positive cells after 7 days of expansion in culture [327] and a total loss of iron after

5-8 cell divisions in rapidly dividing HeLa cells [328]. On the other hand, duration of traceability increases with slower proliferating cells, and SPIO could be detected after 44 days in non-dividing human MSCs [328]. Accordingly, our group previously reported traceability in vivo of SPIO labeled human MSCs in a subcutaneous mouse model for at least seven weeks post labeling [318]. In additional experiments we found a marked decrease in SPIO-generated signal voids from proliferating labeled hBMSCs over a period of 14 days in vitro, representing 5-6 cell divisions (data not shown). How long SPIO labeled cells can be detected in an intra-articular environment remains to be studied in an *in vivo* model.

Another point regarding accurate detection of labeled cells is the fact that cell death will not result in the disappearance of the label. SPIOs released from these cells could lead to misinterpretation of MR images. Pawelczyk et al. have previously shown approximately 10 - 20 % of local macrophages to become SPIO positive after injection of SPIO-labeled BMSCs in a subcutaneous inflammation mouse model [322]. Synovium contains the majority of macrophages present in synovial joints. We observed the vast majority of $SPIO^+$ cells to be non-originally labeled after applying dead, labeled cells on synovial explants. In the case of cell implantation in a cartilage defect, SPIO re-uptake by host cells might be of less consequence because there is no direct contact between the cells seeded in the defect and the synovial membrane. Nonetheless, before clinical application we recommend *in vivo* animal studies using intra-articular cell tracking by MRI in combination with histological confirmation of the localization of originally labeled cells. This would enable the correct interpretation of obtained MR images.

Besides visualizing cells, we were able to quantify beforehand known cell concentrations implanted in cartilage defects in a clinically relevant cell-concentration range. To our knowledge, this has not been reported before in a model comprising biological tissue. Rad et al. were unsuccessful in translating their SPIO-labeled cell quantification experiments to an *ex vivo* setting [329], and Politi et al. did not validate their *ex vivo* quantification results afterwards [330]. Since the original concentration of applied cells is known in an ACI procedure, and seeded in a confined volume, it is possible to quantify the total amount of labeled cells and follow them in the same patient in subsequent MRI sessions. Since both hBMSCs and chondrocytes can be labeled using SPIO [331,332], these findings indicate the potential of SPIO labeling for tracking cells in numerous orthopedic treatments and many other regenerative medicine fields [333]. Cell visualization and especially quantification using MRI can be a major step towards improvement in all these applications.

In summary, we consider SPIO labeling to hold great potential for clinically translatable cell tracking using MRI in cell-based cartilage repair. In an experimental setting this would show the distribution and diffusion of the applied cells, thereby elucidating the regenerative mechanisms and providing opportunities to improve current repair strategies. The use of MRI has the advantage of simultaneous cell tracking and evaluation of cartilage regeneration in one MRI session.

Acknowledgements

The authors are grateful to N. Kops, J.L.M. Koevoet, S.T. van Tiel and J. van der Slegt for their technical assistance; to M. Hoogduijn for his assistance in obtaining and analyzing the CBA data; to A. Uijtterdijk for kindly providing porcine materials; to J.H. Waarsing for his support with statistical analyses; and to U. Woroniecka and B. Wolterbeek for their assistance with the ICP-OES measurements. The monoclonal antibody developed by T. Linsenmayer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA. Furthermore, the authors gratefully acknowledge the support of the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science; and the ENCITE consortium of the European Community under the 7th framework programme.

Chapter 8

Human bone marrow mesenchymal stem cells embedded in alginate beads retain long-term immunomodulatory properties

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Manuscript in preparation

Abstract

Background: Bone marrow mesenchymal stem cells (BMSCs) are multipotent cells with activation dependent immunomodulatory properties. To investigate their immunomodulatory properties, most studies use short term cultures of cells in monolayer. The environment of BMSCs could influence their immune regulatory capacity and it is unclear how the behaviour of BMSC in monolayers relates to their in vivo effects. For application in chronic inflammatory conditions BMSCs have to retain long-term immunomodulatory properties.

Methods: We studied the gene expression and secretion of immunomodulatory and growth factors by BMSCs in a control and in an inflammatory situation (IFN□/TNF□ supplementation of 50 ng/ml each). Cells were grown in monolayer for short-term (3 days) experiments or encapsulated in alginate beads in a short-term and long-term culture (3 and 30 days). DNA content of the beads was determined as a measure of cell survival. Gene expression was measured by RT-PCR and cell secretions were measured using enzyme-linked immunosorbent assays.

Results: Embedded in alginate, BMSCs maintained their immunomodulatory properties during 30 days of culture, expressing IL-6, IDO, TGF□1, VEGF, TIMP1 and TIMP2. We measured IL-6 and TIMP2 levels and IDO activity in the medium. Approximately 50% of cells survived the 30 day culture period. The response of cells in alginate during a short-term or long-term culture to IFN□/TNF□ was similar to the response in short-term monolayer culture.

Conclusions: Our data indicate that BMSCs embedded in alginate beads may act as a continuous immunomodulatory and trophic factor release system for several weeks, making this an interesting system to investigate for application in chronic diseases.

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that have been found in several tissues such as bone marrow, adipose tissue, synovium, deciduous teeth, umbilical cord blood and blood vessels[337], [338], [339], [340]. MSCs have been used as therapeutic agents in experimental models of tissue injuries[341], [342], [343], [344], [345]. These studies show MSCs-dependent improvement of damaged tissues without a significant contribution of engrafted and differentiated cells[346], [347], [348], [349], [350], [351]. This finding suggests that next to their capacity to differentiate into cells of mesodermal lineage, including osteoblasts, chondrocytes, myocytes and adipocytes, MSCs have the ability to stimulate endogenous tissues repair. This repair is orchestrated through the secretion of soluble factors that altered the tissue microenvironment[352].

Additionally, MSCs were shown to be able to modulate the immune response. This property is very useful in cases where inflammation plays an important role in tissue destruction like immune disorders or graft versus host disease (GvHD)[353]. Immune suppression by MSCs requires activation by proinflammatory cytokines such as interferon (IFN)γ, tumor necrosis factor (TNF)α, interleukin (IL)1α or IL1β[294], [354], [355]. In vitro, MSCs can inhibit antibody production by B cells[356], the generation and function of antigen presenting cells[357] and T lymphocyte proliferation and pro-inflammatory cytokine production[358], [359]. In vivo, MSC were shown to be able to reduce immune activity in autoimmune enteropathy[360], prolong heart and skin allograft survival[361], [362] and improve experimental colitis[363]. The responsible mechanisms of this immune modulation are based on multiple factors including prostaglandin E2 (PGE2) and indoleamine 2, 3-dioxygenase (IDO), nitric oxide (NO) or cytokines such as [364] IL-6 [357], [365], [239]. Also, growth factors such as transforming growth factor β1 (TGFβ1) and vascular endotelial growth factor (VEGF)[366] secreted by MSCs, have demonstrated to influence tissue repair as well as immunological processes [367].

Most studies consider the immunomodulatory properties of MSCs in monolayer culture. However, cells in monolayer, can differ considerably in their morphology, cell adhesion, cell cycle, and differentiation from those in three-dimensional (3D) environments in vitro or in vivo[368], [369], [370], [371], [372]. Indeed, the systemic administration of MSCs is characterized by early death of grafted cells or only small numbers of cells reach the target [347], [348], [349], [350], [351].

Additionally, analyses of the immunomodulatory properties of MSCs are mostly performed in short-term experiments. In a clinical application when MSC would be used to modulate more chronic inflammatory reactions they have to be introduced in the area and the immunomodulatory properties have to be guaranteed for several weeks at least, to achieve a prolonged improvement of the pathology.

Alginate is a natural and useful polymer for cell encapsulation because of its biocompatibility and an in vivo stability[373], [374], [375]. Inclusion in alginate beads is an easy way to prevent mechanical stress, but also, to diminish the negative influence of the injured environment on grafted cells, manipulate and deliver the cells near the injury and to set a better tracking of the phenotype of the grafted cells[376].

Therefore, the purpose of this study was to investigate the expression and secretion of immunomodulatory and growth factors by bone marrow MSCs (BMSCs) encapsulated in alginate beads under control and under inflammatory conditions (50 ng/mL TNFα and 50 ng/mL IFNγ) in a long-term culture model. Furthermore, we investigated the behaviour of the cells after several weeks of encapsulation and test the ability of the embedded cells to maintain their multilineage differentiation characteristics.

Material and methods

BMSCs isolation and expansion

Human BMSCs were isolated from heparinized femoral-shaft marrow aspirate from 4 patients undergoing a total hip arthroplasty (after written informed consent with approval of the Medical Ethical Committee of Erasmus MC protocol # MEC-2004-142). BMSCs were isolated according to procedures described before[157]. Briefly, bone marrow aspirate was plated out in basal medium (DMEM low glucose medium (Invitrogen, Carlsbad, CA) with 15% fetal calf serum (FCS; selected batch Lonza, Verviers, Belgium), 50 µg/mL gentamycine (Invitrogen, Carlsbad, CA), 1.5 µg/mL fungizone (Invitrogen, Carlsbad, CA), 1 ng/mL FGF2 (Instruchemie B.V. Delfzijl, the Netherlands) and $1x10^{-4}$M vitamin C (Sigma) and after 24 hours, non-adherent cells were removed with 2% FCS in PBS. Adherent cells were cultured and upon passaging seeded at a density of 2300 nc/cm^2 in basal medium and trypsinized (Invitrogen, Carlsbad, CA) at subconfluence. Cells from passage 3 were used for experiments.

BMSCs encapsulation

4 million/mL BMSCs were encapsulated in 1.2% low viscosity alginate (Keltone LV; Kelco, Surrey, UK) and beads were created by dripping through a 23˝ gauge needle into a CaCl2 solution. After 10 minutes the beads were washed twice with physiological saline and once with control medium ((DMEM low glucose medium with 10% FCS, 50 µg/mL gentamycine, 1.5 µg/mL fungizone).

Stimulation of immunomodulatory properties

BMSCs encapsulated in alginate were incubated in control medium for a period of 48 hours to homogenize the culture. To activate the immunomodulatory properties, BMSCs were incubated 24 hours in control or cytokine medium which consist of control

medium supplemented with IFNγ and TNFα (50 ng/mL each, PeproTech, London, UK). As controls, subconfluent BMSCs in monolayer were preconditioned 48 hours in control medium and then incubated with control or cytokine medium for 24 hours.

To evaluate whether BMSCs could still be activated after long-term culture in alginate, the encapsulated BMSCs were precultured for 30 days in preconditioning medium and then cultured in control or cytokine medium for 24 hours. Beads were harvested 24 hours after treatment for gene expression analyses and medium was harvested and stored at -80ºC for analyses of secreted factors. Agarose coated plates were used to avoid cell growing out of alginate beads.

DNA Content

To determinate cell survival after embedding in alginate beads in a course time t = 0, 7, 14, 21, 30 days we measured the amount of DNA. Three samples of three beads from different culture wells were digested overnight at 56°C in papain digestion buffer (200 µg/mL papain (Sigma, St. Louis, MO, USA) in 50 mM ethylene diamine tetraacetate (EDTA) (Sigma) and 5 mM l-cystein hydrochloride (Sigma). The amount of DNA in each papain-digested sample were analyzed on the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA) using an extinction filter of 340 nm and an emission filter of 590 nm by means of an ethidium bromide assay (Sigma) with calf thymus DNA as a standard[377].

Differentiation assays.

To evaluate if the BMSCs maintained their multilineage differentiation capacity after they have been embedded in alginate for 30 days, we released the cells from alginate using sodium citrate/EDTA (Sigma) and performed osteogenic and adipogenic differentiation during 21 days. For osteogenesis cells were seeded at 3,000 cells/cm2 and cultured in medium containing DMEM high glucose (Invitrogen), 10% FCS, β-glycerophosphate 10 mM (Sigma), dexamethasone 0.1 µM (Sigma) and L-ascorbic acid 2 phosphate 0.5 mM (Sigma). For adipogenic differentiation cells were seeded at 20,000 cells/cm2 and cultured in medium containing DMEM high glucose, 10% FCS, dexamethasone 1 µM, indo-methacin 0.2 mM, insulin 0.01 mg/mL, and 3-isobutyl-l-methyl-xanthine 0.5 mM (all from Sigma). All media contained 50 µg/mL gentamycine and 1.5 µg/mL fungizone. Histology evaluation was performed with Von Kossa staining (Sigma) for osteogenic differentiation and oil-red O (Sigma) for adipose differentiation.

Gene expression analyses

To isolate RNA, 10 alginate beads were pooled and dissolved in 150 µL/bead 55 mM sodium citric acid and spun down for 8 min at 1,400 rpm at 4°C. Cell pellets were resuspended in 1 mL RNABee (Tel-test, firendswood, TX, USA). For monolayer BMSCs cultures, 2 well plates from a 6 well plate were suspended in 1 mL RNABee. After addition of 0.2 mL/mL chloroform, samples were spun down for 15 min at 12000 rpm. Total RNA was isolated from the supernatant using the Qiagen RNA Micro Kit according to

the manufacturer's instructions (Qiagen, Hilden, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop ND1000; Isogen Life Science, IJsselstein, The Netherlands). cDNA was obtained according to manufacturer's instructions using using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Gene expression analysis was performed using ABI7000 for cycling. Ct values were corrected by the best housekeeper (BKI), which was calculated by the average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC) and hypoxanthine phosphoribosyltransferase (HPRT). IL-6, tissue inhibitor of metalloproteinases (TIMP2), IDO, TGFβ1, TIMP1 and VEGF were analyzed in BMSCs.

RT-PCR primer nucleotide sequences were as follows: GAPDH Fw: ATGGGGAAGGTGAAGGTCG; Rv: TAAAAGCAGCCCTGGTGACC. UBC Fw: ATTTGGGTCGCGGTTCTTG; Rv: TGCCTTGACATTCTCGATGGT. HPRT Fw: TATGGACAGGACTGAACGTCTTG; Rv: CACACAGAGGGCTACAATGTG . IL6 Fw: TCGAGCCCACCGGGAACGAA; Rv: GCAGGGAAGGCAGCAGGCAA. TIMP2 Fw: ATGGTGGGTTCTCTGGTG; Rv: CGGTACCACGCACAGGA. IDO assay-on-demand (Hs00158027.m1, Applied Biosystems, Capelle a/d IJssel, the Netherlands). TGFβ1 Fw: GTGACAGCAGGGATAACACACTG; Rv: CATGAATGGTGGCCAGGTC. TIMP1 Fw: TGCCGCATCGCCGAGAT; Rv: ATGGTGGGTTCTCTGGTG. VEGF Fw: GAGTGCCCACTGAGG; Rv: GCCTCGGCTTGTCAC. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[91].

Enzyme-Linked ImmunoSorbent Assay

TIMP2 and IL6 protein levels were measured in stimulated and non-stimulated MSC-conditioned media from three donors by means of ELISAs (R&D systems, Abingdon, UK) according to the protocol supplied by the manufacturer. All factors were corrected for the amounts present in standard MSC culture media. To determine the amount of IDO enzymatic activity in BMSCs media, the level of its metabolite, kynurenine, was measured spectrophotometrically as described before[248]. In brief, 100 µl of 30% trichloroacetic acid (Sigma-Aldrich, St. Louis, USA) was added to 200 µl of culture supernatant, which was incubated at 50°C for 30 min, and then centrifuged at 10,000 × g for 5 minutes. 75 µl of supernatant was then added to an equal volume of Ehrlich's reagent (100 mg P-dimethylbenzaldehyde and 5 mL glacial acetic acid; Sigma St. Louis, USA) and optical density was measured at 490 nm.

Statistics

Analyses were performed by two way ANOVA with repeated measure using GraphPad Prism 5.00. A P value < 0.05 was considered statistically significant.

Results

1. BMSCs retain their immunological properties in alginate

First, to check the immunomodulatory properties of BMSCs in alginate, we compared the BMSCs' response to TNFα and IFNγ exposition between monolayer and alginate beads. BMSCs embedded in alginate beads showed very similar alterations in the gene expression profile in response to TNFα/IFNγ as BMSCs in monolayer cultures (Fig. 1). We found an upregulation of IL-6 and IDO mRNA ($p<0.05$) in response to TNFα/IFNγ in monolayer and alginate culture. TIMP1 expression was not changed by cytokines in both conditions. TGFβ1 was downregulated in both conditions but only reached significance in alginate cultures ($p<0.05$), whereas TIMP2 and VEGF were only significantly downregulated in monolayer cultures ($p<0.05$).

To check whether BMSCs in alginate maintained their capacity to exert an immunological response for a longer period, we performed 24 hours stimulation with control and cytokine medium in BMSCs embedded in alginate beads that were 30 days precultured. BMSCs were able to respond in the inflammatory medium likewise monolayer or only 48 hours preconditioning alginate beads. After 30 days in alginate, TNFα/IFNγ upregulated IL-6 and IDO ($p<0.05$), downregulated TGFβ1, TIMP1 and TIMP2 ($p<0.05$), while VEGF was not significantly altered. (Fig. 1). Also, there seems to be higher TGFβ1 expression in the control BMSCs in 30d alginate beads compared to 48h.

2. Immunomodulatory and growth factors secretion by BMSCs cultured in alginate beads.

Then we analyzed BMSCs´ secretion of IL-6 and TIMP2, together with their IDO activity after a short and long-term culture in alginate beads using monolayer as a control group. BMSCs secreted all the measured factors after 30 days culture in alginate beads (Table I). IDO activity and IL-6 secretion were increased by TNFα/IFNγ, in accordance with gene expression analysis, albeit IL-6 didn´t reach significance in the alginate cultures due to the variances between donors. TIMP2 secretion was not affected by the cytokines in any of the three conditions.

Because we observed a decrease in levels of secreted factors after 30 days in alginate we evaluated the effect of the alginate inclusion on BMSCs survival by measurements of the DNA content of the alginate beads at 0, 7, 14, 21 and 30 days of culture. DNA content appeared to decline after BMSCs inclusion in alginate beads ($p<0.001$) leaving approximately the 40% of the BMSCs at 30 days (Fig. 2). Apparently the remaining cells could still respond to inflammatory cytokines by secretion of factors.

3. Differentiation capacity of BMSCs after encapsulation in alginate.

To characterize the effect of alginate on BMSCs, we released the cells of one donor after 3 or 30 days and performed adipogenic and osteogenic differentiation assays

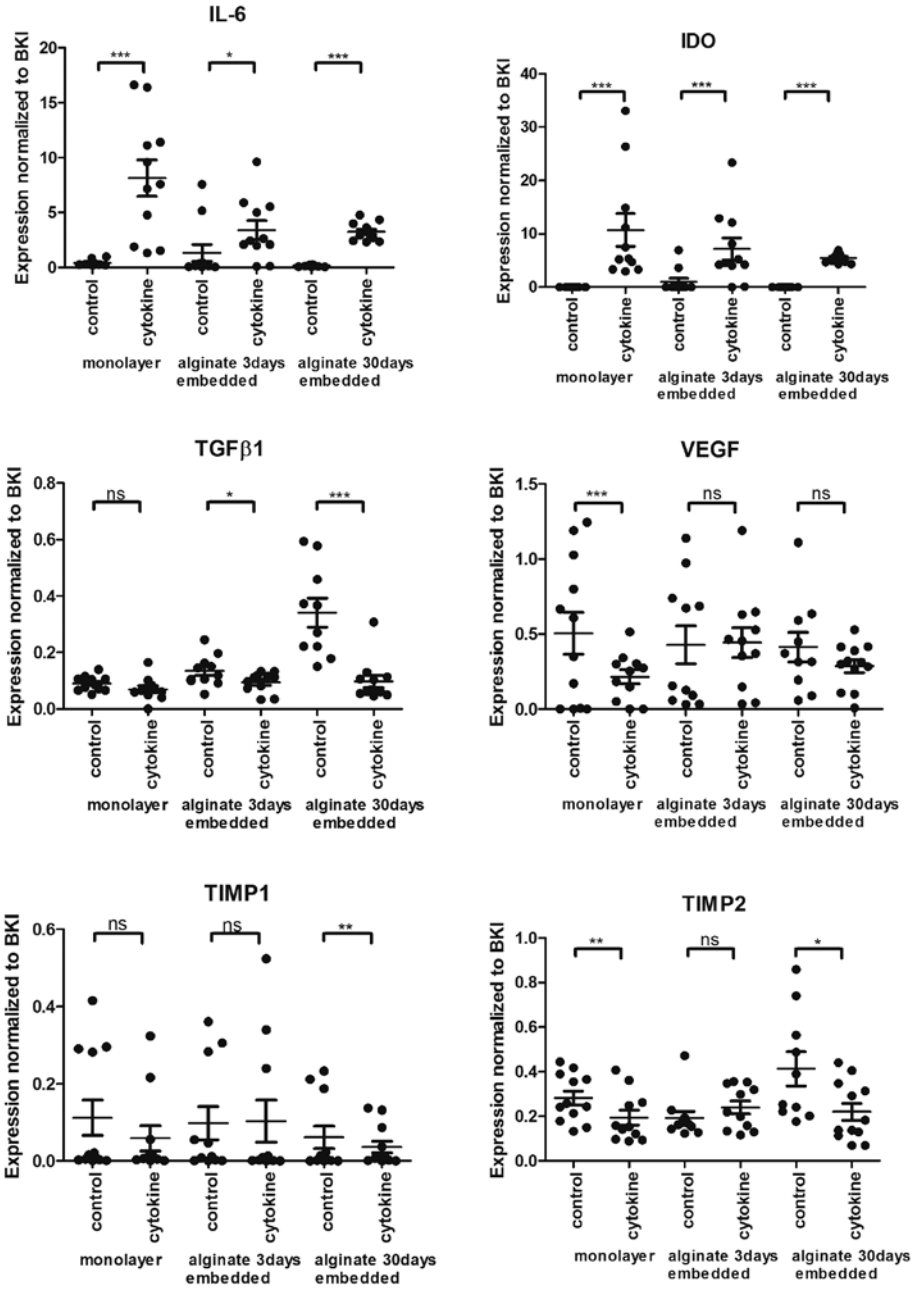

Fig. 1. Gene expression of immunomodulatory genes by human BMSCs with or without stimulation by cytokines (TNFa/IFNy) in monolayer or embedded in alginate beads after 3 days or 30 days in alginate. Data expressed as mean±SEM of 4 donors with duplicate or triplicate samples per donor. All individual data points were presented as dots, outliers (one in TGFβ1 and one in TIMP2 graph) were left out of the graphical representation but included in mean, SEM and statistical analyses. BKI: best house keeper index, an average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC) and hypoxanthine phosphoribosyltransferase (HPRT); IL-6: Interleukin 6; IDO: indoleamine 2, 3-dioxygenase; TGFβ1: transforming growth factor β1; VEGF: vascular endotelial growth factor; TIMP-1: tissue inhibitor of metalloproteinases 1; TIMP-2: tissue inhibitor of metalloproteinases 2. * p<0.05 ** p<0.01, *** p<0.001.

Table I. Secretion from BMSCs in monolayer cultures or embedded in alginate beads for 3 or 30 days.

	Monolayer		Alginate			
	3 days		3 days		30 days	
	Control	Cytokine	Control	Cytokine	Control	Cytokine
IL-6 pg/mL	5495 ± 553	53594 ± 3335 *	1650 ± 1271	36044 ± 23304	u.d.	4148 ± 2439
TIMP2 pg/mL	26181 ± 5099	27750 ± 3917	15541 ± 8027	15291 ± 7669	9483 ± 6913	8635 ± 5518
Kynurenine (IDO activity) ng/mL	440 ± 158	13069 ± 1012 **	690 ± 631	11531 ± 102 *	1430 ± 1430	7661 ± 1548 *

IL6 (pg/mL) and TIMP-2 (pg/mL) secretion measured in the medium by ELISA, IDO enzymatic activity was measured spectrofotometrically by means of kynurenine level (ng/ml). Data expressed as mean±SEM of 3 donors with triplicate samples per donor. * $p<0.05$ ** $p<0.01$ comparing control and cytokine medium. u.d. = under detection limit

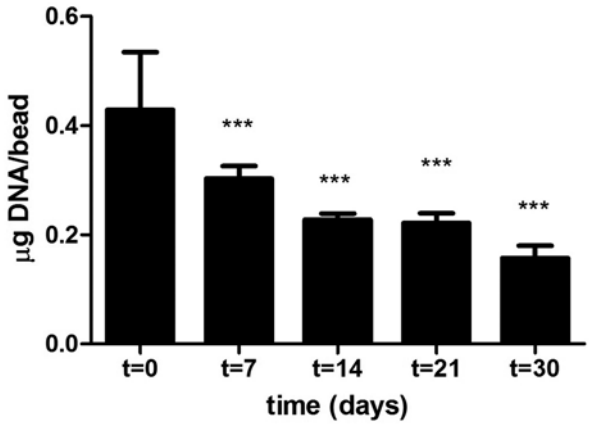

Fig. 2. DNA content of BMSC-alginate beads in a course time experiment. Data expressed as mean±SEM of three donors with triplicate samples per time point per donor. *** p<0.001 compared to t=0.

and compared it to BMSCs that were not embedded in alginate. BMSCs released from alginate were able to differentiate in osteogenic and adipogenic, although after inclusion in alginate, both, at 3 and at 30 days, these capacities were diminished (Fig. 3).

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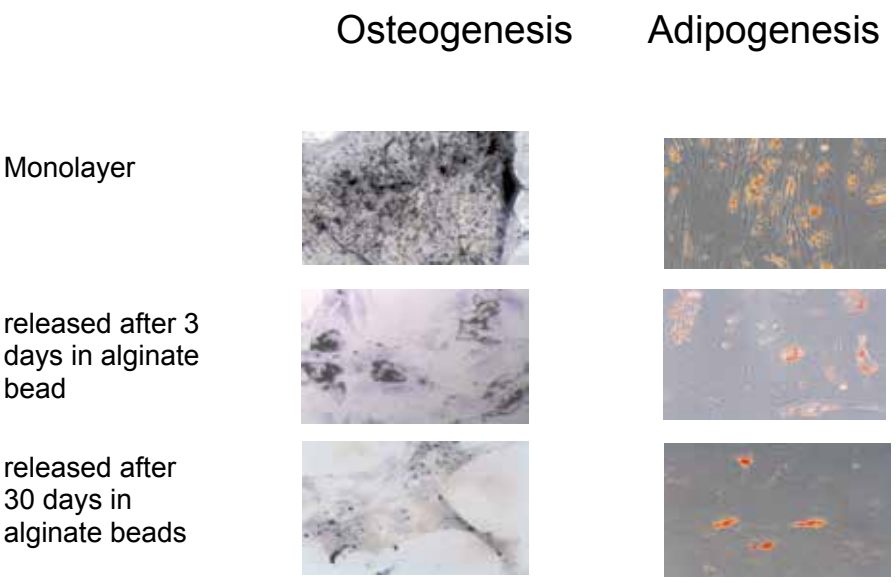

Fig. 3. Osteogenic and adipogenic differentiation of BMSCs after release from alginate. Representative pictures are shown. Von Kossa staining is used for osteogenic differentiation with black indicating calcium-phosphate crystals. Oil-red O staining is used for adipogenic differentiation with red indicating lipid drops. Original magnification 100X

Discussion

the immunomodulatory effects of BMSCs and the enhancement of this function after pro-inflammatory stimuli in monolayer cultures is well established. However, little is known about their behavior in a 3D matrix such as alginate. In this study, we show the maintenance of the immunomodulatory capacities of BMSCs during a long term, 3D culture in alginate beads. Here, we have analyzed the gene expression analysis of BMSCs embedded in alginate, at short and long culture period and found that alginate is able to retain BMSCs alive and capable of responding to an inflammatory stimulus with secretion of immunomodulatory factors even after a long period of culture.

BMSCs are known to be able to influence immune and wound healing processes by secretion of several factors [378], [44], [379]. Therefore BMSCs have received much attention and have been clinically applied, for example in graft versus host disease (GvHD), osteogenesis imperfecta or glycogen storage disease[380]. Studies that used cell tracking methods could not demonstrate the long-term presence of these cells after applications [381,382], [383]. This would limit effectiveness of the application of these cells for chronic diseases. Here we demonstrate that BMSCs encapsulated in alginate beads upregulated gene expression of IDO and IL-6 after IFNγ and TNFα treatment similar to BMSCs in monolayer and confirming a large body of previous data in monolayer culture[239] [384] [385]. These data open the possibility of a long-term release system of immunomodulatory factors by application of BMSCs encapsulated in alginate.

For this study we have selected IL6, TIMPs, TGFβ, VEGF and IDO as factors secreted by MSCs that are known to play a role in modulation of inflammation. IDO in BMSCs promotes immunosuppression and improves experimental autoimmune encephalomyelitis (EAE)[386], [387], [388]. IL-6, on the other hand, could be anti-inflammatory, controlling the extent of local and systemic acute inflammatory response[389], [390], whereas in chronic inflammatory diseases such as collagen-induced arthritis, murine colitis or EAE it could be proinflammatory[391], [392]. Also, the observed maintenance of TIMP1 and TIMP2 gene expression may act locally and control the MMP-induced breakdown of the extracellular matrix[393]. In contrast with other studies, a downregulation of VEGF gene expression after pro-inflammatory treatment was found and this finding is in concordance with the downregulation of TGFβ1. In the immunomodulation field, TGFβ1 has been largely associated with immunosurveillance suppression mechanism[394], [395] and its long-term overexpression leads to severe hyperplasia in normal epidermis or oral mucosa[396], [397,398]. VEGF levels in BMSCs correlate with angiogenesis required for tissue repair but it could also drive inflammation[399]. Summarizing, our data support the idea that BMSCs retain the capacity to secrete modulating factors after being encapsulated in alginate and cultured up to 30 days.

For this first study we have encapsulated our BMSCs in 1.2% low viscosity alginate, solidified by dripping beads in $CaCl_2$ solution using a 23G needle to study the effect of secretion of immunomodulatory factors. This is the system we use in our laboratory to culture BMSCs or chondrocytes to form cartilage matrix[108], [400]. However, we appeared to lose more than half of the cells over time and the remaining cells had a slightly reduced capacity to differentiate osteogenically or adipogenically. This is in contrast to other studies that have shown a higher viability of BMSCs throughout 21 days of culture in alginate[401] and the increase of the expression of the osteogenesis markers osteocalcin and osteomodulin, and the adipogenesis marker PPARγ[402], [403], [404], [405], [406] . These differences may be due to the different concentration of BMSCs used. Most studies have used a final concentration of $1x10^6$ – $2x10^6$ BMSCs/mL, whereas we have used $4x10^6$ BMSCs/mL. This amount of cells may be excessive to amount of growth factors distributed in a gradient trough the alginate bead in a long term culture. Also, we did not add any other factors to the culture system. Additional experiments demonstrated that addition of ascorbic acid, to avoid oxidative stress on BMSCs in culture, did reduce cell loss (data not shown). Furthermore, size of the beads, concentration and type of alginate and BMSCs concentration need to be optimized depending on the final goal, since these aspects have been found to be important in the proliferative capacities and release profile of the BMSCs[407], [401]. Moreover, reduction of the size of the beads will be beneficial for clinical application by injection later on. It has been suggested that alginate beads could be used safe and effectively, to deliver stem cells percutaneously with minimal loss of viability[401], [408]. The application of BMSCs in alginate beads may be useful for several chronic inflammatory diseases, where the activity of BMSCs should be present for weeks or months.

Conclusion

In conclusion, our results indicate that after a long-term culture, BMSCs embedded in alginate beads preserve their ability to modulate their microenvironment by secreting several factors such as IDO, IL-6, TIMP-1, TIMP-2, TGFβ1 and VEGF. BMSCs embedded in alginate beads may act as a continuous immunomodulatory and trophic factor release system for several weeks, making this an interesting system to investigate for application in chronic diseases such as rheumatoid arthritis, osteoarthritis, tendinitis, EAE, diabetes mellitus or myocardial infarction.

Acknowledgements
We would like to thanks to Nicole Kops for her help and technical assistance. The authors also gratefully acknowledge the scholarship travel grant to E. Villafuertes conceded by the Osteoarthritis Research Society International (OARSI).

Chapter 9

Summary and general discussion

Osteoarthritis (OA) is a disabling joint disease with a high prevalence and incidence [1]. The numbers of patients which are prone to develop osteoarthritis is expected to increase due to an ageing population in combination with an obesity epidemic [1]. Next to this, older people have an enhanced wish for (joint) mobility compared to earlier days. To this day, OA treatment includes pain medication and lifestyle changes like weight loss and regular exercise. These treatments are symptomatic treatments which are not able to durably cure the disease [409]. If these conservative measures fail, the final treatment option is joint replacement. Unfortunately, joint prostheses have a limited lifespan and as a result they do not provide a final solution for OA. For this thesis we studied the working mechanisms and efficacy of two biological treatments as a long term disease modifying osteoarthritis drug (DMOAD). As a first treatment we used platelet-rich plasma releasate (PRPr) a preparation of bioactive factors indirectly derived from cells. The second treatment was cell therapy using bone marrow-derived cells. We hypothesized that by using and stimulating one's own biological repair mechanisms, it would be possible to break or slow down the vicious circle of cartilage degeneration that is characteristic for OA.

In **chapter 2** we evaluated the potential of PRPr that was isolated by means of centrifugation from patients own blood. [111]. Platelets are small cell fragments derived from megakaryocytes, containing many bioactive factors. These can potentially reduce the effects of inflammatory mediators on osteoarthritic chondrocytes. PRPr inhibited multiple interleukin-1 beta (IL-1 beta) induced effects in OA chondrocytes. Addition of PRPr to chondrocytes beneficially affected genes involved in the formation and degradation of extracellular matrix, as well as genes related to inflammation. PRPr diminished IL-1 beta induced inhibition of collagen type II (COL2A1) and aggrecan (ACAN) gene expression. These are two important genes involved in cartilage matrix formation [410]. PRPr also reduced IL-1 beta induced increase of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4 and prostaglandin-endoperoxide synthase (PTGS)2 gene expression. The products of these genes are involved in cartilage matrix degradation and inflammation respectively. In order to find a possible pathway through which PRPr exerts these effects, we studied the effects of PRPr on nuclear factor kappa B (NFkB) activation. NFkB is a protein family that controls apoptosis, inflammation and other immune responses, and is thus involved in many pathologic OA processes [96,116,117]. PRPr fully counteracted IL-1 beta induced nuclear factor kappa B (NFkB) activation back to control levels with no IL-1 beta.

This study demonstrated the capacity of a combination of bioactive factors to alter pathologic processes in OA chondrocytes. This could potentially improve the application of PRP in a clinical setting, for instance the timing or frequency of administration. A limitation of this study was the use of a single pro-inflammatory factor to induce inflammation on a single cell-type in a short term in vitro model. OA pathophysiology on the other hand, is a complex interplay of many factors and tissues with a dynamic and chronic time course [82]. Therefore, further in vivo studies are needed to show long term

beneficial PRP effects on diseased cartilage or joints as a whole. Next to the limitations of our model, another drawback of the use of PRP as an OA treatment is the fact that injected biological compounds are known to reside in the joint for a limited time-span only [411]. We searched for a way to provide a durable presence of disease modifying factors in the joint. Since mesenchymal stem cells (MSCs) are able to secrete a broad panel of immunomodulatory factors and growth factors, we further focussed on this source of biological treatment as a durable DMOAD.

Friedenstein et al. were the first to describe colony-forming cells with osteogenic capacities derived from bone marrow [41]. Since then, a vast amount of research has been conducted concerning these multi-potent bone marrow-derived cells which were later denominated mesenchymal stem cells (MSCs) by Caplan et al. [412]. More recently, progenitor cells with a high resemblance to bone marrow-derived MSCs have been isolated from many other musculoskeletal tissues and adipose tissue. In **Chapter 3** we reviewed the natural presence and characteristics of progenitor cells in various other tissues found in the joint, including adipose tissue, periosteum, perichondrium, tendons, ligaments, muscle, cartilage, bone and synovial membrane or -fluid. Cells from these various tissues are generally isolated by mincing the tissue followed by enzymatic digestion. In general, these cells have small dissimilarities from one another, but generally are very comparable to bone marrow-derived MSCs. Cell yields from these tissues and proliferation capacities of these cells appear to be within the same order of magnitude. Furthermore, cells derived from the various musculoskeletal tissues have all been shown to have a multi-lineage differentiation potential, although they in general do show some preference for differentiating towards the tissue they were originally derived from.

Regenerative capacities of local stem cells are based on at least two features. Firstly they have the ability to differentiate into mature tissue cells, thereby contributing to new tissue formation [42]. A second, more recently discovered feature of stem cells is the secretion of trophic factors that may be responsible for other mechanisms of stem cell-mediated tissue repair [43]. These trophic factors can alter the diseased microenvironment towards an anti-inflammatory and regenerative state. Moreover, these factors are capable of stimulating locally present progenitor cells to repair OA damage or by attracting circulating endogenous progenitor cells, thereby further contributing to tissue repair [238]. Overall, we concluded that musculoskeletal stem cells derived from various sources posses a huge capacity for application in regenerative medicine. Better characterization of musculoskeletal stem cells and more knowledge about lineage differentiation is required to fully understand the potential of each individual source of cells for each of the different applications. In this respect, not only differentiation into mature tissue cells, but also the secretion of trophic factors and the influence of host microenvironment on cell fate and function deserve more study. By far, the most knowledge has been gathered concerning bone marrow-derived MSCs. These cells have already been used in more than 100 clinical trials [413] without reports of serious adverse side ef-

fects. Since one of the main aims of this thesis was to direct our studies towards clinical translation, this is the cell-type we chose for our further research.

Various pre-clinical and some initial clinical studies have already been performed applying intra-articularly injected MSCs to OA joints [54,55,237]. Animal studies have shown beneficial effects of MSCs on cartilage morphology and histology in various OA models [47,54,55]. Interestingly, studies using cell tracking in cartilage repair show only limited cartilage formation by chondrogenic differentiation of the injected MSCs [54,55]. Instead, the applied cells are mostly retrieved from other articular structures, such as the synovium. Apparently, the intra-articularly injected MSCs only occasionally differentiate into chondrocytes to actively produce extracellular matrix. This implies a different OA modifying mechanism, such as the previously mentioned paracrine effects of MSCs by secreting bioactive factors. Paracrine effects of MSCs have already been demonstrated in other applications including cardiovascular regenerative medicine and organ transplantation. In these fields, factors secreted by MSCs have been shown to increase ventricular function after ischemic myocardial injury and to reduce the occurence and severeness of graft versus host disease, respectively [414,415]. Whether factors secreted by MSCs are also capable of influencing osteoarthritic environments was not known. Therefore, in **chapter 4**, we studied the influence of MSC-derived factors on OA cartilage and synovial explants in vitro. We prepared MSC-conditioned medium by stimulating primary human MSCs, to secrete bioactive factors, with tumor necrosis factor alpha (TNF□) and interferon gamma (IFN□). The stimulated MSCs produced IL-6, hepatocyte growth factor (HGF), tissue inhibitor of MMP (TIMP)2 and transforming growth factor beta (TGF□)-1 and displayed a high enzymatic indoleamine 2,3-dioxygenase (IDO) activity. IDO is an immunomodulatory enzyme with an anti-inflammatory function. Selection of this panel of factors as study targets was based on the fact that they are known to be secreted by MSCs at high levels and their known involvement in general inflammation processes or joint metabolism [250,252]. Synovial explants exposed to MSC-conditioned medium showed decreased expression of the inflammation- or matrix degradation-related genes interleukin-1 beta (IL-1□), matrix metalloproteinase (MMP)1 and MMP13. On the other hand, the anti-inflammation-related gene suppressor of cytokine signalling (SOCS)1 was upregulated. In cartilage, expression of the anti-inflammation-related gene IL-1 receptor antagonist (IL-1RA) was upregulated. IL-1RA is a competitive non-functional binder to the IL-1 receptor and is already clinically used as a drug (Anakinra) to treat rheumatoid arthritis [416]. In addition, genes related to matrix degradation (ADAMTS5) as well as matrix formation (COL2A1) were downregulated. Additionally, MSC-conditioned medium reduced nitric oxide (NO) production in cartilage explants and the presence of the inhibitor of nuclear factor kappa B alpha (IκBa) was increased in synoviocytes and chondrocytes treated with MSC-conditioned medium. IκBa is the protein inhibiting the activation of NFkB, a protein family controlling apoptosis, inflammation and other immune responses. Based on these results, we concluded that MSCs in an inflammatory environment secrete factors which cause multiple anti-

inflammatory effects and influence genes involved in matrix turnover in OA synovium and cartilage explants.

A limitation of our study, as in our PRP studies, was the use of a short term *in vitro* culture model to evaluate the paracrine effects of MSCs. We did use a more complex *in vitro* model than the one used in chapter 2 by performing experiments on cartilage and synovium as well as a pilot experiment where we combined both tissues in the same model. In this way, we resembled more closely the *in vivo* situation. Nevertheless, further *in vivo* studies are needed to demonstrate the efficacy of MSC injection as a treatment for OA. Next to the use of an *in vitro* model, another limitation of our study was the need to stimulate MSCs with supra-physiological concentrations of inflammatory factors in order to initiate an immunomodulatory function. Whether the level of inflammatory and catabolic factors in OA joints *in-vivo* is sufficient to elicit a regenerative response in MSCs remains to be elucidated, although we recently investigated *in-vitro* effects of bioactive factors in synovial fluid of OA patients on MSC immunomodulation. In this study, we found that synovial fluid upregulated the expression of genes in MSCs with an anti-inflammatory function and that synovial fluid stimulated MSCs to condition medium in a way that suppressed lymphocyte proliferation *in vitro* [417]. MSC-conditioned medium undoubtedly contained many more factors than the ones we have measured in chapter 4. The whole panel of bio-active factors probably worked in concert to achieve the anti-osteoarthritic effects observed in this study. Several anti-inflammatory effects could be caused by the direct secretion of anti-inflammatory factors by MSCs, or by their influence on macrophages and the subsequent effect on their secretion of immunomodulatory factors [418]. Next to these MSC-secreted bioactive factors, it has more recently been shown that the regenerative potential of MSCs might be related to cellular transfer of mitochondria and other cytosolic elements [419,420]. The anti-osteoarthritic effect of this aspect has to be further studied using different *in-vitro* models. Since we observed clear paracrine effects of MSCs and given that intra-articularly injected MSCs have been shown to survive in an intra-articular environment for up to at least four to six weeks [54,55], we believe that they could provide the ultimate long term delivery of a cocktail of OA modifying elements.

As a next step, we studied the effects of intra-articularly injected bone marrow-derived cells in a rat OA model in **chapter 5.** Since OA is a complex disease consisting of many features, we evaluated effects of cellular therapies on pain, cartilage structure and bone alterations as well as inflammation. We used intra-articular injection of monoiodoacetate (MIA) as an OA model. With the aim to maximize the options for clinical translation, we evaluated the efficacy of different cell-types. Firstly, rat MSCs were used as a species specific treatment to maximize crosstalk via soluble factors or cell-cell contact and to prevent immunogenic reactions as much as possible. Secondly, human MSCs derived from OA patient were injected in OA induced rat knees to evaluate the effect of cells derived from aged and diseased donors. We hypothesized that human

MSCs would be immune-privileged and therefore would not cause an inflammatory reaction and retain their immunomodulatory capacities [291]. Finally, freshly isolated rat bone marrow mononuclear cells were used as a one-step treatment for OA. This is a cell fraction which contains approximately 0.001% MSCs and other progenitor cells as well [276,421]. This cell fraction could have a high regenerative potential, and makes extensive culture procedures, which are necessary to obtain MSCs, unnecessary. All therapies were well tolerated by the animals without any external signs of inflammation, such as swelling or erythema. Animals treated with rat MSCs distributed significantly more weight to the affected limb after treatment than before treatment, indicating a decreased pain sensation. None of the other treatments significantly affected weight distribution to the affected limb. Regarding structural changes in the joint, MIA injected knees had significant cartilage damage and subchondral bone alterations compared to contralateral control knees. Rats treated with rat MSCs displayed the least MIA induced changes, albeit no statistically significant differences between treatment groups were observed. Rat MSCs and human MSCs induced a trend towards increased synovial inflammation. The treatments applied in this study resulted in some slight therapeutic effects. Although rat MSCs were able to reduce pain sensation compared to pre-treatment animals, no statistically significant difference was observed compared to saline treated animals. This absence of significance could have been, at least partly, due to a lack of power or the OA model we used. Other groups, on the other hand, do report positive effects of intra-articularly injected MSCs on cartilage quality and other outcome parameters using different OA models, like meniscus resection or cruciate ligament transection [47,55,57]. The results of these studies indicated an indirect protective effect of the MSCs and did not point towards actual regeneration of cartilage. The fact that we used an OA model consisting of direct chemical cartilage damage, without mechanical joint instability, could explain the modest effects of our cellular treatments. This is in accordance with Frisbie et al., who found no effect of MSCs on cartilage quality in a horse osteochondral defect OA model [49]. In addition, Matsumoto et al. found just a trend towards improved cartilage quality after injection of muscle-derived stem cells (MDSCs) in a rat MIA OA model [54]. The effects of MDSCs became only clear after they were transduced with bone morphogenetic protein 4, which is involved in bone and cartilage development, and sFlt-1, a vascular endothelial growth factor antagonist. OA models like intra-articular MIA injection affect the entire joint without applying mechanical instability, in that way resembling the majority of clinical OA patients. The fact that in such models repeatedly very modest effects of stem cell therapies are reported, and that the effects are increased after genetic cell manipulation, indicates that further optimization is required before the introduction of large scale clinical application. The lack of clear effects in our study could have had other reasons as well, including, the mild degree of inflammation upon MSC injection or the fact that we used non-autologous cells. We concluded that additional studies are warranted, with a focus on cell tracking, to further optimize cellular therapies for OA treatment.

Since a better understanding of the fate of the cells after intra-articular injection provides better insight in their regenerative role, and thereby options to increase their efficacy, we continued our studies by adapting a cell tracking method based on MRI for intra-articular use. To permit possible future clinical studies using this technique, we aimed at setting-up a clinically applicable cell tracking method. Cell labeling with superparamagnetic iron oxides (SPIOs) allows cell tracking using magnetic resonance imaging (MRI) [422]. SPIO particles influence a magnetic field, thereby generating signal voids on MR images. Once SPIOs are endocytosed by cells, these cells can be detected by MRI. The use of SPIOs enables cell labeling using only clinically applied substances and this technique has already entered the clinical arena in experimental settings in other fields [62,63,64,316]. In **chapter 6** we compared two SPIOs, ferumoxides and ferucarbotran, for cell labeling. To label non-phagocytic cells with ferumoxides, the particles need to be complexed with a transfection agent like protamine sulfate, which is clinically applied as heparin antidote [303]. Ferucarbotran does not need to be complexed prior to cell labeling [298]. For clinical application, an effectively and specifically labeled cell preparation is highly desired. This means that labeled cells should contain a high amount of intracellular SPIO and a negligible amount of extracellular SPIO. We tested both SPIOs not only on MSCs but also on chondrocytes, since both cell-types are being used in cartilage regenerative medicine [216,423]. Cell labeling using ferumoxides-protamine sulfate resulted in higher labeling efficiencies and a higher iron load per cell for both cell types, as compared to ferucarbotran. Furthermore, ferumoxides-protamine sulfate labeling caused a lower amount of extracellular iron attached to the cell membranes. Therefore, we considered labeling with ferumoxides-protamine sulfate the superior approach to develop into a clinically applicable cell-tracking method.

We further evaluated this method by determining several aspects regarding safety and MRI traceability in **chapter 7** in order to fine-tune this technique for in-vivo use in an intra-articular environment. We studied SPIO for labeling MSCs regarding effectivity, cell viability, long term metabolic cell activity, chondrogenic differentiation and MSC secretion profile. We additionally examined the capacity of synovial cells to endocytose SPIO from dead, labeled cells, together with the use of magnetic resonance imaging (MRI) for intra-articular visualization and quantification of SPIO labeled cells. SPIO labeling was effective and did not impair any of the studied safety aspects, including MSC secretion profile. SPIO from dead, labeled cells could be taken up by synovial cells. This implicates, since SPIO from originally labeled cells can be taken up by other cells, that labeled cells as visualized by MRI do not necessarily indicate originally labeled cells. Therefore, prior to clinical application we recommended *in vivo* animal studies using intra-articular cell tracking by MRI in combination with another tracer to localize originally labeled cells. This would enable the correct interpretation of obtained MR images. Intra-articular SPIO-labeled cells could accurately be visualized by MRI in a clinically relevant sized joint model using clinically applied cell doses. This was determined for injected cells as well as cells implanted in focal defects, two approaches to cell-based cartilage

regenerative medicine [58,423]. In the latter approach, chondrocytes or MSCs are directly placed in a cartilage defect in order to locally generate extracellular, cartilage-like matrix. Finally, for the local implantation approach we were able to quantify different amounts of labeled cells implanted in cartilage defects using MR-based relaxometry. Based on these results we concluded that SPIO labeling appears to be safe without influencing cell behavior. SPIO labeled cells can be visualized in an intra-articular environment and quantified when seeded in cartilage defects. In an experimental setting this would show the distribution and diffusion of the applied cells, thereby elucidating the regenerative mechanisms and providing opportunities to improve current repair strategies. Several techniques using MRI have been developed to evaluate cartilage structure and quality [424,425]. The use of MRI has the advantage of simultaneous cell tracking and evaluation of cartilage regeneration in one MRI session.

Since limited cell survival might be one of the aspects compromising long term effects of cell therapies, we studied a method to increase their longevity after application. In **chapter 8** we encapsulated MSCs in alginate beads, enabling a long-lasting interplay between MSCs and their environment in the diseased joint. This interplay is necessary to initiate the regenerative function of MSCs, since MSCs need to be stimulated in order to exert their immunomodulatory role [242,243]. Once stimulated, MSCs are able to modulate chronic disease processes and structurally modify the pathophysiologic vicious circle in OA. The alginate beads are permeable to small soluble factors as secreted by MSCs and diseased tissues, yet they can protect MSCs from hostile interactions with the immune system by providing a physical barrier for invasion of host immune cells [426]. Next to this, the alginate will prevent escape of the MSCs from the intra-articular space as well as cell mortality due to non-adherence after application in the joint. We found that MSCs encapsulated in beads maintained their immunomodulatory properties and responded to inflammatory stimuli during 30 days of culture in vitro. However, we appeared to lose slightly more than half of the cells over time and the remaining cells had a reduced capacity to differentiate into the osteogenic and adipogenic lineages. We consider the diminished differentiation capacities of relatively low importance, since we regard their immunomodulatory capacity of more importance for their regenerative role and this is not dependent on their differentiation potential. This could even be an advantage, reducing the risk of osteochondral differentiation and thereby the formation of small cartilage fragments, known as loose bodies, in the joint. Nevertheless, size of the beads, concentration and type of alginate and BMSCs concentration need to be further optimized. These aspects are important in the survival, proliferative capacities and release profile of the BMSCs in terms of cellular interaction and diffusion properties of cellular nutrients as well as secretions [401,407]. Our data indicated that MSCs embedded in alginate beads may act as a continuous immunomodulatory or trophic factor release system for several weeks, making this a promising approach towards a durable DMOAD.

Concluding, in this thesis we demonstrated that biological treatments, by means of bioactive factors or cell therapies, may provide a promising future alternative for the symptomatic treatments currently available for OA. We found that bioactive factors concentrated from blood (PRP), as well as factors secreted by MSCs, have beneficial effects on various osteoarthritic processes *in vitro*. The use of MSCs has the potential advantage of providing a more durable treatment than the use of PRP, since MSCs are able to secrete bioactive factors for a longer period of time. Next to this, MSCs are locally present in multiple joint tissues [267] and are able to react to the joint environment, as shown by the increased presence of MSCs in synovium and synovial fluid after joint injury [45,46]. This suggests that the increased intra-articular presence of MSCs is part of a natural healing process. The administration of MSCs in an osteoarthritic joint could be a therapy for OA mimicking and enhancing this natural healing process and thereby provide a natural and autologous treatment for OA. At this moment, merely injecting MSCs into osteoarthritic joints shows promising, yet modest effects on pain and structural outcome measures. Further studies are warranted to further optimize these treatments. Cell-tracking is a crucial tool to elucidate the regenerative mechanisms and provide insight into cell distribution and survival. Cell tracking of SPIO labeled cells using MRI allows accurate cell visualization with a high spatial resolution and in some circumstances even quantification of applied cells. These tracking tools enable additional improvement and optimization of current cell therapies. Encapsulating the cells in alginate beads could improve their intra-articular longevity after application, allowing modification of osteoarthritic processes for a longer period of time. This would eventually advance intra-articular cell therapy into a durably, effective, disease modifying osteoarthritic drug (DMOAD).

Chapter 10

Future perspectives

Future studies based on this thesis can be roughly divided into three (interconnected) aspects: 1) optimizing cell tracking techniques and thereby generating better insight in the relevant anti-osteoarthritic working mechanisms; 2) improving monitoring of various osteoarthritic processes to evaluate therapeutic efficacy, and 3) using this knowledge of cell function and osteoarthritic processes to further optimize the efficacy of the therapy and to develop new therapies. These components will be addressed hereafter.

Cell tracking

MRI is a great modality for high-resolution intra-articular cell tracking. Unfortunately, SPIO label is not necessarily associated with living cells, since it remains present after cells have died [322]. Cell tracking using a dual imaging technique, for instance optical imaging together with MRI, allows high resolution visualization of cells in combination with quantification of viable cells. For this purpose MSCs can be transduced with a luciferase gene construct [427]. Luciferase acts as a reporter in living cells and can be detected with bioluminescence imaging techniques. Activation of its substrate D-luciferin requires the presence of ATP [428], which is only produced in living cells. The signal emitted is proportional to the number of living cells [429]. We already performed a pilot experiment using this combined technique of optical imaging with MRI, which indicated that cell number decreases significantly within two weeks after injection in the joint. These experiments should be further extended to see if cell survival is related to, for instance, inflammation or other micro environmental circumstances. It could even be the case that, in line with other reports, MSCs locally reside in a quiescent state after inflammation diminishes, but become active and proliferate once a new episode of inflammation occurs [137]. Certain imaging markers could even be linked to a particular gene of interest [430], which could teach us whether cells have a merely anabolic role via the secretion of growth factors, an anti-catabolic role via the secretion of factors counteracting degradative enzymes, or more an anti-inflammatory function by the secretion of anti-inflammatory factors. Likely, different roles are being played at different stages of the disease and by different subsets of MSCs. Visualizing this complicated but finely tuned interplay would give true insight into cell activity over time.

Osteoarthritis monitoring

Since OA is a multifactorial disease consisting of many simultaneous processes, assessment of the effect of cellular therapies on structural joint aspects, joint inflammation as well as pain in various animal OA models and after application in early and later stages of the disease is essential in optimizing cellular treatments.

Structural cartilage changes can be determined by micro computed tomography (μCT) or MRI. Techniques based on the diffusion of a contrast agent into cartilage have been developed, which can be used to visualize the amount of proteoglycans present in the cartilage. For small animals μCT arthography can be used and for humans delayed gadolinium enhanced MRI of cartilage (dGEMRIC) [279,424]. The amount of proteoglycans is an indicator of structural cartilage quality and thereby a good outcome measure for therapeutic efficacy. CT can also be used to evaluate subchondral bone changes in terms of sclerosis, porosity and osteophyte formation [431]. Regarding MRI, new sequences are being developed to visualize structural cartilage characteristics without the need for applying a contrast agent, thereby further reducing the exposure of patients to potentially harmful interventions [425,432].

Synovitis is another OA aspect which can be evaluated using MRI [433]. For this application, new sequences are being developed that could make use of a contrast agent redundant as well. Macrophage activity can more specifically be used as a measure of inflammation and can be visualized and quantified using probes for positron emission tomography (PET), single photon emission computed tomography (SPECT) or optical imaging [434]. This can be used to evaluate the effect of inflammation on applied MSCs, as well as the subsequent effect of MSCs on inflammation. In addition, histological techniques can be used to verify these data and to further distinguish between macrophage subtypes [268]. Currently, macrophages are broadly classified into two main groups designated M1 and M2. In short, M1 macrophages represent pro-inflammatory cells, whereas M2 macrophages have a more regenerative and immune modulatory role. As mentioned before, MSCs are able to influence macrophage subtype differentiation [418]. The distinction between macrophage subtypes can be used to gain further insight into the effect of MSCs on joint inflammation. Next to these imaging options, various techniques, like enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (CBA) based on flow cytometry, exist to measure different cytokines. These cytokines can be determined in blood or in synovial fluid, giving further insight in inflammatory processes.

Pain can be measured using several methods and is a complicated phenomenon consisting of inflammatory and neuropathic aspects [287,288]. The effect of MSC therapy on pain or other clinical outcome measures are difficult to assess in animal OA studies, and therefore they are not extensively documented. Nevertheless, pain is the most important clinical outcome measure and it is known that pain is not clinically associated with cartilage damage [435], although correlations between pain levels and the amount of synovitis and intra-articular cytokine levels have been demonstrated [436]. Further evaluation of multiple pain aspects, including weight bearing, mechanical allodynia and thermal hypersensitivity, is needed to assess the capacity of MSCs to alleviate osteoarthritic pain.

Finally, sensitive markers for MSCs or other progenitor cells would allow (histological) detection of endogenous repair cells who could also account for various regenerative processes [437]. If a large part of the regenerative capacities of MSCs is based on the activation of endogenous cells, this knowledge can be used to enhance this feature by, for instance, selection of MSC-subtypes. Another option is to determine the factor responsible for endogenous repair cell recruitment and activation [221] and integrate or add this factor to cellular therapies.

Therapeutic efficacy

To increase therapeutic efficacy various adjustments can be made. Since MSCs comprise a heterogeneous cell population, cell selection techniques can be developed to isolate the subpopulation with the greatest regenerative potential [438]. This has to be based on insight into their specific mode of regenerative action. Potentially, this can be combined with a form of pre-treatment of cells prior to application, to ensure that they are optimally instructed to fulfil their regenerative action [439]. This could be performed by pre-treatment with bioactive factors [439] or even genetic modifications [54], although the latter implies many extra hurdles for clinical application. Other adjustments include the timing and mode of application. It could be that injecting a low dose of cells for multiple times has advantages over a single injection of a high cell dose for instance. Regarding the mode of application many adaptations can be made, including the encapsulation of MSCs in alginate beads to improve the survival and retention time of cells in the joint, enabling a durable modification of multiple osteoarthritic processes by MSCs [426]. Of course, the specific encapsulation material can be endlessly varied, and must be tailored to the specific anti-osteoarthritic actions of MSCs.

Beyond cell-based therapies

As argued before, MSC injection into damaged joints is an approach mimicking and enhancing natural repair mechanisms [46]. Nevertheless, if we assume that this repair mechanism is not based on the formation of new tissue by differentiated MSCs but by the secretion of trophic factors, identification of the responsible factors must be achievable. By exactly finding out what cells secrete at what time point and by what this is triggered, we could mimic this process without the need for actual cell application. A future perspective could then be to isolate and combine the relevant bioactive factors, enabling the production of a so-called anti-osteoarthritic cocktail. Maybe a few different cocktails are needed depending on the stage of the disease or certain patient-specific characteristics, which will have to be further determined. To allow long term presence

of bioactive factors they can be incorporated in various available slow-release systems [440]. The effect might not last as long as actual cell therapy, but due to the off-the-shelf nature of this approach the treatment could be repeated once cost-effectiveness has been established. A disadvantage of this approach is the fact that this therapy will inevitably be less tailored than individual autologous cell therapy, but it has the huge advantages of standardization of the medicinal product (and thereby the expected effects) and a much higher chance of broad clinical application in terms of safety, availability and costs. Furthermore, the relevant factors could be applied in a supra-physiological concentration if desired. At the same time, MSC-secreted factors with a known negative effect on cartilage, like VEGF for instance [441], could be specifically excluded from the cocktail. This way, we could develop biological cell therapy through understanding of cellular mechanisms and isolation of the beneficial bioactive factors to evolve into the ultimate durable cell-free DMOAD.

Chapter 11

Appendices

Nederlandse samenvatting

Dankwoord

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PhD Portfolio Summary

List of publications

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Nederlandse samenvatting

Richting celtherapie als behandeling voor artrose

Artrose is een veel voorkomende degeneratieve gewrichtsaandoening en wordt gekenmerkt door een catabool en inflammatoir gewrichtsmilieu. Het gehele gewricht speelt een rol bij artrose, waaronder het kraakbeen, synovium en het subchondrale bot. Tot op heden zijn alleen symptomatische behandelingen beschikbaar voor artrose, zoals pijnmedicatie, fysiotherapie en levensstijl veranderingen zoals gewichtsverlies. Deze behandelingen zijn niet in staat om artrose te genezen en kunnen daarmee geen langdurige oplossing brengen. Als deze conservatieve methoden falen, is de definitieve behandeling een gewrichtsvervangende operatie. Helaas hebben de gebruikte protheses een beperkte levensduur van 10 à 15 jaar, en zijn dus ook niet in staat om een permanente oplossing te vormen voor artrose, vooral bij jonge paitenten. In dit proefschrift heben we het werkingsmechanisme en de effectiviteit onderzocht van twee biologische therapieen en hun capaciteit om artrotische processen te veranderen om daarmee de ziekteverschijnselen te voorkomen of verminderen. Hiertoe hebben we als eerste therapie plaatjes-rijk plasma (PRP) onderzocht. De bloedplaatjes in PRP zijn kleine celfragmenten die van megakaryocyten komen. Deze plaatjes kunnen worden geïsoleerd uit bloed door middel van centrifugatie en vervolgens worden gestimuleerd om bioactieve factoren uit te scheiden. Als tweede optie hebben wij mesenchymale stamcellen uit het beenmerg onderzocht. Stamcellen komen in meerdere weefsels voor en spelen een belangrijke rol bij natuurlijke homeostase en regeneratie van weefsels. Zij zijn in staat om nieuw weefsel te vormen en ook om allerlei factoren uit te scheiden die de lokale omgeving beïnvloeden. Onze hypothese bij deze biologische behandelopties was dat door het stimuleren van de patient zijn eigen herstel mechanismen, het mogelijk zou zijn om de vicieuze degeneratieve cirkel van artrotische processen te doorbreken.

In **hoofdstuk 2** hebben we de capaciteit onderzocht van factoren uit PRP om de effecten van ontsteking op humane artrotische chondrocyten te verminderen. De factoren uit PRP waren in staat om de effecten van interleukine-1 beta, een ontstekingsfactor, te verminderen op genen gerelateerd aan ontsteking en de opbouw en afbraak van kraakbeenmatrix. De factoren uit PRP verminderden de activatie van nucleaire factor kappa B, dit zou een mogelijke route kunnen zijn via welke deze factoren hun effecten teweeg brengen. Deze studie toonde aan dat een combinatie van bioactieve factoren in staat was om pathologische processen in artrotische chondrocyten te veranderen. Een beperking van deze mogelijke therapie is het feit dat geinjecteerde stoffen slechts een beperkte tijd in de knie aanwezig blijven. Aangezien mesenchymale stamcellen in staat zijn om langdurig een breed scala aan bioactieve factoren uit te scheiden, hebben we ons verder hier op gericht als een langere termijn therapie voor artrose.

Friedenstein en zijn collega's hebben als eerste kolonie-vormende cellen beschreven die verkregen werden uit beenmerg. Later werden deze cellen tot mesenchymale stamcellen (MSCs) benoemd door Caplan en zijn collega's. In **hoofdstuk 3** geven we een literatuuroverzicht van de karakteristieken van de natuurlijk aanwezige voorlopercellen of stamcellen in verschillende weefsels in het gewricht, waaronder het gewrichtsvlies, vetweefsel, de pezen, spieren en het bot. Over het algemeen tonen de cellen verkregen uit deze verschillende weefsels minimale verschillen, en zijn ze heel vergelijkbaar met stamcellen uit het beenmerg wat betreft proliferatie- en differentiatie capaciteiten. Aangezien verreweg de meeste kennis en ervaring is verkregen met betrekking tot stamcellen uit het beenmerg, is dit het celtype waar we ons verdere onderzoek mee hebben verricht. Dit om de eventuele klinische translatie van ons onderzoek zo eenvoudig mogelijk te houden. Vanaf nu zullen wij aan deze cellen refereren als MSCs.

De regeneratieve capaciteit van MSCs is op zijn minst gebaseerd op twee aspecten. Ten eerste hebben ze de capaciteit om te differentiëren naar volwassen weefselcel, bijvoorbeeld een kraakbeencel, om zo nieuwe (kraakbeen)matrix te vormen. Een tweede aspect is het feit dat ze een breed scala aan groei- en immunomodulatoire factoren kunnen uitscheiden, een paracriene functie die het lokale micro-milieu kan beïnvloeden. Eerdere dierstudies die gebruik maken van technieken om MSCs te vervolgen in een beschadigd gewricht laten zien dat de MSCs maar beperkt chondrogeen differentiëren en nieuwe kraakbeenmatrix vormen. Vanwege die reden hebben wij in **hoofdstuk 4** onderzocht in hoeverre de paracriene effecten van MSCs een rol spelen bij het beïnvloeden van artrotische processen in kraakbeen en synovium. Het gewrichtsvlies, (synovium) speelt met name een belangrijke rol in bij ontsteking van een gewricht. Het bleek dat de groei- en immunomodulatoire factoren die worden uitgescheiden door MSCs, anti-inflammatoire en anti-catabole effecten hebben op genexpressie niveau in artrotisch synovium. In artrotisch kraakbeen werden anti-inflammatoire effecten gezien op genexpressie niveau alsmede een remming van opbouw en afbraak van kraakbeen matrix. Ook met andere assays werden anti-inflammatoire effecten gezien, met remming van nucleaire factor kappa B activatie als een mogelijk werkingsmechanisme. Gebaseerd op deze resultaten hebben we geconcludeerd dat MSCs in staat zijn om factoren uit te scheiden die meerdere anti-inflammatoire effecten hebben en genen beïnvloeden die zijn betrokken bij matrix opbouw en –afbraak in artrotisch synovium en kraakbeen. Gezien het feit dat MSCs ten minste zes weken kunnen overleven in een gewricht, zouden zij de ultieme therapie kunnen zijn die voor een langere termijn artrotische processen kan beinvloeden.

Als volgende stap hebben we in **hoofdstuk 5** de effecten bestudeerd van verschillende celtypen, verkregen uit het beenmerg, op een model voor artrose in ratten. We hebben gekeken naar de effecten op pijn, kraakbeen- en botstructuur en ontsteking. Als celtypen hebben we ratten MSCs gebruikt, humane MSCs (om de klinische transleerbaarheid te vergroten) en de mononucleaire fractie van ratten beenmergcellen. Dit

laatste met als doel om te onderzoeken of het kweken van cellen overbodig zou kunnen worden waardoor translatie naar de kliniek makkelijker zou zijn. Wij zagen dat, na het induceren van artrose, dieren die met ratten MSCs werden behandeld meer gewicht op hun aangedane poot zetten na behandeling met celinjectie dan ervoor. Dit is indicatief voor een verminderde pijn sensatie na behandeling. In de overige groepen en de controle groep werd dit effect niet gezien. Wij zagen geen effecten van enige behandeling op kraakbeenschade, subchondraal bot of ontsteking in het gewricht. Op basis van deze resultaten hebben wij gekozen om verder onderzoek te doen naar het labelen en vervolgen van cellen om meer inzicht te krijgen in hun functie in het gewricht en zo mogelijk hun effectiviteit te vergroten.

Met het doel om meer inzicht te krijgen in celfunctie en lokalisatie in het gewricht hebben we onze studies gecontinueerd door een cel volg-methode gebaseerd op MRI, geschikt te maken voor intra-articulair gebruik. Wederom met het oog op klinische translatie hebben we gekozen voor een methode door middel van cel labeling met superparamagnetisch ijzer oxide (SPIO), een methode die reeds experimenteel in de kliniek gebruikt wordt in andere vakgebieden. In **hoofdstuk 6** hebben we twee SPIOs vergeleken, ferumoxides en ferucarbotran, voor het intracellulair labelen van cellen. Het bleek dat ferumoxides, in combinatie met protamine als transfectie mediator, leidde tot meer intracellulair en minder extracellulair SPIO. Deze methode hebben wij dus het meest geschikt bevonden om cellen te labelen en te vervolgen in het gewricht, gezien het feit dat dit de hoogste betrouwbaarheid geeft dat de signalen gezien op MRI daadwerkelijk overeenkomen met gelabelde cellen.

Deze methode hebben we verder ontwikkeld door verschillende aspecten te onderzoeken met betrekking tot effectiviteit, veiligheid en visualiseerbaarheid door MRI in **hoofdstuk 7**. Onze studies lieten geen nadelige effecten zien van de labeling methode op levensvatbaarheid van de MSCs, hun chondrogene differentiatie en de uitgescheidde groei- en immunomodulatoire factoren. SPIO uit dode, gelabelde cellen kon worden opgenomen door synovium, wat betekent dat gelabelde cellen die worden gevisualiseerd door MRI niet automatisch origineel gelabelde cellen zijn. Wij hebben daarom geadviseerd om eerst dierstudies te verrichten met een dubbele labeling techniek om het MRI signaal te kunnen valideren. Intra-articulair ingebrachte cellen gelabeld met SPIO konden adequaat gevisualiseerd worden met de MRI. Wanneer de cellen geimplanteerd werden in een lokaal kraakbeendefect was het mogelijk om verschillende hoeveelheden cellen te quantificeren. Gebaseerd op deze resultaten hebben wij geconcludeerd dat cellabeling met SPIO veilig is, dat de cellen intra-articulair gevisualiseerd kunnen worden als zij in het gewricht worden geïnjecteerd en gekwantificeerd als zij worden geïmplanteerd in een lokaal kraakbeendefect. De MRI heeft als voordeel dat tegelijkertijd eventuele kraakbeenregeneratie beoordeeld kan worden.

Aangezien beperkte cel overleving één van de aspecten is die de lange termijn effecten van stamceltherapie kan verminderen, hebben we een methode bestudeerd om hun overleving intra-articulair te verbeteren. In **hoofdstuk 8** hebben we MSCs in alginaat bolletjes ingesloten zodat zij beschermd zijn tegen cellen van het immuunsysteem, maar nog steeds hun bioactieve factoren kunnen uitscheiden. We zagen dat MSCs in deze alginaat bolletjes hun immunomodulatoire capaciteiten behielden gedurende ten minste 30 dagen *in vitro*. Wel moeten er nog aspecten geoptimaliseerd worden, zoals alginaat- en MSC concentratie, alsmede de grootte van de bolletjes, om de continue uitscheiding van bioactieve factoren door MSCs te verbeteren.

Concluderend hebben we in dit proefschrift aangetoond dat biologische behandelingen, in de vorm van bioactieve factoren of celtherapie, een veelbelovend alternatief kan worden voor de huidige symptomatische behandelingen die beschikbaar zijn voor artrose. We hebben ontdekt dat bioactieve factoren geisoleerd uit bloed (PRP), zowel als factoren uitgescheiden door MSCs, positieve effecten hebben op verschillende artrotische processen *in vitro*. Het gebruik van MSCs heeft mogelijk als voordeel dat ze gedurende een langere periode factoren kunnen uitscheiden, en dus een langduriger artrose modulerend effect kunnen hebben. Door stamcellen in een artrotisch gewricht te injecteren in een proefdiermodel werden slechts bescheiden effecten gezien op pijn en structurele uitkomstmaten. Meer onderzoek is nodig om deze effecten te verbeteren. Het labelen en vervolgen van cellen met MRI is een methode die gebruikt kan worden om inzicht te krijgen in hun werkingsmechnisme, om zo de effectiviteit van stamceltherapie te vergroten. Het insluiten van MSCs in alginaatbolletjes kan hun overleving in het gewricht vergroten en daarmee de duur van het therapeutische effect. Deze ontwikkelingen zouden intra-articulaire stamceltherapie kunnen maken tot een effectieve therapie die langdurig artrotische processen kan modificeren.

Dankwoord

Als sluitstuk mijn dank voor alle mensen die mij geholpen hebben bij de totstandkoming van dit proefschrift. Zeker bij een promotie die tot stand komt op basis van een project van twee afdelingen is teamwork onontbeerlijk. Een aantal mensen wil ik in het bijzonder bedanken.

Prof. Dr. G.J.V.M. van Osch, beste Gerjo, als promotor en dagelijks begeleider gedurende de mijn promotie was jij altijd biojzonder laagdrempelig te bereiken. Deze laagdrempeligheid, jouw enthousiasme en jouw kritische blik hebben mij voor 4 jaar enorm gestimuleerd. Daarnaast is de snelheid waarmee je reageert op vragen of e-mails ronduit verbazingwekkend. Ik ben enorm blij dat ik onder een begeleider als jij heb mogen promoveren, een waar voorrecht. Ik hoop in de toekomst nog veel met je samen te werken.

Prof. Dr. ir. Weinans, beste Harrie, jij bent mijn schoolvoorbeeld van 'de professor' gebleken: intelligente vragen, een zeer brede interesse en kennis, gecombineerd met een bovengemiddeld chaotische werkwijze. Jouw prikkelende, kritische en toch 'openstaand voor alles' manier van wetenschap bedrijven heeft mijn projecten en wetenschappelijke vorming bijzonder positief beïnvloed.

Dr. M.R. Bernsen, beste Monique, ook jij bent als co-promotor bijzonder nauw betrokken geweest bij mij onderzoek en de begeleiding daarvan. Je enthousiasme en inzicht tonen veel overeenkomsten met die van Gerjo, maar de werkwijze en een aantal persoonlijke kenmerken verschillen dag en nacht. Dit maakte jouw begeleiding voor mij bijzonder waardevol; jouw kritische schrijf-aanwijzingen hebben mijn publicaties zonder uitzondering flink verbeterd.

Dr. E. Farrell, dear Eric, you were my first daily supervisor, and probably the best I could have ever wished for. You taught me a massive amount of useful, and useless, stuff during my first year. At the same time, you are socially a tireless centipede, which really enlightened my presence in Rotterdam. You're also responsible for probably the most efficient didactic moment during my PhD. After reading the first version of my first manuscript you did not bother to go into any detail but simply replied: "Cut every sentence in half and just make it better". I sincerely hope we will share a bright future in both a professional and personal way.

Prof. Dr. J.A.N. Verhaar, beste professor, de manier waarop u kliniek en wetenschap met elkaar combineert heb ik altijd bewonderenswaardig gevonden. Dank voor uw begeleiding en de mogelijkheden die u me geboden heeft.

Prof. Dr. Krestin, dank voor uw ondersteuning en aanvullingen op mijn manuscripten. Het goedkeurende oordeel van u de radiologische wetenschapscommissie 1 jaar na het

starten van mijn promotie heeft mij gedurende de rest van het traject enorme steun en vertrouwen gegeven.

Dr. P.K. Bos, beste Koen, jouw visie in klinische translatie is een belangrijke leidraad geweest voor de richting van mijn onderzoek. Dank voor je steun en je lange termijnvisie op de klinische toepassing van stamceltherapie in de orthopaedie.

Beste Nicole, Kopseflops, Opperdepop, etc. Jij bent zonder twijfel de collega in mijn leven waar ik de meeste bijnamen ooit voor heb bedacht. Ik denk dat dit een goede reflectie is voor jouw veelzijdigheid als persoon, alsmede de hoeveelheid aan verschillen in werkwijze die wij er op nahouden. Onze samenwerking werd exponentieel beter in die 4 jaar, en jouw klaterende lach was een verlichting op de werkvloer. Dank voor al het werk dat je voor me verzet hebt.

Beste Wendy, Sandra en Gaby, als analisten van de afdelingen orthopaedie en radiologie hebben jullie mij gedurende mijn gehele promotie geweldig ondersteund. Dank voor al jullie hulp en gezellige momenten.

Dear Gyula and Piotr, you were the brains behind the physical and technical part of the MR imaging in my thesis, which enabled me to perform cell-tracking at a level that would have been way out of my reach without you. Many thanks for your help and for sharing your knowledge with me.

Beste collega (onderzoeker)'s, beste Michiel, YBJ, Roberto, Erwin, Maarten, Joost, Jasper, Rintje, Sandra, Ruud, Olav, Tom, Anna, Wu, Femke, Robert-Jan, Marloes, Mieke, Nienke, Johan, Job, Belle, Ton, en alle anderen, heel veel dank voor jullie samenwerking en gezelligheid.

Dirk en Stefan, mijn beide paranimfen. Jullie zijn beiden fantastische (assistent)orthopeden en hebben mij beiden in een ander gedeelte van de opleiding meegemaakt. Dirk tijdens mijn studie, Stefan tijdens deze promotie, beiden even waardevol. Zowel sociaal als qua professionele instelling denk ik dat wij drieën een grote gelijkenis vertonen. Geweldig dat ik samen met jullie een vergelijkbare weg bewandel! Ik dank jullie bij voorbaat voor de steun die jullie mij op dit toneel gaan bieden.

En dan mijn familie, lieve PaPaul, MaSylvia, grote broer Jasper, schoonzus Marie-Christine en aan de ander kant Paul, Roos, Marije, Rutger en Floor. Dank voor jullie steun en interesse. Jullie zijn een (schoon)familie waar enorm veel warmte van uit gaat en die altijd vol belangstelling naar mijn onderzoeksresultaten en avonturen hebben geluisterd. Pa en ma, jullie weten precies wat promoveren inhoudt. Jullie lichte verontwaardiging toen jullie erachter kwamen dat ik mijn eerste artikel niet ter correctie aan jullie had gestuurd alvorens het werd gepubliceerd vond ik schattig en geweldig tegelijk. Dank

voor alle support en steun die ik al mijn hele leven van jullie ontvang, dit proefschrift kan weer mooi op het “voor een kind niet slecht, ook al is het maar een arts” rijtje worden bijgeschreven.

Lieve Felien, als waarachtige Mini-Me vind ik je geweldig en fantastisch. De schuld aan je ouders betaal je terug aan je eigen kinderen, en in die geest heb je mij op wetenschappelijk gebied ruimschoots de gelegenheid geboden om hieraan te voldoen. Ik hou van je, en hoop nog heel veel met je te gaan beleven.

Lieve Wendy, als een moderne ‘vrouw van de dokter’ heb jij jezelf gewoon lekker verder ontwikkeld gedurende mijn promotie op zowel persoonlijk als professioneel gebied. Inmiddels bij jij zelfs ‘de baas’ geworden, iets wat ik nog niet kan zeggen. Je was altijd geïnteresseerd en ondersteunend in mijn onderzoek, maar vond het heerlijk als ik op congres was om je eigen ritme er op na te kunnen houden. Dank voor al jouw liefde, je kritische houding en aandeel in onze avonturen, van jou leer ik het meest!”

Curriculum vitae

Gerben Matthijs van Buul werd geboren op 28 augustus 1982 te Leiderdorp. Hij werd grootgebracht in het waterrijke Woubrugge waar hij naar basisschool “Esselyckerwoude” ging. Hij vervolgde zijn opleiding op het Stedelijk Gymnasium Leiden, waar hij in 2000 cum laude zijn diploma behaalde. In datzelfde jaar ging hij geneeskunde studeren in de domstad aan de Universiteit Utrecht. In 2003-2004 heeft hij een studievrij jaar gehad om zich in te zetten als praeses van de geneeskundige faculteitsvereniging MSFU “Sams”. Hierna zette hij zijn co-schappen voort en combineerde dit waar mogelijk met zijn liefde voor reizen. Zijn co-schap kindergeneeskunde volgde hij in Mauritius en zijn co-schap gynaecologie in Aruba. In 2007 volbracht hij zijn semi-arts stage gecombineerd met wetenschappelijk onderzoek bij de afdeling orthopaedie van het UMC Utrecht onder leiding van prof. W.J.A. Dhert en prof. D.B. Saris. Hier werd de basis gelegd voor zijn interesse in basaal wetenschappelijk onderzoek met een translationele inslag. Dit vertaalde zich in de start van zijn promotie in 2008 aan de Erasumus Universiteit in Rotterdam onder begeleiding van onder andere zijn promotoren prof. G.J.V.M. van Osch en prof. H. Weinans. In 2012 startte hij met zijn vooropleiding chirurgie in het Reinier de Graaf Gasthuis in Delft onder leiding van opleider Dr. M. van der Elst. In 2013 is hij begonnen met zijn opleiding tot orthopaedisch chirurg die hij in het Erasmus MC Rotterdam en het Reinier de Graaf Gasthuis zal volgen. In 2010 is hij getrouwd met Wendy van Buul-Arah en samen hebben zij Felien als stralende dochter.

PhD portfolio summary

Summary of PhD training and teaching activities

Name PhD student: G.M. van Buul	PhD period: 01-03-2008 – 01-01-2012
Erasmus MC Department: Orthopaedics & Radiology	Promotor(s): Prof. G.J.V.M. van Osch, Prof. H. Weinans
Research School: Molmed	Supervisor: Dr. M.R. Bernsen

1. PhD training

	Year	Workload
General academic skills		
- Biomedical English Writing and Communication	‘09	4.0
- Laboratory animal science, artikel 9	‘09	4.0
Research skills		
- Literature meeting orthopedic lab	’08-‘12	4.0
- Erasmus MC radiology lunch meeting	’08-‘12	2.0
In-depth courses (e.g. Research school, Medical Training)		
- 2nd Animal Imaging Workshop: Applied Molecular Imaging Erasmuc MC	‘08	1.0
- Laboratory Skills Course: International Cartilage Repair Society	‘08	1.0
- Statistics: introduction to data analysis: Erasmus MC	‘09	2.0
- Statistics: regression analysis: Erasmus MC	‘09	2.0
- Statistics: Basic introduction on SPSS	‘09	1.0
(Inter)national conferences: poster presentations		
- Clinically applicable cell tracking using Super Paramagnetic Iron Oxides (SPIO), International Stem Cell Symposium: Amsterdam, the Netherlands	‘08	1.0
- Cell labeling using Superparamagnetic Iron Oxide (SPIO) has no effect on chondrocyte behavior: implications for clinically applicable cell tracking using MRI , European Society of Molecular Imaging: Antalya, Turkey	‘09	1.0
- Clinically applicable hBMSC tracking in cartilage repair using MRIMSC in Solid Organ Transplantation: Rotterdam, the Netherlands	‘09	1.0
- Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI, Osteoarthritis Research Society International: Montreal, Canada	‘10	1.0
- Cell labeling using Superparamagnetic Iron Oxide (SPIO) has no effect on chondrocyte behavior: implications for clinically applicable cell tracking using MRI , European Congress of Radiology: Vienna, Austria	‘10	1.0

- Feasibility of superparamagnetic iron oxides for clinical intra-articular cell tracking, European Congress of Radiology: Vienna, Austria	'10	1.0
- Clinically applicable cell tracking by MRI in cartilage repair using Superparamagnetic Iron Oxide (SPIO), European Molecular Imaging Meeting, Warsaw, Poland.	'10	1.0
- Clinically applicable cell tracking in cartilage repair using MRI, Tissue Engineering & Regenerative Medicine International Society, Galway, Ireland.	'10	1.0
- Cell tracking for cartilage repair using Superparamagnetic Iron Oxides: clinical potential, Osteoarthritis Research Society International, Brussels, Belgium.	'10	1.0
- Clinically applicable cell tracking in cartilage repair using MRI, International Cartilage Repair Society, Barcelona, Spain.	'10	1.0
- Platelet Rich Plasma inhibits IL-1b induced catabolic processes in osteoarthritic chondrocytes, Orthopedic Research Society, Long Beach, USA.	'11	1.0
- Platelet-rich Plasma releaste inhibits catabolic processes in osteoarthritic chondrocytes, Molecular Medicine Day, Rotterdam, the Netherlands.	'11	1.0
- Platelet-Rich Plasma has anti-catabolic properties in Osteoarthritic Chondrocytes, Tissue Engineering & Regenerative Medicine International Society, Granada, Spain.	'11	1.0
- Mesenchymal stem cells exert paracrine effects on osteoarthritic cartilage and synovium, Tissue Engineering & Regenerative Medicine International Society, Granada, Spain.	'11	1.0
- Mesenchymal stem cells have anti-catabolic and anti-inflammatory effects on osteoarthritic cartilage and synovium, International Society for Cellular Therapy, Rotterdam, the Netherlands.	'11 '11	1.0
- Clinical feasibility of cell tracking for cartilage repair using MRI, International Society for Cellular Therapy, Rotterdam, the Netherlands.		1.0
- Platelet-Rich Plasma has anti-inflammatory properties in Osteoarthritic Chondrocytes, Osteoarthritis Research Society International, San Diego, USA.	'11	1.0
- Mesenchymal stem cell therapy in a monoiodoacetate induced rat model of osteoarthritis, Osteoarthritis Research Society International, Barcelona, Spain	'12	1.0
(Inter)national conferences: podium presentations		
- Clinically applicable cell tracking using super paramagnetic iron oxides (SPIO) in an osteoarhtitis (OA) model, Radiology days: Rotterdam, the Netherlands	'08	1.0

- Cell tracking using super paramagnetic iron oxides in cell therapy for cartilage regeneration, Term symposium: Egmond aan Zee, the Netherlands	'08	1.0
- Clinically relevant cell tracking using Superparamagnetic Iron Oxides has no effect on chondrocyte behaviour, International Cartilage Repair Society: Miami, USA	'09	1.0
- Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI, European Society for Magnetic Resonance in Medicine and Biology: Barcelona, Spain	'09	1.0
- Clinically applicable cell tracking in cartilage repair using MRI, Molmed day: Rotterdam, the Netherlands	'10	1.0
- Clinically applicable cell tracking in cartilage repair; suitability of superparamagnetic iron oxide labeling, Orthopedic Research Society: New Orleans, USA	'10	1.0
- Clinically applicable cell tracking in cartilage repair using Superparamagnetic Iron Oxides (SPIO), Radiological Society of North America, Chicago, USA.	'10	1.0
- Clinically applicable cell tracking by MRI in cartilage repair, Biomedical Materials & Translational excellence in Regenerative Medicine Meeting, Ermelo, the Netherlands	'10	1.0
- Mesenchymal stem cells exert paracrine effects on osteoarthritic cartilage and synovium, Osteoarthritis Research Society International, San Diego, USA.	'11	1.0
- Mesenchymale stamcellen remmen ontstekingsprocessen en kraakbeen afbraak in artrotisch synovium en kraakbeen, Nederlandse Orthopedie Vereniging, the Hague, the Netherlands	'12	1.0
- Mesenchymal stem cells exert paracrine effects on osteoarthritic cartilage and synovium, International Cartilage Repair Society, Montreal, Canada	'12	1.0
Invited lectures		
- Cell tracking and quantification using SPIO in cartilage repair by MRI, Catholic Univerisity Leuven, Leuven, Belgium.	'11	1.0
- Stem cells in osteoarthritis, Santander Summer School, Santander, Spain.	'11	1.0

2. Teaching activities

Lecturing		
Supervising practicals and excursions		
- Teaching cartilage/OA histology	’08 – ‘12	1.0
Supervising Master’s theses		
- Supervising medical students (Jasper van de Slegt and Maarten Leijs) in scientific period	’09 & ‘11	8.0

List of Publications

Leijs MJ, van Buul GM, Lubberts E, Bos PK, Verhaar JA, Hoogduijn MJ, van Osch GJ. Effect of Arthritic Synovial Fluids on the Expression of Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro Study. Frontiers in Immunology 2012;3:231.

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van Buul GM, Koevoet WL, Kops N, Bos PK, Verhaar JA, Weinans H, Bernsen MR, van Osch GJ. Platelet-rich plasma releasate inhibits inflammatory processes in osteoarthritic chondrocytes. American Journal of Sports Medicine. 2011 Nov;39(11):2362-70.

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