# In Vitro Models For Cell-Based Cartilage Regeneration


The printing of this thesis was financially supported by:
Erasmus MC, department of Orthopaedics
Erasmus Universiteit Rotterdam
Anna Fonds, Leiden
Nederlandse Vereniging voor Matrixbiologie

The research leading to the results described in this thesis was financially supported by the European Union's 7th Framework Programme under grant agreement no. NMP3-SL-2010-245993. The research in this thesis was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.
The word clouds displayed in thesis were generated using wordle.net


**ISBN:** 978-94-91487-12-5

**Lay-out by:** Ridderprint BV, Ridderkerk, the Netherlands
**Printed by:** Ridderprint BV, Ridderkerk, the Netherlands
**Publisher:** YourThesis published by Medix Publishers BV,
Keizersgracht 317A, 1016 EE Amsterdam, the Netherlands

De digitale versie van dit proefschrift is te vinden in de YourThesis app en kan gelezen worden op een tablet of smartphone. De app kan gedownload worden in de App Store en de Google Play store of middels het scannen van onderstaande QR-code.

# In Vitro Models For Cell-Based Cartilage Regeneration

In vitro modellen voor kraakbeenregeneratie met cellen

**Proefschrift**

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

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
donderdag 27 maart 2014 om 15:30 uur

door

**Marie Louise de Vries – van Melle**
geboren te Deventer

**Promotiecommissie:**

**Promotoren:**

Prof. dr. G.J.V.M. van Osch
Prof. dr. J.A.N. Verhaar

**Overige leden:**

Prof. dr. J.M.W. Hazes
Prof. dr. D.F.E. Huylebroeck
Prof. dr. S.K. Bulstra

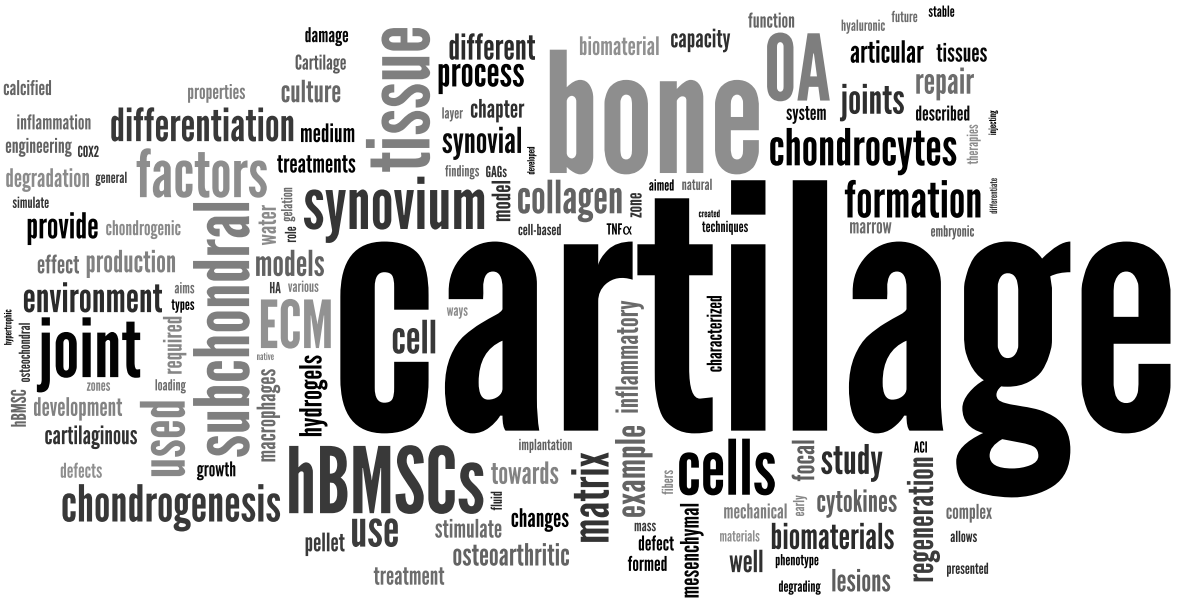
**Copromotor:**

Dr. P.M. van der Kraan

# Table of contents

# Chapter 1

## Introduction



Covering the ends of the long bones in articular joints, cartilage allows smooth, gliding movement of the joints and distributes load evenly across the joint surface. Lesions in the articular cartilage are a major cause of discomfort and disability, especially in the aging population [1]. Cartilage damage can be sub-divided roughly in 2 categories: focal cartilage defects that are for example due to trauma and degenerative cartilage damage in diseases such as osteoarthritis (OA). It is generally believed that untreated focal cartilage defects progress towards OA [2-4]. Apart from the quest for treatments for OA, orthopaedic research is also focusing on the prevention of early OA by developing treatments for focal cartilage lesions.

**The extracellular matrix of cartilage**

Cartilage is an exceptional tissue in many ways. The general statement that all cells in the body reside a maximum of 200 μm away from a blood vessel is not applicable for cartilage; it is an avascular and non-innervated tissue [5]. It consists of a complex extracellular matrix (ECM) in which the cells – chondrocytes – reside. The cartilage ECM consists mainly of collagen fibers and proteoglycans (PGs), respectively making up 60% and 30% of the dry weight. Together, collagen and PGs are responsible for the specific mechanical properties that are required for the function of cartilage. Over 90% of the collagen in the cartilage ECM consists of collagen type 2. Within the cartilaginous ECM, collagen fibers provide tensile strength [6, 7]. PGs are large molecules composed of a core protein, such as aggrecan, with sulphated glycosaminoglycan (GAG) side chains. Aggregates of PGs can form onto a hyaluronic acid (HA) backbone. GAGs are negatively charged and are responsible for the attraction of water into the cartilage matrix, thereby providing the sponge-like function of cartilage [6]. Upon compression, water is forced out of the cartilage matrix, which is attracted back into the cartilage after release of compression [8, 9]. The GAGs attract water to such extent that the volume of unloaded cartilage consists for 60 to 80% of water [10]. The ECM of cartilage is organized in three zones: the superficial zone, the medial zone and the deep zone. The collagen fibers are aligned in different orientations in the superficial zone than in the other zones and the distribution of chondrocytes within the ECM varies over the different zones [11]. This zonal organization increases difficulty of regeneration of articular cartilage.

**Other joint tissues: the synovium and subchondral bone**

Apart from cartilage, articular joints consist of more tissues among which the synovium and the subchondral bone. The synovium is a membrane of only a couple of cell layers in thickness that seals the joint capsule. The two main cell types residing in the synovium are synovial fibroblasts and macrophages. Synovial fibroblasts have a function in lubricating the joint by the production of molecules such as hyaluronic acid and lubricin that make up the synovial fluid. Apart from its lubricating function, the synovial fluid also nourishes chondrocytes,

which rely on the diffusion of nutrients into the cartilage [12]. Synovial macrophages secrete various growth factors and cytokines into the synovial fluid. Depending on the subtype of the macrophages, these can either be pro-inflammatory factors such as TNFα, IL1β and IL6 or anti-inflammatory factors like IL10 and CCL18 [13, 14]. The subchondral bone is separated from the cartilage by the calcified cartilage layer. In this transitional layer, hypertrophic chondrocytes reside in a calcified ECM as remnants of the embryonic process of endochondral bone formation, in which a cartilaginous template is calcified and remodeled to bone [15, 16]. The subchondral bone consists of a dense bone matrix that distributes the mechanical load on the cartilage over the underlying trabecular bone [17, 18]. Homeostasis of bone is characterized by continuous remodeling of the bone ECM by matrix degrading osteoclasts and matrix depositing osteoblasts. This process causes the release of growth factors and cytokines that are embedded within the bone ECM. Trabecular bone houses the bone marrow in which haematopoietic stem cells as well as mesenchymal stem cells reside.

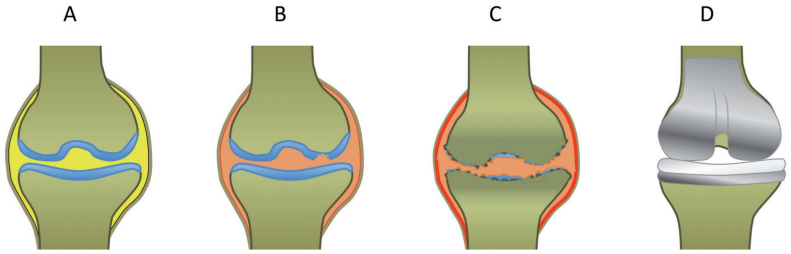



**Figure 1. Schematic representation of the knee joint.**
A. Healthy knee joint, cartilage is indicated in blue, synovial fluid in yellow and the synovial membrane as a black lining encapsulating the joint; B. Knee joint with a focal cartilage lesion, the synovial membrane indicated in red secretes inflammatory factors into the synovial fluid; C. Knee joint with end stage OA, the cartilage is severly eroded and subchondral bone changes occur such as thickening of the subchondral plate indicated by the color gradient in the bone, and the formation of osteophytes; D. Artificial knee joint after total knee replacement surgery.

### Osteoarthritis and inflammation

In contrast to what is often stated, OA cannot be simply described as cartilage wear. OA is a progressive disease that is characterized by complex interactions of cartilage, bone, the synovium and systemic factors [19, 20]. The most well known feature of OA is degradation of the articular cartilage, which can lead to full exposure of the subchondral bone [21]. Apart from this, the subchondral bone changes, which is characterized by outgrowths known as osteophytes and the formation of sclerotic bone of poor quality [22]. Often this is accompanied by thickening of the synovium as well as synovial inflammation [13, 23]. For a long time it was thought that the degradation and loss of articular cartilage were a direct cause of the changes of the subchondral bone in OA. According to current opinions, changes

in both cartilage and subchondral bone are accompanying each other [24-27]. Pre-existing cartilage or joint damage can lead to changes in joint surface loading [28]. This in turn causes the production of inflammatory cytokines like TNFα and ECM degrading mediators such as matrix metalloproteinases (MMPs) [14]. Together, this leads to a negative feedback loop caused by the complex crosstalk between cartilage, subchondral bone and synovium.

Many factors are known to play a role in the development and progression of OA. Obesity leads to increased joint loading and increased susceptibility for inflammation [29]. Meniscal tears or ligament ruptures are known to increase the risk of early OA due to changes in distribution of mechanical loading [30]. Every violation of joint integrity, from trauma to surgery, elicits a certain inflammatory response and as mentioned, inflammation plays a role in OA. Inflammatory cytokines such as IL1β and TNFα that are produced by activated macrophages and other immune cells elicit responses in the joint tissues that can stimulate the production of matrix degrading enzymes, such as matrix metalloproteinases (MMPs), that induce degradation of collagen and GAGs [31-34]. In turn, these matrix-degrading enzymes can stimulate the production of more inflammatory cytokines such as IL6 [14, 35, 36]. Also cyclooxygenase 2 (COX2) is upregulated in this process, causing conversion of arachidonic acid to prostaglandin endoperoxide H2 (PGE2) [37]. Inhibition of COX2 by non-steroid anti-inflammatory drugs (NSAIDS) prevents the production of PGE2, which is a widely used treatment to reduce inflammation-related symptoms. However, NSAIDS are known to negatively affect cartilage regeneration [38].

Currently there is no treatment for OA other than symptomatic therapies such as physical therapy, hyaluronic acid injections and NSAIDs that inhibit inflammatory factors like COX2 [39-42]. Ultimately, the only treatment option is a total joint replacement. Replaced joints have a limited lifetime. Revision surgeries in which parts of the artificial joint surfaces are replaced again are far less successful than the initial surgery [43]. This poses problems for younger OA patients, stressing the importance of the search for therapies that can prevent or treat OA.

**Current treatments for cartilage lesions**

Due to its avascular nature, damaged cartilage does not have an intrinsic healing or regenerative capacity [44-46]. Generally, cartilage lesions in which the subchondral bone is not damaged do not repair, where fibrocartilaginous tissue is formed in lesions in which the subchondral bone is reached [47, 48]. Current surgical treatments for focal cartilage lesions are either based on expanded chondrocytes, such as in autologous chondrocyte implantation (ACI), or on puncturing the subchondral bone, such as Pridie drilling and microfracturing. In ACI and its derivatives, chondrocytes are harvested from a non-weight bearing joint

area, expanded in culture and placed back into the cartilage lesion either underneath a membrane or seeded onto a biomaterial [49-54]. In microfracturing, after careful removal of the calcified cartilage layer that separates the cartilage from the subchondral bone, a tapered awl is used to puncture holes in the subchondral bone after which a bone marrow clot forms in the cartilage lesion [55-58]. The formation of repair tissue in this technique is addressed to mesenchymal stem cells present in the bone marrow that either differentiate themselves or attract other precursor cells [47]. Both techniques have their drawbacks such as the limited availability of chondrocytes and their tendency to dedifferentiate upon in vitro expansion for ACI, poor integration with surrounding tissue and the formation of mainly fibrocartilaginous repair tissue in both approaches [52, 59-68]. So far, it is unknown whether these techniques can delay or prevent the early development of OA.

**Mesenchymal stem cells**

Several cell sources can be considered for use in cell-based cartilage regeneration. As mentioned before, the use of chondrocytes has several drawbacks. Another option that allows the use of autologous cells is provided by human bone marrow derived mesenchymal stem cells (hBMSCs). In the 1960's these cells were identified by Friedenstein based on their capacity to form colonies in culture [69, 70]. In the late 1990's, the capacity of these cells to differentiate towards different tissues of mesenchymal origin such as cartilage, bone and adipose tissue, was first described [71-73]. Being recognized for this multilineage differentiation capacity as well as their in vitro expandability, hBMSCs are widely studied for various tissue engineering purposes.

To stimulate chondrogenic differentiation of hBMSCs in vitro, the cells need to be placed in a 3D environment and a specific differentiation medium containing TGFβ is required [72, 73]. An issue that can compromise the use of hBMSCs for cartilage tissue engineering is that hBMSCs tend to undergo terminal differentiation, which means that cartilaginous tissue formed by hBMSCs is not stable [74]. The cells become hypertrophic and the formed tissue tends to mineralize in the presence of a phosphate source [74]. This process is characterized by the production of hypertrophy-related factors such as collagen type X, MMP13 and alkaline phosphatase [74-76]. Hereby, terminal differentiation of hBMSCs resembles the embryonic process of endochondral bone formation [76]. Evidently, this phenomenon is highly undesired in cartilage tissue engineering [77]. In order to obtain stable tissue engineered cartilage using hBMSCs, this issue needs to be addressed.

**Biomaterials**

For chondrogenesis of hBMSCs, a 3D environment is required. The simplest way to provide a 3D environment is by pellet mass culture, in which a high-density cell pellet is created by

centrifugation [74, 75, 78-83]. Another option is to use a biomaterial as a scaffold. A wide variety of biomaterials have been developed, which can be categorized in different ways. There are synthetic biomaterials, for example polyurethane, polyethyleneglycol and polylactic acid, and natural biomaterials for example collagen scaffolds, fibrin, alginate, decellularized ECM and starch-based scaffolds [84-94]. Hybrid materials consist of a combination of synthetic and natural biomaterials, for example the HA-pNIPAM used in this thesis, that consists of the natural HA onto which pNIPAM sidechains are grafted [95]. Other ways of categorization are for example based on porosity or biodegradability. Biomaterials can be selected on multiple criteria; one can for example select a biomaterial that provides mechanical properties similar to native cartilage or one can prefer a material that allows deposition of large amounts of ECM that can later substitute the biomaterial. The ideal biomaterial should have both of these properties.

This thesis focuses on the use of hydrogels, which can provide interesting features such as easy cell encapsulation and their similarity to cartilage ECM in their high water content. Another advantage of the use of hydrogels is that it is possible to fill the cartilage defect perfectly, in contrast to biomaterials that have a defined shape [86, 90]. This enables better integration of newly formed cartilage into the surrounding tissue. Also less invasive surgical treatment of cartilage defects can be applied by injecting the hydrogel into the cartilage defect followed by in situ gelation.

For use in cell-based cartilage regeneration, hydrogels should meet certain criteria. For example, the material and the gelation process should not compromise cell viability, which excludes materials that gelate in the presence of toxic radicals or materials that need high temperatures for gelation. Several hydrogels are already approved for use in other clinical applications, which illustrates that translation of cell-based cartilage tissue engineering using hydrogels towards treatment of patients is a serious option [96, 97].

**Models for chondrogenesis**

A widely used model to study chondrogenesis in vitro is by pellet mass culture, in which a high cell density pellet is created by centrifugation [74, 75, 78-83]. This system mimics the mesenchymal condensation that takes place in embryonic limb formation. Since in this process chondrogenic differentiation is followed by hypertrophy and ultimately by bone formation, resulting in the formation of joints and bones, it is possible that the pellet mass culture system stimulates terminal differentiation of hBMSCs rather than stable cartilage formation [77].

Co-culture of hBMSCs with chondrocytes has been used to stimulate chondrogenesis of hBMSCs [98]. This can be done by creation of pellets consisting of both cell types or by using

transwell systems, in which different cell types can be cultured without cell-cell contact while allowing diffusion of nutrients and growth factors throughout the culture system. Conditioned medium from chondrocytes or cartilage explants containing growth factors secreted by these cells can also be used to stimulate hBMSC chondrogenesis. All these approaches exclude the effects of the subchondral bone and synovium and are for that reason a simplification of the complex biological environment within in the joint.

Cartilage defect models in dogs, goats or horses are used to study cartilage repair in vivo [99]. Cartilage damage can be induced surgically by creating grooves or punctures in the cartilage by scratching with a needle or using a biopsy punch. To simulate general cartilage degradation rather than focal cartilage lesions, the destruction can be induced surgically or chemically in mice or rats by anterior cruciate ligament transection or injecting collagenase, papain or monoiodoacetate [100-104]. Strenuous running of rats is also used to induce cartilage degradation [103]. Mouse strains that spontaneously develop OA can be used in studies on prevention of OA, or on the effect of for example diet on the development of OA [105, 106]. To validate in vitro findings, tissue engineered cartilaginous constructs can be implanted in various defect models to study orthotopic cartilage formation and to observe how these cartilaginous constructs are incorporated in the native cartilage. To study chondrogenic differentiation separate from joint influences of the host, ectopic implantation is required. Often, subcutaneous implantation is used for this purpose.

Since translation towards future clinical application needs extensive proof of functionality and safety, performing animal studies is inevitable. The development of representative in vitro models can help to drastically reduce the amount of animals required. Such in vitro models would allow careful selection of successful techniques and conditions, ensuring that only the most promising options are studied in animal models.

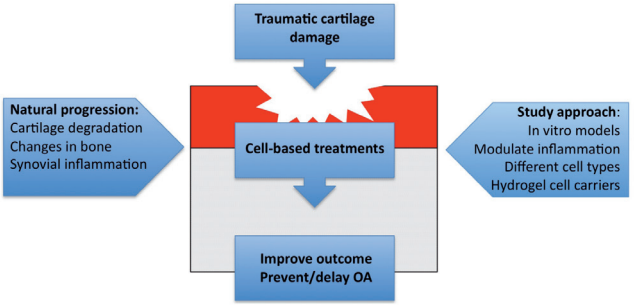



**Figure 2. Schematic representation of the aim and the experimental approach described in this thesis.**

## Aim and outline of this thesis

The research described in this thesis aims to ultimately improve cartilage regeneration using hBMSCs. To achieve this, we developed models to simulate a joint-like environment in vitro. These models were used to study the effect of the different tissues present in the synovial joint on hBMSC chondrogenesis and to study several hydrogels for their capacity to support hBMSC chondrogenesis.

**Chapter 2** aims to provide a review of the history of current cartilage repair strategies as well as a vision on prospects for future therapies for cartilage repair and regeneration in the field of regenerative medicine.

The synovium plays an important role in joint physiology and pathology. Conditioned medium prepared from synovium from osteoarthritic joints was used as a model system to study the effect of osteoarthritic factors secreted by synovium on chondrogenesis of hBMSCs. In **chapter 3**, we aimed to modulate the negative effect of osteoarthritic factors by inhibition of two pathways in hBMSCs that are known to be involved in the signalling of inflammatory cytokines: TAK1 and JAK.

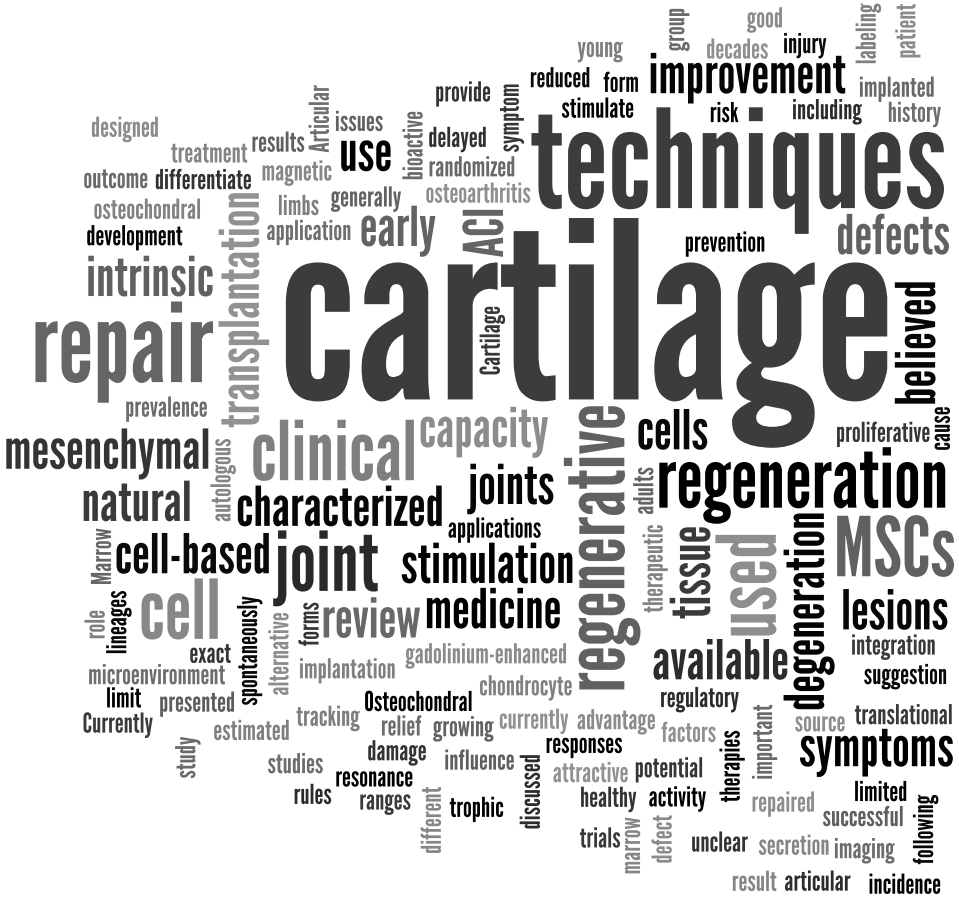
In **chapter 4**, it is hypothesized that the negative effect of osteoarthritic synovial factors on hBMSC chondrogenesis can be attributed to the phenotype of macrophages in the synovium. We aim to study this by using conditioned medium prepared from synovium from osteoarthritic joints as well as medium conditioned by peripheral blood derived monocytes that were stimulated towards an inflammatory phenotype or towards a tissue repair phenotype. This can provide clues on the development of treatments for osteoarthritic joints that target the synovium to provide an optimal environment allowing cartilage regeneration by hBMSCs.

In **chapter 5**, we aimed to develop and validate a representative and reproducible model to simulate focal cartilage defects in a joint-like environment in vitro. **Chapter 6 and 7** focus on the use of the osteochondral model presented in **chapter 5. Chapter 6** focuses on the mechanisms involved in chondrogenic differentiation of hBMSCs in an osteochondral environment. The aim was to identify the separate roles of cartilage and bone in this process. Using the same osteochondral model, **chapter 7** aimed to provide a comparison of different biomaterials to support chondrogenesis of hBMSCs. The conventional biomaterials alginate and fibrin are compared with a sophisticated hydrogel based on HA with thermoresponsive properties.

**Chapter 8** aims to place the findings presented in this thesis in general context and to address the future consequences of the findings presented in this thesis.

# Chapter 2

## Articular cartilage repair and the evolving role of regenerative medicine




P.K. Bos, M.L. van Melle, G.J.V.M. van Osch

Open Access Surgery 2010. 3

## Abstract

Among the growing applications of regenerative medicine, clinical articular cartilage repair, now used for two decades, forms a successful example of translational medicine. Cartilage is characterized by a limited intrinsic repair capacity following injury. Articular cartilage defects cause symptoms, are not spontaneously repaired and are generally thought to result in early osteoarthritis (OA). Marrow stimulation techniques, osteochondral transplantation, cell-based therapies such as autologous chondrocyte implantation (ACI) and use of mesenchymal stem cells (MSCs) are used for tissue regeneration, symptom relief and prevention of further joint degeneration. The exact incidence of cartilage defects and the natural outcome of joints with these lesions are unclear. Currently available cartilage repair techniques are designed for defect treatment in otherwise healthy joints and limbs, mostly in young adults. Estimated from the presented natural history studies in this review, the prevalence of cartilage lesions in this patient group ranges from 5 to 11 %. The background and results from currently available RCTs of the three mostly used cartilage repair techniques are outlined in this review. Osteochondral transplantation, marrow stimulation and ACI show improvement of symptoms with an advantage for cell-based techniques, but only a suggestion that risk for joint degeneration can be reduced. MSCs, characterized by their good proliferative capacity and the potential to differentiate into different mesenchymal lineages, form an attractive alternative cell source for cartilage regeneration. Moreover, MSCs provide a regenerative microenvironment by the secretion of bioactive factors. This trophic activity is thought to limit damage and stimulate intrinsic regenerative responses. Finally, important clinical issues are discussed, including techniques to study the role of implanted cells in tissue regeneration using cell labeling and cell tracking, improvement of cartilage integration, use of delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) for early judgment of joint degeneration/regeneration and the influence of regulatory rules for therapeutic application development.

## Introduction to articular cartilage repair

Cartilage is characterized by a limited intrinsic repair capacity following injury. Articular cartilage lesions are frequently associated with symptoms such as pain, effusion, locking phenomena and disturbed function. Moreover, these lesions are generally thought to progress to early osteoarthritis (OA)[2-4]. Regenerative medicine, including in situ induction of cartilage tissue, use of tissue engineered cartilage constructs or cell-based therapies (autologous chondrocytes or mesenchymal stem cells) are used for tissue regeneration, symptom relief and prevention of further degeneration.

In his editorial, accompanying Brittberg's pioneering paper in the New England Journal of Medicine on autologous chondrocyte implantation (ACI) in 1994 [49], Mankin stated: "It should be clear that cartilage does not yield its secrets easily and that inducing cartilage to heal is not simple. The tissue is difficult to work with, injuries to joint surface - whether traumatic or degenerative - are unforgiving, and the progression to osteoarthritis is sometimes so slow that we delude ourselves into thinking we are doing better than we are. It is important, however, to keep trying." [107]. Clinical and basic scientists have made much progress since 1994 in understanding cartilage disease and degeneration and have made progress in biological repair of it. However, Mankin's remarks illustrate that the translation from basic knowledge and experimental treatments towards *successful* and *durable* repair of cartilage defects and osteoarthritic joints is difficult. Goals of treatment of cartilage pathology are symptom relief, improvement of joint congruence by restoring the joint surface with the best possible tissue, *and* prevention further joint degeneration.

Articular cartilage is a highly organized avascular tissue composed of chondrocytes embedded within an extracellular matrix of collagens, proteoglycans and noncollagenous proteins. It makes painless, low friction movement of synovial joints possible. Hyaline cartilage covers the subchondral bone and forms the articulating surface of synovial joints. It functions as a mechanical shock absorber and distributes the applied load over the subchondral bone. The regeneration capacity of articular cartilage following injury is considered to be limited. Partial-thickness articular cartilage defects, limited to the cartilage itself, are not repaired and full thickness defects are repaired with fibrocartilage [47], which has inferior biological and biomechanical properties compared to hyaline cartilage [48].

In this review we will start with a description of the epidemiology and natural history of cartilage lesions and provide an overview of current regenerative cartilage repair techniques, outcome from randomized clinical trials and give insight in new developments with use of mesenchymal stem cells (MSCs) and tissue derived progenitor cells.

## Epidemiology and natural history of cartilage injury

The exact incidence of symptomatic chondral lesions and the natural outcome of joints with (osteo)chondral lesions is not well defined. A prospective study of 1,000 consecutive knee arthroscopies revealed ICRS grade III and IV chondral lesions [108], with an area of at least 1cm$^2$ in patients younger than 40, 45 and 50 years of 5.3%, 6.1% and 7.1% respectively [109]. The mean (osteo)chondral defect area was 2.1cm$^2$. Another prospective study of 993 knee artroscopies [110] in patients with a median age of 35 years, shows 11% full-thickness articular cartilage defect (ICRS grade III and IV), 6% had a size of more than 2cm$^2$. Prospective arthroscopic evaluation of traumatic knee haemarthrosis patients showed 8-20% (osteo) chondral lesions, frequently associated with injury to the anterior cruciate ligament [111-113]. Curl et al. retrospectively reviewed 31,516 knee arthroscopies of patients in all age groups and reported chondral lesions in 19,827 (63%) of patients, with a mean of 2.7 lesions per knee. The incidence of grade III lesions was 41% and grade IV lesions 19%. In the younger population (age < 40 years) however, the incidence of unipolar grade IV lesions of the femoral condyle was only 5% [114]. Thus, the prevalence of isolated (osteo)chondral defects ranges from 5 to 11% in the young patients up to 63% in the overall patients.

It is unclear which chondral lesions give symptoms, many of the detected lesions are asymptomatic and therefore the exact incidence in the general population is unknown. It is likely that symptoms and joint degeneration are dependant on lesion size, location and patient characteristics.

Shelbourne et al. [115] reported a series of 125 Outerbridge grade III and IV[116] cartilage defects (mean size 1.7cm$^2$, 60 medial, 65 lateral compartment, intact menisci), discovered during 2,770 anterior cruciate ligament (ACL) reconstructive procedures. These authors showed, at a mean follow up of 8.7 years, very little difference in clinical outcome following ACL repair between patients with a chondral defect and those without a defect. There was no difference between groups with regard to radiological degenerative changes. However, it may take up to more than 20 years before clinical and radiological degenerative changes come forward. Linden showed in a retrospective radiological study on osteochondritis dissecans of the knee in adult patients, with a 32.5 +/-7.5 year follow up, mild radiological deterioration in 14/44 joints and severe changes in 29/44 joints [117].

In a recent natural history study, Widuchowski et al [118]. retrospectively analysed 4,121 consecutive knee arthroscopies. In the patient group below 35 years there were 37 single isolated Outerbridge grade 3 and 4 lesions within the weight-bearing areas of the femoraltibial compartments and the patella. At a mean follow up of 15.3 years the authors

found no difference in OA severity between the injured and uninjured knees, indicating that severe isolated chondral defects may have limited influence on the development of knee OA. Gelber et al. [119] followed 1,321 former medical students, with joint injuries with median follow up of 36 years and found for knee injuries 13.9% progressed to fully developed knee OA by the age of 65 compared to 6% in controls without joint injury. In this frequently cited article however, from the 111 isolated knee injuries just 8 sustained an isolated cartilage injury. More severe injuries, including tibial plateau fractures, knee dislocation and open fractures are considered to have a higher risk for OA development.

In conclusion, most authors assume that cartilage lesions, frequently associated with other articular injuries progress to joint degeneration. However, it is difficult to exactly predict which lesions will benefit from cartilage repair. Whether a cartilage lesion causes progression towards OA may depend on lesion size, location, pre-injury joint degeneration, limb alignment and other patient characteristics. The available articular cartilage repair techniques are not designed to treat degenerative joint disease, i.e. OA. However, several authors have used the described techniques or combinations of it to treat degenerative joint disease [120, 121]. They used, for example, microfracturing [120] and/or meniscus transplantation or joint realignment procedures for knee OA cases [121]. To date there are no randomized clinical studies reporting the outcome of treatment of these patient groups.

Osteoarthritis(OA) is affecting nearly 27 million or 12.1% of the adult population in the United States in 2008 [122]. OA is the fifth leading cause of disability in older Americans after cardiovascular, cerebrovascular and pulmonary diseases [123]. It is estimated that the number of adults in the United States with arthritis disease will reach up to 67 million people, or 25% of the population, by 2030 [124]. Successful repair techniques for isolated cartilage defects in otherwise healthy joints may, in the future, be translated to treatment of more extensive joint degeneration, such as OA. Biological repair and possible disease modification, with the use of regenerative medicine techniques, may thereby decrease the expected medical and economic burden.

## Current clinical methods of repair

The primary goal in articular cartilage repair procedures should be defect filling and restoration of the articular surface with the best possible repair tissue. Long lasting biomechanical properties resembling that of hyaline cartilage and a full integration with the surrounding articular cartilage should result in pain free movement and prevent early joint degeneration. Surgical treatment options for cartilage repair include symptomatic

treatments like debridement and lavage, osteochondral autograft transplantation (OAT), marrow stimulation techniques (Pridie drilling or microfracture), autologous chondrocyte implantation (ACI), and tissue engineering techniques using cells and biomaterials to replace damaged or lost cartilage and bone.

Focusing on articular cartilage repair we can distinguish 3 main techniques for biological repair of cartilage defects: osteochondral transplantation (OAT or mosaicplasty), subchondral marrow stimulation (Pridie drilling or microfracture) and ACI.
In OAT or mosaicplasty, introduced in the 1990's, autologous osteochondral biopsy plugs are harvested from relatively non-weight bearing areas of the joint and subsequently implanted in a mosaic-like pattern in debrided cartilage defects [125, 126]. OAT or mosaicplasty, popularised by Hangody [125] and Bobic [126], is recommended for defects limited to between 1 and 4 $cm^2$ [127]. For these small to medium sized defects good results have been reported in terms of function scores and histology for follow-up up to seven years post-treatment [125, 127]. Drawbacks of this technique are limited availability of donor tissue as well as donor site morbidity [125, 127, 128]. In this review we will focus on the two techniques that can be characterized as regenerative medicine: the microfracture technique and ACI with or without matrix augmentation.

**Microfracture**
Already in the 1950's, it was hypothesized that accessing the bone marrow could be helpful in the repair of cartilage defects. The general hypothesis behind all marrow stimulation techniques is that MSCs present in the bone marrow are responsible for the formation of fibrocartilaginous tissue that fills the initial defect [47, 129]. Marrow stimulation in the microfracture technique is achieved by cortical penetration with an awl, in Pridie drilling a drill or Kirchner-wire is used. The microfracture technique was introduced in the 1980's and is considered as an evolved form of Pridie drilling, generally accepted to result in clinically more favorable outcomes, which is addressed to the absence of thermal damage in the microfracture technique, although the two techniques have never been compared directly [47, 48, 107, 109, 130]. After debridement of the defect, conical holes of 0.5 to 1mm in diameter and 4mm deep are punched all over the defect at a distance of 3 to 4mm apart with specialized tapered awls. Consequently, a blood clot fills the defect followed by ingrowth of bone marrow cells [55-58, 131].

Using the equine medial femorotibial joint as a model for the medial femoral condyle of the human knee, many aspects of the microfracture technique have been studied [99, 132-136]. A finding that was subsequently translated into clinical practice was the importance of the removal of the calcified cartilage layer prior to creation of the holes [132, 135]. Insufficient

removal can lead to dislocation of the clot, where damaging the subchondral plate can result in overgrowth of subchondral bone, causing decrease of repair tissue volume and compromised mechanical properties [132, 135, 137, 138].

For optimal results patients should preferably be younger than 45 years of age, have a body mass index below 30 and experience of symptoms (activity related pain, swelling, locking, catching) for less than one year. The defect should be isolated from other lesions and should be smaller than 4cm$^2$. An intact rim of cartilage should surround the defect, to ensure that the bone marrow clot stays in place [57]. Contraindications are e.g. degenerative joint changes, axial malalignment >5$^o$ for femoral condyle defects, tumors, infections, meniscus pathology that requires treatment and high-grade ligament instabilities [57, 132]. Microfracture is often coupled to a specific rehabilitation program [139]. Initially, weight bearing is avoided, followed by controlled partial weight bearing, to provide nutrients and to provide mechanical stimuli [57, 58, 139-142]. Return to full pre-morbid activities is generally achieved at 15 to 18 months post surgery [139]. Improvement in terms of pain and function is widely reported up to 24 months post surgery, however the long-term durability is debated; return of complaints is generally expected. Upon histological analysis of biopsies taken two years post-treatment 69% of treated lesions was found to consist of mainly fibrocartilaginous tissue, where 11% predominantly contained hyaline cartilage [59]. The fibrocartilaginous repair tissue contains more collagen type 1 as well as less proteoglycan compared to native articular cartilage, indicating that the biochemical and biomechanical properties are not equal to those of the native articular cartilage [99, 133-135, 143].

Many augmentation strategies are currently investigated in order to improve the long-term outcome of the microfracture technique e.g. autologous matrix induced chondrogenesis (AMIC) involving collagen type I/III scaffolds (Geistlich Pharma AG, Switzerland) or chitosan-glycerol phosphate based BST-CarGel® scaffold (Biosyntech Inc, Canada). Good results were reported for the AMIC collagen type I/III scaffold technique[144]. Improvement of repair tissue quality and ICRS II scores compared to conventional microfracture have been found for chitosan-glycerol phosphate based BST-CarGel, which is currently evaluated in a multi-center clinical trial [145-148]. Other augmentation strategies consist of hyaluronic acid injections or biomaterials with incorporated growth factors [56, 149-152]. Most of these strategies are currently in pre-clinical stages [151].

**Autologous chondrocyte implantation**

The ACI technique for clinical treatment of human cartilage defects was first reported in 1994 [49]. In summary, a cartilage biopsy is taken arthroscopically from a non-weight bearing area of the joint. Cartilage biopsies are enzymatically digested to isolate chondrocytes. The

chondrocytes are expanded in monolayer culture. In a second open procedure that can take place six weeks up to 18 months after the biopsy, a periosteal flap, harvested from the tibia is placed over the cartilage defect, fixed with sutures and/or sealed with fibrin glue after which a solution of expanded chondrocytes is injected underneath the flap [49, 50]. This first generation of ACI is also known as ACI-P, based on the use of the periosteal flap. Leakage of cells and uneven distribution of chondrocytes was reported, as well as hypertrophy of the periosteal flap [50, 153]. In the second generation ACI procedure, a collagen membrane is used to replace the periosteal flap, which is also known as ACI-C [50]. Several tissue engineering based approaches are classified as the third generation of ACI, which means that cells are cultured on a biodegradable membrane or scaffold prior to placement into the defect. Examples are matrix-induced ACI (MACI): chondrocytes are pre-cultured on a porcine collagen type I/III membrane *or* characterized chondrocyte implantation (CCI) marketed as ChondroCelect® (TiGenix NV, Belgium), in which autologous chondrocytes are characterized based on specific marker proteins and expansion is standardized [51, 52]. With these emerging tissue engineering strategies, the possibility to perform ACI procedures arthroscopically gains more interest [51, 53, 154]. The presence of cartilage damage at the opposing surface of the joint is a contraindication for ACI [50]. Malalignment and ligamentous instability should be corrected prior to treatment [50, 155]. For optimal results, prevalence of symptoms should be less than two years and the cartilage defect should be an isolated focal lesion [50]. As for microfracture, an intact rim of cartilage should surround the defect to allow suturing of the periosteal flap, membrane or scaffold. Damaging the subchondral bone is to be avoided to prevent the formation of fibrocartilaginous tissue due to bone marrow invasion [50]. In contrast to the microfracture technique, for ACI no relationship between defect size and clinical outcome was found, which implies that ACI can be applied for cartilage defects of all sizes [156]. ACI treatment of cartilage defects is, as for microfracture followed by a rehabilitation program involving restricted weight bearing and use of continuous passive motion. Return to daily activities and light sports is generally achieved at four to six months post-treatment [50, 157]. Good to excellent outcomes have been reported for long term (up to seven years) follow-up of femoral condyle lesions treated with ACI, with indications of clinical outcome improvement over the years [157]. Histological biopsy studies[158] have shown that ACI repair can result in repair tissue of varying morphology, ranging from predominantly hyaline (22%) through mixed (48%) to predominantly fibrocartilage. The success rate of ACI reported in long-term durability studies varies from 69% at ten year follow up[159] to 84% at 7.4 year follow up[157]. Failures are reported within the first years following treatment; good results at short-term follow up are generally sustained at long-term follow up[157, 159].

ACI was also found to result in improved clinical outcome when applied in patients with large cartilage defects that failed to repair in a previous treatment [160]. However, this finding is

debated; poor clinical outcome has been reported for patients undergoing ACI or MACI after failed mosaicplasty or ACI [61]. A limitation in ACI and its derivatives is the fact that chondrocytes tend to dedifferentiate during monolayer expansion, which decreases their extracellular cartilage matrix formation potential [68]. For ACI a widely used animal model lacks due to reported variations in expansion and other characteristics of autologous chondrocytes over different species [161].

Among the various types of cartilage, of interest are two distinct different cartilage types in the growing skeleton: (1) growth plate cartilage and (2) hyaline or articular cartilage. In growth plate cartilage chondrocytes proliferate, go into hypertrophy and terminally differentiate. This process of maturation leads to cell death, followed by calcification of the tissue and chondrocytes are replaced by osteoblasts. In the growing joint hyaline cartilage chondrocytes proliferate and form the extracellular matrix, resulting in adult hyaline cartilage, with chondrocytes lying in low densities in a tight extracellular matrix. For ACI regenerative cartilage medicine, one of the challenges is to culture expand cells to sufficient numbers for tissue regeneration while preventing hypertrophy, terminal differentiation and calcification of the repair tissue[162].

## Randomized clinical trials

Many case studies and clinical trials on the above-described techniques have been published the last two decades. In addition several (systematic) reviews on ACI or cartilage repair techniques in general have been published during the last years[3, 56, 79, 132, 143, 155, 163-169] expressing the growing interest of the community in the possibilities of these techniques. For this review we decided to highlight the Randomized Clinical Trials (RCTs). The microfracture technique, popularised by Steadman, is often used as a first treatment for cartilage defects and has become the control treatment in several prospective studies evaluating other, more extensive surgical interventions such as ACI. We aim to provide an overview of RCTs involving ACI or its derivatives, microfracture or both. The study and patient characteristics of all available RCTs are given in table 1. The outcome parameters and RCT quality scores are summarized in table 2. The quality of presented RCTs comparing the different cartilage repair techniques was assessed according to Jadad et al.[170].

Horas et al. conducted a prospective RCT in which femoral condyle lesions of 40 patients were treated either with ACI-P or OAT [62]. For both treatments improvement of Lysholm Knee Scoring Scale (LKSS) scores compared to pre-operative levels was reported, however the increase was significantly slower for ACI-P treated patients compared to OAT treated patients at follow up after 6, 12 and 24 months [62].

**Table 1. Overview of RCTs on cartilage defect repair techniques.**

| Author | Treatments {n) | Patient age (years) | Defect size {cm²) | longest follow up (months) | Outcome measures |
|---|---|---|---|---|---|
| Horas et al [62] | ACI-P (20)<br>OAT (20) | 31.4 (18-42)<br>35.4 (21 -44) | 3.9 (3.2-5.6) | 24 | LKSS<br>Tegner<br>SEM<br>Histology |
| Bentley et al [63] | ACI-P &C (58)<br>MP (42) | 30.9 (16-49)<br>31.6 (20-48) | 4.66 (1-12)<br>4.66 (1-12) | 12 | Cincinnati<br>Stanmore<br>ICRS<br>Histology |
| Bartlett et al [61] | ACI-C (44)<br>MACI (47) | 33.7 (15-49)<br>33.4 (17-47) | 6 (1.5-16)<br>6.1 (1-22) | 12 | Cincinnati<br>Stanmore<br>VAS<br>Histology |
| Dozin et al [64] | ACI-P (22)<br>OAT (22) | 29.6 (±7.3)<br>27.9 (±8.1) | 1.97 (±0.43)<br>1.9 (±0.45) | 36 | LKSS<br>IKDC |
| Gudas et al [65] | OAT (28)<br>MF (29) | 24.6 (±6.54)<br>24.3 (±6.8) | 2.8 (±0.65)<br>2.77 (±0.68) | 36 | ICRS<br>HSS<br>MRI<br>Histology |
| Gooding et al [66] | ACI-P (33)<br>ACI-C (35) | 30.5 (15-52)<br>30.5 (16-49) | 4.54 (1-12) | 24 | Cincinnati<br>ICRS<br>Histology |
| Knutsen et al [59,60] | ACI-P (40)<br>MF (40) | 33.3<br>31.1 | 5.1<br>4.5 | 60 | LKSS<br>Kellgren & Lawrence<br>ICRS<br>Tegner<br>SF-36<br>Histology |
| Saris et al [52,67] | CCI (57)<br>MF (61) | 33.9 (±8.5)<br>33.9 (±8.6) | 2.5 (1-5) | 36 | KOOS<br>MRI |

Characteristics on treatment groups, amounts of patients per treatment, defect sizes, duration of post-treatment follow-up as well as the applied outcome measures are presented.
ACI-P: first generation ACI (with periosteal coverage); ACI-C: second generation ACI (with collagen coverage); MACI: matrix-induced ACI; OAT: osteochondral autograft transplantation; MP: mosaicplasty; MF: microfracture; CCI: characterized chondrocyte implantation. LKSS: Lysholm Knee Scoring Scale; SEM: scanning electron microscopy; ICRS: International Cartilage Repair Society score; VAS: Visual Analogue Scale for pain; IKDC: International Knee Documentation Committee scale; HSS: Hospital for Special Surgery knee score questionnaire; KOOS: Knee injury and Osteoarthritis Outcome Score.

In 2003 Bentley et al. reported their findings of a prospective RCT in which 100 patients with symptomatic lesions of the articular cartilage of the knee were treated either with ACI-P/C or mosaicplasty [63]. One year post-treatment 82% of the ACI-P/C treated group showed good or excellent results in ICRS grade arthroscopic results against 34% of the mosaicplasty treated group. Upon functional assessment (Cincinnati and Stanmore scores) 88% of the ACI-P/C treated group had good or excellent results, where in the mosaicplasty 69% was reported. There needs to be noted that in this study patients with cartilage lesions over 4cm² in size were treated with mosaicplasty. Also, the rehabilitation program was equal for both treatment

**Table 2. Quality assessment and summary of the main conclusion of the RCTs.**

| Author | Treatments (n) | RCT quality score | Results |
|---|---|---|---|
| Horas et al [62] | ACI-P (20)<br>OAT (20) | 3 | Meyer & Tegner: equal results<br>LKSS improvement both treatments, slower for ACI-P |
| Bentley et al [63] | ACI-P &C (58)<br>MP (42) | 2 | ICRS score good/excellent: ACI-P 82%, MP 34%<br>Cincinnatti/Stanmore good/excellent: ACI-P 88%, MP 69% Clinical outcome: ACI-P > MP |
| Bartlett et al [61] | ACI-C (44)<br>MACI (47) | 1 | Cincinnati/Stanmore, VAS, histology: equal results |
| Dozin et al [64] | ACI-P (22)<br>OAT (22) | 3 | 31.8% no further treatment needed following debridement<br>Complete clinical recovery: ACI-P 68%, OAT 88%<br>Clinical outcome: equal |
| Gudas et al [65] | OAT (28)<br>MF (29) | 2 | ICRS & HSS good/excellent: OAT 96%, MF 52%<br>Clinical outcome: OAT > MF |
| Gooding et al [66] | ACI-P (33)<br>ACI-C (35) | 2 | Cincinnati, ICRS, histology: equal results<br>Graft hypertrophy: ACI-P 36.4%, ACI-C 0% |
| Knutsen et al [59, 60] | ACI-P (40)<br>MF (40) | 3 | LKSS, K&L, ICRS, Tegner, SF-36, histology: equal results<br>77% satisfactory results & 23% failures in both groups<br>No relation between histology & clinical outcome |
| Saris et al [52, 67] | CCI (57)<br>MF (61) | 3 | KOOS: both significant improvement, CCI > MF |

A summary of the results and conclusions are displayed per RCT. The quality of presented RCTs was assessed using the Jadad score [170] (0-5, a high score is an indication of high quality of the study). The comparison of single surgery approaches (MF, OAT) with double surgery approaches (all ACI) makes double blinding the procedure difficult for these surgical interventions, without double blinding the maximum Jadad score for RCTs is 3.

groups, where different programs are recommended for ACI and mosaicplasty [63, 127].

Bartlett et al. reported in 2004 their results of a prospective RCT studying ACI-C versus MACI [61]. 91 patients were randomized to one of the treatments. No significant differences were observed at one year post-treatment in terms of ICRS scores, histological examination as well as functional Cincinnati knee scores. Bartlett et al. concluded that although no significant differences were found between the two treatments, MACI is technically more attractive due to factors like quicker surgery and the possibility to not use sutures; however more long-term studies are required [61].

ACI-P and mosaicplasty were compared in a multicenter RCT by Dozin et al. in 2005 [64]. Forty-seven patients were treated with arthroscopic debridement and subsequently randomized to one of the treatments. Debridement alone resulted in improvement to such extent that 14 patients (31.8%) were clinically asymptomatic and were not subjected to further treatment. Eventually, 52.3% of the originally included patients were evaluated. For 88% of the patients subjected to mosaicplasty and 68% of the ACI-P treated patients complete clinical recovery was reported. The two methods were found to be clinically equivalent.

OAT and microfracture were compared in a prospective RCT in competitive or well-trained athletes by Gudas et al. in 2006 [65]. 57 patients with full thickness cartilage lesions or single osteochondritis dissecans (OCD) were randomized to either OAT or microfracture. The recommended continuous passive motion rehabilitation program was not applied. At 6, 12, 24 and 36 months post-treatment patients were evaluated. 93% of the OAT and 52% of the microfracture treated group returned to pre-symptomatic sports level at four to eight months post-treatment. In terms of Hospital for Special Surgery (HSS) and ICRS scores, MRI, histology and clinical assessment significantly better results were reported for OAT compared to microfracture treated athletes [65].

In 2006 Gooding et al. compared ACI-P with ACI-C in a prospective RCT including 68 patients [66]. In 36.4% of the ACI-P versus none in the ACI-C treated patients graft hypertrophy occurred and shaving was required at one year post-treatment. No significant differences between the two treatments were found in terms Cincinnati, ICRS as well as histology two years post-treatment [66].

Knutsen et al. reported their findings at five years in 2007 of a multicenter RCT comparing ACI-P with microfracture in 80 patients with single cartilage defects of the femoral condyle of the knee [59, 60]. After 5 years, 23% failures were reported in both groups, defined as reoperation required due to symptoms as a result of lack of healing after the initial treatment. Shaving or trimming was necessary in 25% of the ACI-P and 10% of the microfracture treated patients [59]. This was not considered as failure. No significant differences were found between both treatments in terms of clinical and radiographical outcome. Interestingly, no relation between histological findings and clinical outcome was observed [60].

The results of a multicenter RCT comparing CCI (ChondroCelect) to microfracture 36 months post-treatment were reported by Saris et al. in 2008 and 2009 [52, 67]. 118 patients with symptomatic lesions of the femoral condyle of the knee were randomized to one of the treatments. Based on findings in characterization of the autologous chondrocytes, six patients were not subjected to CCI treatment. It is not known whether these chondrocytes classified as not usable actually can result in compromised repair tissue. It was found that the longer the duration of the symptoms, the higher the improvement of KOOS scores of CCI treated patients compared to microfracture treated patients. 36 months post-treatment, the clinical outcome in terms of KOOS scores showed significantly better results for CCI compared to microfracture treated patients [67].

Evaluating the currently available RCTs, with in mind the two main objectives of articular cartilage repair, symptom relief and prevention of joint degeneration, we can conclude

that all therapies show initial improvement measured with functional outcome scores. The comparison of single surgery approaches (microfracture, OAT) with double surgery approaches (all ACI) makes double blinding the procedure difficult for these RCTs. Therefore non of the RCTs reached the maximum Jada RCT quality score of five points. Four out of eight RCTs scored the maximum of 3 points for unblended RCTs and could be regarded as good quality RCTs. Considering prevention of early OA development, histological examinations have shown variable results, with a general suggestion of better tissue quality following ACI compared to microfracture and OAT. Arguments to assume that microfracture and OAT may result in worse long-term outcome and earlier OA development are fibrocartilage formation, donor-site morbidity and the persistence of gaps between osteochondral plugs and surrounding cartilage. However, in general ACI is a two-stage procedure (harvesting of cartilage and a second open/arthroscopic implantation procedure) that may also lead to complications. For example an increased inflammatory response and negative influences on joint propriocepsis following two procedures performed shortly after each other may also increase the risk for early OA development. The above-mentioned natural outcome studies have shown that it takes a long time for untreated cartilage defects to lead to detectable OA. Therefore, it may even take longer to judge whether extensive cell-based interventions protect joints from degenerating. Moreover, marrow stimulation shows good improvement in short to mid-term follow up. This may already be sufficient to protect joints from degeneration. Use of MRI techniques, such as dGEMRIC or sensitive biomarkers for OA development are examples by which we can improve early judgment of cartilage repair tissue.

## Mesenchymal stem cells for cartilage repair

All the above-mentioned techniques make use of chondrocytes for articular cartilage repair with the exception of microfracture, where MSCs are accredited for the formation of repair tissue. For the treatment of cartilage defects cells are needed in substantial amounts to fill the gap and to produce extracellular matrix of sufficient strength in a relatively short time compared to cartilage development in a growing joint. This requires in vitro cell expansion of harvested and enzymatically liberated chondrocytes with the risk of dedifferentiation and loss of re-differentiation capacity after expansion.

MSCs can be an attractive alternative cell source. MSCs have a good proliferative capacity in culture and have the potential to differentiate into different mesenchymal lineages, such as bone, cartilage, tendon, muscle and fat [71]. Chondrogenic differentiation is achieved when the cells, after expansion, are allowed to form three-dimensional aggregates in a chemically defined medium containing Transforming Growth Factor-β (TGF-β) and dexamethasone [72].

These multipotent progenitor cells can be derived from several tissues, including bone marrow [171], adipose tissue [172], joint-related tissues like synovial membrane [173] and infrapatellar fat [174]. Furthermore, it has been shown that articular cartilage contains progenitor cells with the capacity to regenerate cartilage in vitro [175]. In another study undifferentiated progenitor cells were isolated from 7 day old calf articular cartilage [176].

Next to their capacity to form repair tissue, MSCs have shown to secrete a large spectrum of bioactive molecules in culture, including TGF-β, interleukin (IL)-10, IL-6, lymphocyte inhibitor factor (LIF), cyclooxygenase-1(COX-1) and COX-2 (for review see [177]). These molecules are immunosuppressive; therefore the secreted bioactive molecules are thought to provide a regenerative microenvironment for injured or ischemic adult tissues. This regenerative microenvironment referred to as trophic activity, provided by the presence of MSCs, limits the damage sustained by injury or ischemia and stimulates intrinsic regenerative responses [178].
For similar reasons, MSCs have been shown to be a promising cell population for immunomodulatory therapy as they can modulate T-lymphocyte reaction both in vitro and in vivo [179]. LeBlanc et al. [180] showed that ex-vivo expanded allogeneic MSCs were immunosuppressive, reversed established graft versus host disease (GVHD) and prolonged graft survival in patients after bone marrow transplantation. MSC infusions have also been tested as a possible method to induce immunologic tolerance or to reduce the need for pharmacologic immunosuppression for organ transplantation [179].

Furthermore, from cardiovascular research we have learned that the microenvironment provided by injected MSCs, and not the initially thought transdifferentiation of MSCs into contractile cardiomyocytes, reduces the development of heart failure following myocardial infarction [181]. From this point of view, one can hypothesize that a part of the observed effects of current cartilage repair techniques depend on this trophic activity. Undifferentiated or dedifferentiated cells, actors in the observed repair with microfracture and ACI techniques, may not only inhabit the cartilage defects and produce the necessary extracellular matrix, but also provide a regenerative microenvironment. This may partly explain why investigators have found no distinct relation between repair cartilage histology and functional outcome [60]. Future studies have to reveal whether we can further improve these techniques by optimizing trophic activity. Catabolic conditions in joints with cartilage defects and/or OA may be stopped or reversed by the continuous presence of MSC trophic activity.

Animal studies, using a combination of MSCs combined with different biomaterials and growth factors have shown promising results (for review see [182]). There are few clinical case-studies reporting the results of bone marrow derived MSCs for cartilage defect

repair. The MSCs are implanted in cartilage defects, seeded in collagen and covered with periosteum [96]. Others have injected culture expanded MSCs percutaneously into the knee in an attempt to regenerate cartilage in OA patients [183]. Currently, it is not known whether MSC treatments can give results similar to ACI or microfracture treatment [79]. A recent observational cohort study compared bone marrow derived MSCs to chondrocytes and found no differences in clinical outcome scores [184]. The authors concluded that bone marrow derived MSCs were as effective as chondrocytes for articular cartilage repair, with the advantage of 1 fewer knee intervention and minimized donor-site morbidity.

## Clinical issues and future perspectives

### Which cells are responsible for repair?

The presented cartilage repair techniques are designed to replace damaged articular cartilage, by supplying or attracting cells in sufficient amounts that produce extracellular matrix and thereby fill the gap. There is evidence to support the idea that the implanted culture expanded chondrocytes or MSCs are relevant for cell-based therapies [185, 186]. In order to further optimize cell-based therapies we need to know whether the implanted cells can be accredited for repair tissue formation by cell tracing in the joint. The fate of cells following in vivo implantation in humans and their exact role in regeneration remains unclear. It may appear that other cells are relevant for repair tissue formation, cells such as periosteal progenitor cells in ACI-P, ingrowth of subchondral marrow-cells or synovial progenitor cells. Studies undertaken to determine the fate of implanted chondrocytes for *in vivo* follow up include retroviral green fluorescence protein (GFP) marking of cells [187], PKH26 fluorescent labeling of chondrocytes [185] and the use of 'physicochemical labels' such as magnetic nanoparticles [188, 189]. An important advantage of magnetic nanoparticles like 'superparamagnetic iron oxide' (SPIO)-labeling over other labeling techniques is that it enables clinical non-invasive in vivo cell tracking using MRI, without the need for harvesting biopsies. This allows for continuous follow-up of biological repair of articular cartilage without influencing the repair tissue or jeopardizing the patient with repeated interventions.

### Integrative cartilage repair

An important prerequisite for durable repair of cartilage lesions is the integration of regenerated or transplanted cartilage with the surrounding native cartilage at the recipient site. Integrative cartilage repair is probably hindered by the lack of matrix-producing cells in wound edges caused by chondrocyte death induced by wounding of cartilage. In vitro experiments have shown a rapid onset of cell death in experimentally wounded hyaline cartilage [190, 191]. The acellularity is probably caused by a combination of chondrocyte loss

from lesion edges, cartilage avascularity, absence of necrotic tissue removal and the inability of chondrocytes to migrate through the tight extracellular matrix. Approaches to improve cell density at wound edges include use of cells in the interface region, enzymatic treatment and use of immature constructs. Silverman et al. studied the adhesion between native cartilage discs, using fibrin glue polymer alone or mixed with fresh articular chondrocytes. Their results demonstrated that adhesion of cartilage to cartilage can be improved by an increased amount of chondrocytes in the interface region [192].

The age and cell density of regenerated tissue influence the histological integration and its biomechanical strength. An increased cell density in the interface region by using immature constructs compared with mature constructs in an integration study was shown to improve biomechanical bonding strength and histological integration [193]. In other studies highly purified collagenase treatment was used, that resulted in an increased wound edge cell density. This “vitalization” of wound edges with vital, matrix-producing cells at the cartilage–cartilage interface was shown to improve histological and biomechanical integrative cartilage repair [190, 194, 195]. Enzymatic removal of proteoglycans or glycosaminoglycan chains from the cartilage lesion edges by using chondroitinase ABC, trypsin or hyaluronidase has shown to improve the initial adhesion of transplanted cells[196] or cartilage to cartilage wound edges [197, 198]

Components of the synovial fluid may have an inhibitory effect on integrative cartilage repair. Proteoglycan 4 (PRG4 or lubricin), present in the synovial fluid, normally acting as a lubricant of articular surfaces was shown to strongly reduce cartilage integration [199].

Integrative cartilage repair appears to be less of a problem for in situ cartilage regeneration (ACI and marrow stimulation) as compared to mature tissue transplantation (OAT or mature tissue engineered constructs). However, cell-based cartilage repair does need improvement of integrative repair if we plan to extend the indications for cartilage repair, for example with defect treatment in older patients or in more degenerative joints. Integration of *in situ* induced neocartilage with older and thus lower cell density cartilage or degenerated cartilage wound edges probably benefits from the above-mentioned interventions.

**Outcome parameters**

Long term follow-up of patients is required in order to determine whether cartilage defect repair prevents patients from OA development, as this can take many years. Clinical outcome scores and histogical grading of biopsies (in the currently available RCTs often from a small part of the study subjects) are now used to follow the repair process. Methods that are both objective and non-invasive, with the ability to follow the repair process

and/or the development and progression of OA in time would be of large clinical value. Currently, delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), in which a gadolinium containing contrast agent (Gd-DPTA$^2$) is injected intravenously followed by MRI, is evolving as a non invasive method to provide information the quality of cartilage and repair tissue [200, 201]. In damaged cartilage, the GAG content and thereby charge is different compared to healthy cartilage, which also affects mechanical properties [202]. These charge changes are detectable using dGEMRIC as a consequence of differences in Gd-DPTA$^2$ uptake in the cartilage [200, 203]. Recently, dGEMRIC has been studied for follow-up of patients treated with ACI or ACI derived treatments [204-206]. For ACI-P treated patients at 9 to 18 years post treatment, the quality of repair tissue was found to be comparable to surrounding native cartilage, however no correlation between dGEMRIC results and KOOS score was found [206]. Studies using dGEMRIC for evaluation ACI or derived treatments are so far conducted in small study populations (5 to 36 patients) [204-206]. To our knowledge, there is only one animal study from which is concluded that dGEMRIC might be useful for microfracture follow-up [207]. It is evident that larger studies and RCTs are required in order to truly assess the potential of dGEMRIC in follow-up of treated cartilage defects and the early detection of development and progression of OA.

**Bioactive materials to improve intrinsic healing capacity**

Earlier in this review we described the use of augmentation of the microfracture technique by using biomaterials. Stimulation of the body's intrinsic healing capacity by the use of bioactive biomaterials is attractive because it can yield an off-the-shelf product. Research focuses on the attraction of cells from the environment (bone marrow, synovium or even the cartilage) into a scaffold material and to stimulate these cells to form cartilage matrix. Many different types of biomaterials, both synthetic and natural, are being developed, modified and evaluated. These biomaterials can be made bioactive by incorporation of growth factors or gene vectors to improve cell ingrowth, cell proliferation or matrix production. Even anti-inflammatory factors can be incorporated to inhibit inflammatory processes, which are known to have negative effects on cartilage repair. Controlled release of these factors in time and maybe even sequential release of a number of factors will be necessary for optimal control of the tissue repair process. Research in this area can be expected to reveal new suitable products to improve cartilage repair in the future.

**Regulatory obstacles**

Stringent regulatory requirements by US Food and Drug Administration (FDA) and the European Advanced Therapy Medicinal Therapy (ATMP) regulations have made industrial development of cell therapeutic applications more difficult. Therefore simpler, and cheaper, one stage methods, where cell culture is avoided, are receiving more and more attention. Examples of these single stage methods are the earlier mentioned bone marrow stimulation

techniques augmented with biomaterials and the cartilage autograft implantation system (CAIS, dePuy) where cartilage is minced, added to a synthetic scaffold and fixed cartilage defects with resorbable staples. More developments are to be expected such as INSTRUCT, a cartilage repair method that is currently undergoing a pilot study in patients where the construct is prepared in the operation theater by combining isolated primary chondrocytes with freshly isolated bone marrow cells seeded in a mechanically functional scaffold (CellCoTEc, the Netherlands).

## Conclusions and future directions

The intrinsic repair capacity of articular cartilage defects is limited and we believe that these lesions contribute to the development of early OA. The goals of cartilage defect repair should always be a combination of symptom relief *and* prevention of early joint degeneration. From natural outcome studies we have learned that it may take many years for isolated cartilage lesions to lead to degenerative changes. However, most cartilage lesions are associated with more extensive joint injuries, contributing to the risk for OA development. For this reason results from RCTs, with selected patients, cannot fully elucidate the value of current cartilage repair techniques for often more extensive injuries. The above-presented RCTs show an improvement of symptoms following use of current repair techniques with an advantage for cell-based techniques, but only a suggestion that risk for joint degeneration can be reduced.

Successful repair techniques for isolated cartilage defects in otherwise healthy joints may, in the future, may be translated to treatment of more extensively degenerated joint diseases such as OA. In degenerative joints, repair of cartilage lesions alone is probably not enough to restore joint function. Because of the progressive nature of OA and the involvement of many different tissues in the joint, this new repair tissue will probably be degraded by a combination of catabolic synovial factors as well as the altered subchondral bone lying underneath the repaired cartilage. It will therefore be necessary to develop a combination of therapies to modulate the degenerative processes, either surgically or pharmacologically, before or at the time of application of a cartilage repair technique.

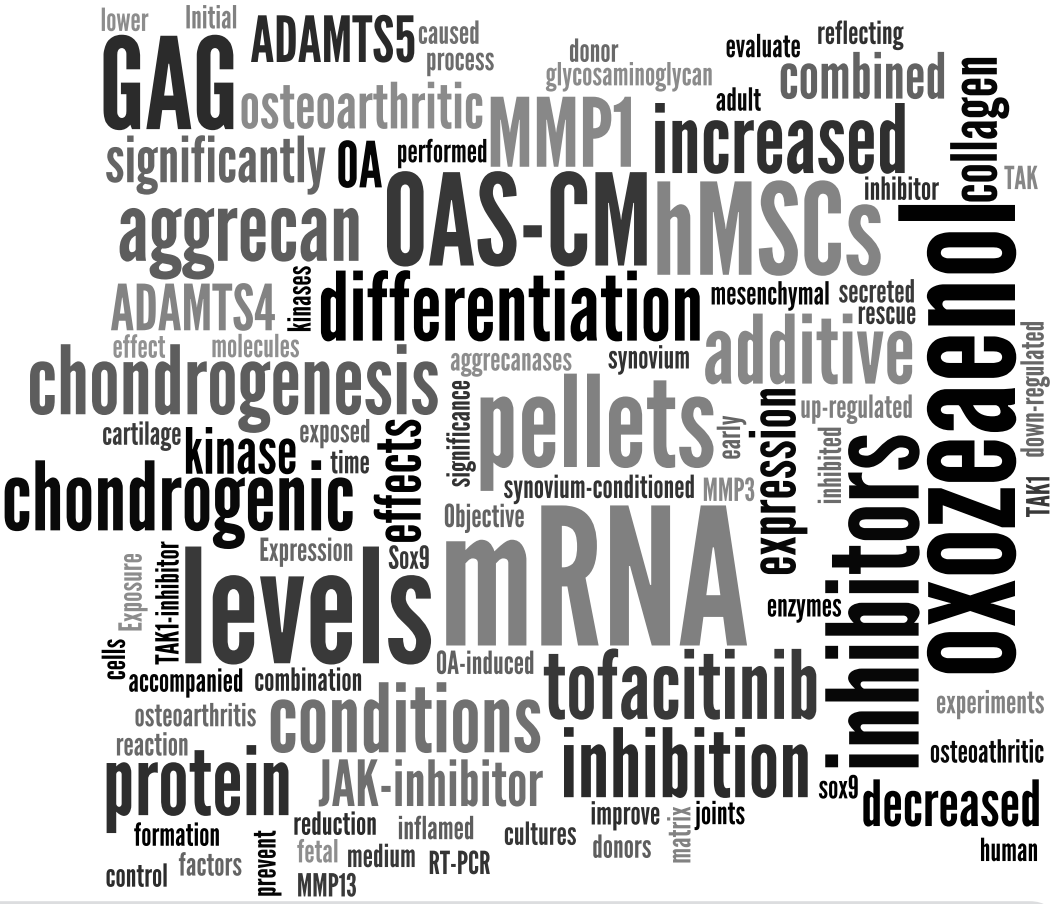
MSCs can be an attractive cell source for cartilage repair, not only because they are easily harvested, have a good proliferative capacity and can differentiate into chondrocytes but also because of their trophic activity. MSCs have shown to secrete a large spectrum of bioactive molecules resulting in a regenerative microenvironment potentially limiting damage and stimulating intrinsic regenerative responses.

## Acknowledgements

M.L. van Melle is supported by a research grant of the Dutch Arthritis Association. The authors declare the absence of competing interests and confirm their independence regarding the content of this manuscript.

# Chapter 3

## Inhibition of TAK1 and/or JAK can rescue impaired chondrogenic differentiation of human mesenchymal stem cells in osteoarthritis-like conditions




H.M. van Beuningen, M.L. de Vries - van Melle, E.L. Vitters,
B.W. Schreurs, W.B. van den Berg, G.J.V.M. van Osch, P.M. van der Kraan

Accepted for publication in Tissue Engineering: Part A, January 2014

## Abstract

**Objective:** To rescue chondrogenic differentiation of human mesenchymal stem cells (hMSCs) in osteoarthritic conditions by inhibition of protein kinases.
**Methods:** hMSCs were cultured in pellets. During early chondrogenic differentiation these were exposed to osteoarthritic synovium-conditioned medium (OAS-CM), combined with the JAK-inhibitor tofacitinib and/or the TAK1-inhibitor oxozeaenol. To evaluate effects on chondrogenesis, the glycosaminoglycan (GAG) content of the pellets was measured at the time chondrogenesis was manifest in control cultures. Moreover, mRNA levels of matrix molecules and enzymes were measured during this process, using real-time polymerase chain reaction (RT-PCR). Initial experiments were performed with hMSCs from a fetal donor and results of these studies were confirmed with hMSCs from adult donors.
**Results:** Exposure to OAS-CM resulted in pellets with a much lower GAG content, reflecting inhibited chondrogenic differentiation. This was accompanied by decreased mRNA levels of aggrecan, type II collagen and Sox9, and increased levels of MMP1, MMP3, MMP13, ADAMTS4 and ADAMTS5. Both tofacitinib (JAK-inhibitor) and oxozeaenol (TAK1 inhibitor) significantly increased the GAG content of the pellets in osteoarthritis (OA)-like conditions. The combination of both protein kinase inhibitors showed an additive effect on GAG content. In agreement with this, in the presence of OAS-CM both tofacitinib and oxozeaenol increased mRNA expression of sox9. Expression of aggrecan and type II collagen was also upregulated, but this only reached significance for aggrecan after TAK1 inhibition. Both inhibitors decreased the mRNA levels of MMP1, 3 and 13 in the presence of OAS-CM. Moreover, oxozeaenol also significantly down-regulated the mRNA levels of aggrecanases ADAMTS4 and ADAMTS5. When combined, the inhibitors caused additive reduction of OA-induced MMP1 mRNA expression. Counteraction of OAS-CM-induced inhibition of chondrogenesis by these protein kinase inhibitors was confirmed with hMSCs from two different adult donors. Both tofacitinib and oxozeaenol significantly improved GAG content in cell pellets from adult donors and their effects were additive in pellets from one of the two donors.
**Conclusions:** Tofacitinib and oxozeaenol partially prevent the inhibition of chondrogenesis by factors secreted by OA synovium. Their effects are additive. This indicates that these protein kinase inhibitors can potentially be used to improve cartilage formation under the conditions occurring in osteoathritic, or otherwise inflamed, joints.

## Introduction

Articular cartilage is nonvascularized and noninnervated and has a limited capacity to repair itself, thereby presenting a major clinical problem. Many efforts are made to tissue engineer cartilage or manipulate the joint to circumvent the incapability of natural repair. For tissue engineering purposes, stem cells are placed in a cartilage defect or stem cell recruitment from the bone marrow is stimulated by penetrating the subchondral bone plate. However, cartilage requiring repair is generally located in a diseased joint and not in a healthy joint. This diseased joint will contain a mixture of factors that potentially will not benefit the chondrogenesis of the mesenchymal stem cells (MSCs) in the defect. Several studies showed that synovial fluid obtained from knees of patients with a traumatic chondral defect can inhibit chondrogenic redifferentiation of monolayer expanded human chondrocytes [208, 209]. However, it should be noted that these studies were performed with differentiated cells rather than true progenitor cells. Krüger et al showed that chondrogenic differentiation of human subchondral progenitor cells is affected by synovial fluid from donors with osteoarthritis (OA) or rheumatoid arthritis (RA) [210]. Previously we have shown that factors secreted by the synovial membrane of OA joints inhibit chondrogenic differentiation of hMSCs, thereby impairing successful tissue engineering [211]. We could not discern a clear relation between the levels of our primary suspects IL-1 and TNF-α in the OA synovium conditioned medium (OAS-CM) and the strength of inhibition of chondrogenesis [211]. So, the exact catabolic factors and pathways responsible for the inhibition of chondrogenesis remain to be identified. As specific knowledge on the nature of these factors is lacking, we aimed to modulate this effect by targeting common signaling pathways.

In the present study we therefore inhibited protein kinases to block catabolic intracellular pathways in hMSCs. We focused on inhibition of Transforming Growth Factor β-activated kinase 1(TAK1) and Janus kinases (JAKs) since TAK1 and JAKs are common routes of several specific, but different, cytokine signaling pathways. The TAK1 inhibitor oxozeaenol, was used in our model of OA-induced inhibition of chondrogenesis, because of the central role of TAK1 in the intracellular signaling of a number of important inflammatory cytokines and growth factors [212, 213]. In response to interleukin-1, tumor necrosis factor-α, toll-like receptor agonists, and TGFβ/BMP it mediates the activation of the nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), and p38 pathways. TAK1 is now defined as an activating kinase for the IkB kinase (IKK) complex, composed of IKKa, IKKb and NF-kB essential modulator (NEMO), via association with TNF receptor-associated factor 2 (TRAF2) and TRAF6 in TNF-α and IL-1–TLR signaling pathways, respectively [214, 215]. We also used the JAK inhibitor tofacitinib (also known as CP-690,550) in our model of OA-induced inhibition of chondrogenesis. Tofacitinib primarily inhibits JAK1 and JAK3 and, to a lesser extent, JAK2.

Most of the STAT-activating cytokine receptors (i.e., type I and type II cytokine receptors) do not have tyrosine kinase activity and instead require JAKs to initiate intercellular signaling. The JAK family of proteins are tyrosine kinases and constitute four members (JAK1, JAK2, JAK3, and TYK2) in mammals [216, 217]. The JAK/STAT signaling pathway is activated by numerous growth factors and cytokines, including members of the interferon (IFN) family, such as IFN γ and IL-10, of the gp130 family, like IL-6, oncostatin M, and leukemia inhibitory factor (LIF), of the γC family, such as IL-2, and the single chain family, like erythropoietin [218-222]. Because JAK activation is needed for signaling through the receptors for cytokines that are integral to lymphocyte function, tofacitinib may be able to modulate many aspects of the immune response [223, 224]. Tofacitinib is in clinical development for the indications of RA, psoriasis, renal transplant prevention, inflammatory bowel disease and dry eye. Phase III studies in RA show its efficacy and safety and tofacitinib is recently approved by the FDA for the treatment of RA [225, 226].

In the present study, we aimed to rescue chondrogenic differentiation of hMSCs in OA-like conditions by blocking the JAK/STAT or/and the TAK1 intracellular pathways. We showed that both protein kinase inhibitors can rescue the chondrogenesis that was impaired by synovial factors, and that jointly they can do more than separately.

## Materials and Methods

### Culture of hMSCs

Initial experiments were performed with hMSCs from a fetal donor (ScienCell Research Laboratories, Carlsbad, California). Passage 2 bone marrow-derived hMSCs were expanded for 12 days, diluting these cells three times each passage. This was performed in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza,Verviers, Belgium). The hMSCs were stored in liquid nitrogen at passage 5. These passage 5 cells were expanded and passaged for another approximate 12 days before using these cells in experiment at passage 7. At this passage, the cells retained their multilineage potential as determined by chondrogenic, osteogenic, and adipogenic differentiation experiments (data not shown).

Besides these fetal cells, we used hMSCs of two adult donors. Bone marrow was aspirated from patients undergoing total hip replacement surgery after informed consent was obtained. The procedures were approved by the local ethical committee of the Erasmus MC, University Medical Center Rotterdam (MEC 2004-142). hMSCs were isolated based on their plastic adherence. The heparinized bone marrow aspirates were seeded at a density of 2-5*$10^5$ cells/$cm^2$ in hMSC expansion medium consisting of Minimum Essential Medium

– alpha (MEM-α, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium), 50µg/ml gentamicine (Gibco), 1.5µg/ml fungizone (Gibco), 1ng/ml fibroblast growth factor 2 (FGF2, AbD Serotec, Kidlington, UK) and 25µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA). Non-adherent cells were washed off after 24 hours and adherent cells were further expanded. At subconfluence, hMSCs were trypsinized and replated at a density of 2,300 cells/cm$^2$. MSC expansion medium was refreshed twice per week. Passage 3 cells were used for experiments.

**Chondrogenic differentiation of hMSCs**

hMSCs from fetal origin or from adult donors were chondrogenically differentiated by culturing in high cell density through pelletation (0.25 x 10$^6$ cells per pellet) in 0.5ml of serum free chondrogenic differentiation medium, consisting of Dulbecco's modified Eagles medium (DMEM, Gibco) supplemented with insulin (6.25 mg/ml), transferrin (6.25 mg/ml), sodium selenite (6.25 ng/ml),prolin (0.4mg/ml), sodium pyruvate (1mg/ml), linoleic acid (5.35 mg/ml), ascorbic acid (50 mg/ml), bovine serum albumin (1mg/ml), and dexamethasone (10$^{-7}$M) (Sigma). This serum free chondrogenic differentiation medium was supplemented with transforming growth factor β type 1(TGFβ1, 10 ng/ml, R&D) and bone morphogenetic protein 2 (BMP2, 50 ng/ml, R&D) [227]. After pelletation the medium was refreshed three times a week, adding fresh growth factors each time.

**Conditioned medium derived from OA synovium**

Synovium was obtained from OA patients undergoing total hip replacement. Pieces of the joint capsule, with the synovial membrane on it, were used to produce OA synovium conditioned medium. In comparison to using monolayers of isolated synoviocytes, this method seemed more optimal because it yields products of all cell types of the OA synovium still embedded in the relatively intact synovial membrane. The synovium was divided into small pieces and cultured in a six-well plate in DMEM with 0.1% BSA (0.3 g tissue/ml). The supernatant was collected after 24h. Debris was removed by centrifugation after which the medium was stored at - 20°C. Two different pools of OAS-CM were used, each consisting of pooled OAS-CM from seven different OA patients. This was stored in small aliquots at -20°C until further use. The mean age of OA patients was 72.4 (SD 9.1) for pool1 and 65.7 (SD 3.7) for pool 2.

**Blocking of chondrogenesis by OAS-CM and use of protein kinase inhibitors to counteract this**

***hMSCs of fetal donor***

At day three after pelletation 10% OAS-CM and protein kinase inhibitors were added. (5Z)-7-Oxozeaenol (Tocris Bioscience, Bristol, UK) and tofacitinib (LC Laboratories) were used at

concentrations of 1µg/ml and 800 ng/ml, respectively. These concentrations were found to be at plateau level in initial dose-response studies based on GAG content of pellets (data not shown). Pellets were pre-incubated with the inhibitors (or the solvent DMSO only) for 10 minutes before the addition of the OAS-CM. The final concentration of all components of the chondrogenic differentiation medium, including the growth factors, was kept equal in all conditions. The media were replaced by fresh medium +/- OAS-CM and/or inhibitors at day 5 after pelletation. After 24 or 72 hours exposure to OAS-CM and/or the inhibitors (days 4 and 6 respectively), pellets were harvested for mRNA isolation. At day 7 after pelletation, pellets were harvested for measurement of GAG content.

***hMSCs of adult donors***

Since it is known that the progression of chondrogenesis in MSCs from adult donors is slower than chondrogenesis of MSCs from fetal donors, the timing of the administration of OAS-CM and protein kinase inhibitors was adjusted. To ensure proper timing of administration, treatment with OAS-CM and protein kinase inhibitors was started at different time points: from day 7, 14 or 21on, the chondrogenic differentiation medium was supplemented with 10%OAS-CM (1:1 mix of pools 1 and 2) and 800 ng/ml tofacitinib, 1µg/ml oxozaeanol or a combination of both inhibitors. The final concentration of all components of the chondrogenic differentiation medium was kept equal in all conditions. Pellets cultured in chondrogenic differentiation medium with or without 10ng/ml TGFβ1 and 50ng/ml BMP2 served respectively as positive and negative controls. Since both inhibitors were dissolved in DMSO, levels of DMSO were equalized over all conditions to which OAS-CM, tofacitinib or oxozeaenol where added. To test whether DMSO had a negative effect on chondrogenesis of hMSCs from adult donors, conditions in which pellets were cultured in chondrogenic medium with TGFβ1 and BMP2 with additional DMSO were included. Medium was refreshed three times per week. At days 7, 14 and 21 (time-points at which treatment of pellets was started) pellets were harvested to evaluate chondrogenesis. The remaining pellets were cultured for 28 days and harvested for GAG quantification.

**Measurement of GAG content in cell pellets**

For measurements of the GAG content each individual cell pellet was first digested overnight with 0.1% papain (Sigma) in digestion buffer (200mM $NaPO_4$, 100mM NaAc, 5mM cysteine HCl, 10mM EDTA, pH 6.4) at 60°C. Then the GAG concentration of the digest was measured using the Farndale assay [228]. This method is based on a shift in the maximum absorbance wavelength of the dye 1,9-dimethylmethylene blue after binding to multiple sulfate groups (metachromasia). The absorbance of dimethylmethylene blue after binding with the sulfate groups in samples of the digest was measured at 535 nm. Each experimental group consisted of three cell pellets.

**RNA isolation and quantitative RT-PCR**

At 24h and 72 h after the start of exposure to OAS-CM and protein kinase inhibitors, pellets were harvested for analysis of mRNA levels. Total RNA was extracted from the cell pellets (2 pellets for each sample) using TRI Reagent (Sigma) according to the manufacturer's protocol. Isolated RNA was DNase treated and reverse transcribed. Primers were designed using the Primer Express software (Applied Biosystems). Reverse transcriptase (RT) primer nucleotide sequences are listed in Table 1. Real-time quantitative PCR was performed using a StepOnePlus sequence detection system (Applied Biosystems). Messenger RNA levels were normalized to the housekeeping gene ribosomal protein S27a (RPS27a) levels, and all conditions and genes were expressed relative to the noncatabolic control condition (ddCt).

**Histological analysis**

Pellets were fixed in phosphate buffered formalin for 7 days, followed by dehydration using an automated tissue-processing apparatus (Tissue Tek VIP; Sakura). Thereafter the pellets were embedded in paraffin and 7 mm tissue sections were prepared. Sections were mounted on Superfrost plus glass (Thermo Scientific), stained with Safranin O and Fast Green and mounted with Permount.

**Statistical analysis**

Normality was verified with Kolmogorov-Smirnov and Shapiro-Willk tests using SPSS 15.0. When necessary, logarithmic transformation was performed to obtain normally distributed data. For unpaired data, the Student's t-test was performed. For normally distributed paired data, a generalized estimated equations model was used. For non-normally distributed paired data, a Kurskall-Wallis test was performed, followed by a Mann-Whitney U test. Correction for multiple testing was performed using Bonferroni correction. P values less than 0.05 (2-tailed) were regarded as statistically significant.

## Results

**Time-course of chondrogenic differentiation of hMSCs from fetal donor**

To induce chondrogenesis of hMSCs from fetal donors, cell pellets were cultured in chondrogenic differentiation medium including TGFβ1 and BMP2 during 7 days. Histological sections (figure 1.) show that at day 3 after pelletation the pellets were still small and did not stain with safranin O. At day 4 after pelletation the first signs of chondrogenic differentiation were visible. At day 7 safranin O-stained histological sections showed that the pellets were strongly enlarged and stained positive for proteoglycans. The exposure to 10% OAS-CM during days 3-7 caused suppression of chondrogenic differentiation (figure 1).

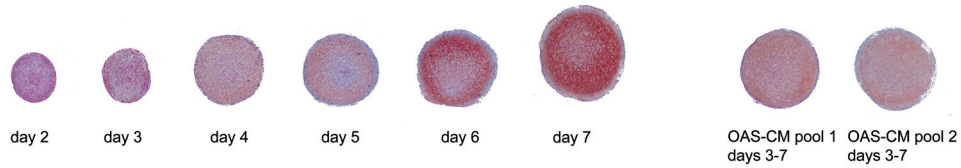



**Figure 1. Histological sections of hMSCs from fetal origin in pellet culture at different stages of chondrogenic differentiation (days after pelletation).**
At day 7, the effect of exposure to OAS-CM from the two different pools is shown. Sections were stained with Safranin O for proteglycan staining and counterstained with Fast Green. Original magnification 50x.

### The TAK1 inhibitor oxozeaenol improves chondrogenesis of hMSCs in OA-like conditions

GAG content of pellets at day 7 after pelletation of hMSCs from fetal origin was used as a measure for chondrogenic differentiation. Exposure to 10% OAS-CM from day 3 on caused a decrease of more than 50% in the GAG content of the pellets at day 7 (figure 2.A). Both pools of OAS-CM that were used, each consisting of OAS-CM from seven different OA patients, suppressed chondrogenesis, albeit stronger with pool 2 than pool 1.

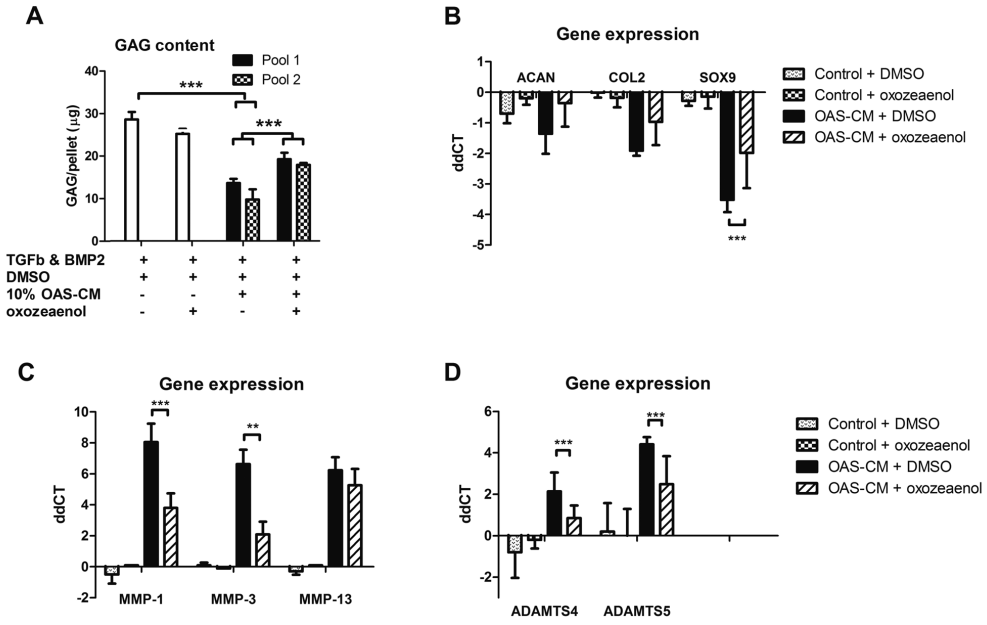



**Figure 2. TAK1 inhibition by oxozeaenol partially counteracts the effect of OAS-CM on fetal hMSCs.**
Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 1µg/ml oxozeaenol from day 3 after pelletation. (a) GAG content of pellets at day 7 after pelletation. Values represent mean +/- SD (n=3). (b-d) mRNA expression (ddCT) was measured at 24h after addition of 10% OAS-CM and/or 1µg/ml oxozeaenol from day 3 after pelletation of hMSCs. Values represent mean +/- SD (n=6). Generalized estimated equations model with correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001.

Supplementation with 1µg/ml oxozeaenol from day 3 on significantly enhanced the GAG content of the pellets exposed to OAS-CM as compared to OAS-CM with only the solvent. The same pattern was found for the 2 different pools of OAS-CM.

For the measurement of mRNA levels using RT-PCR, cell pellets were harvested at day 4, which was 24 h after the start of exposure to OAS-CM +/- inhibitor. The mRNA expressions of the cartilage-specific extracellular matrix (ECM) molecules aggrecan and type II collagen and of the transcription factor sox9, which is involved in chondrocyte differentiation and cartilage formation, were significantly decreased by the OAS-CM (Figure 2B).
Treatment with oxozeaenol counteracted this, although only for sox9 this counteraction reached statistical significance (Figure 2.B). mRNA expressions of all three MMPs and both aggrecanases studied were highly up-regulated by the OAS-CM at day 4 (Figures 2.C and D). 1µg/ml oxozeaenol was able to significantly counteract this effect of the OAS-CM on all these enzymes, except for MMP13. At a later time-point (day 6), when cartilage formation is much more advanced under control conditions, the same patterns were found (data not shown).

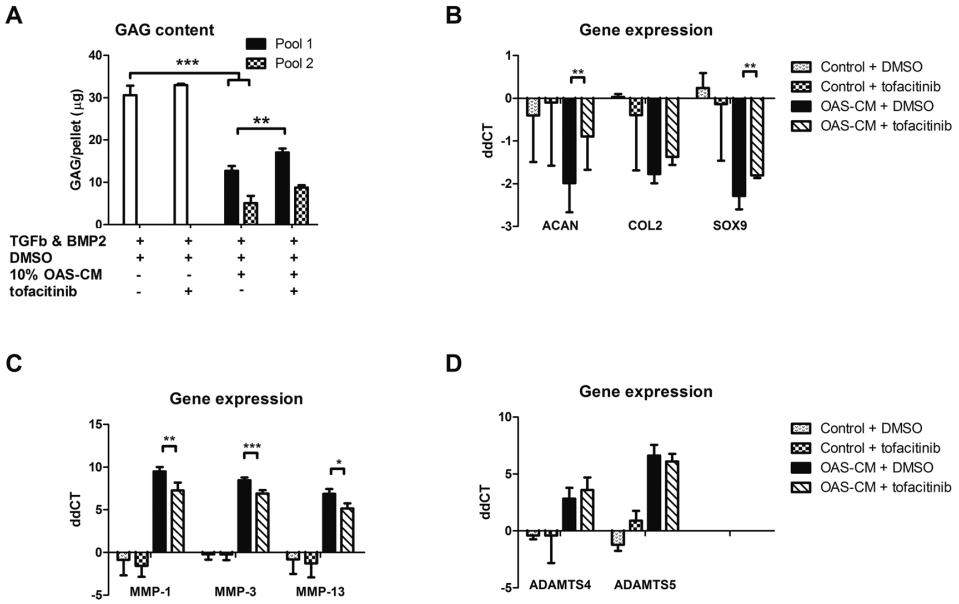



**Figure 3. JAK inhibition by tofacitinib partially counteracts the effects of OAS-CM on fetal hMSCs.**
Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 800ng/ml tofacitinib from day 3 after pelletation. (a) GAG content of pellets at day 7 after pelletation. Values represent mean +/- SD (n=3). (b-e) mRNA expression (ddCT) was measured at 24h after addition of 10% OAS-CM and/or 800ng/ml tofacitinib from day 3 after pelletation of hMSCs. Values represent mean +/- SD (n=6). Generalized estimated equations model with correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001.

**The Jak inhibitor tofacitinib improves chondrogenesis of hMSC in OA-like conditions.**
Supplementation with 800 ng/ml tofacitinib from day 3 on significantly enhanced the GAG content of the pellets exposed to OAS-CM from pool 1 as compared to OAS-CM with only the solvent, where this was not significant for pool 2 (Figure 3.A). In this experiment OAS-CM from pool 2 inhibited chondrogenesis relatively strong, and this could have hindered counteraction by tofacitinib. At day 4, which was 24 h after the start of exposure to OAS-CM +/- inhibitor, the mRNA expressions of aggrecan, type II collagen and sox9 were significantly decreased by the OAS-CM, and treatment with tofacitinib significantly counteracted this, except for type II collagen (Figure 3.B). mRNA expressions of all three MMPs and both aggrecanases were highly up-regulated by the OAS-CM at day 4 (Figures 3.C and D). 800 ng/ml tofacitinib was able to counteract this effect of the OAS-CM on all three MMPs (Figure 3C). In contrast to TAK1 inhibition, JAK inhibition by tofacitinib had no significant effect on the enhanced levels of the aggrecanases ADAMSTS4 and-5 in OA-like conditions (Figure 3.D). At a later time-point (day 6), when cartilage formation is much more advanced under control conditions, the same patterns were found, including clear insensibility of aggrecanase mRNA levels to tofacitinib (data not shown).

**TAK1 and JAK inhibition contribute additively to the rescue of chondrogenesis of hMSC in OA conditions.**
Combinations of oxozeaenol and tofacitinib were applied, to study whether this combination has more effect than addition of a single inhibitor. In this experiment (Figure 4.A) the GAG content of pellets at day seven was again severely decreased after treatment with OAS-CM from day 3 on. The effect of OAS-CM from both pools was significantly decreased by 1µg/ml oxozeaenol and by 800 ng tofacitinib added from day 3 on. Further counteraction of OAS-CM was demonstrated when both inhibitors were combined. GAG content after the combined treatment was significantly increased compared to oxozeaenol or tofacitinib alone. The same pattern was found for the two different pools of OAS-CM. mRNA levels at day 4 were measured using RT-PCR. In figures 4.C-E it was tested whether the combination of the two inhibitors significantly counteracted OAS-CM and whether the effect of the combination was significantly stronger than that of the single inhibitors. In this experiment, up-regulation of the mRNA levels of aggrecan, type II collagen, and sox9 at day 4 (Figure 4.E) did not reach statistical significance, even when both inhibitors were combined. The effects on MMP expression were more clear. OAS-CM induced high mRNA levels of all three MMPs, and these were significantly decreased by the combination of both inhibitors. Only for MMP1 the combination showed stronger counteraction than each of the single inhibitors, indicating that their effects were additive in this respect (Figure 4.C). Down-regulation of MMP3 expression by oxozeaenol alone appeared to be equivalent to the combined effect of both inhibitors. On the other hand, tofacitinib suppressed MMP13 mRNA levels

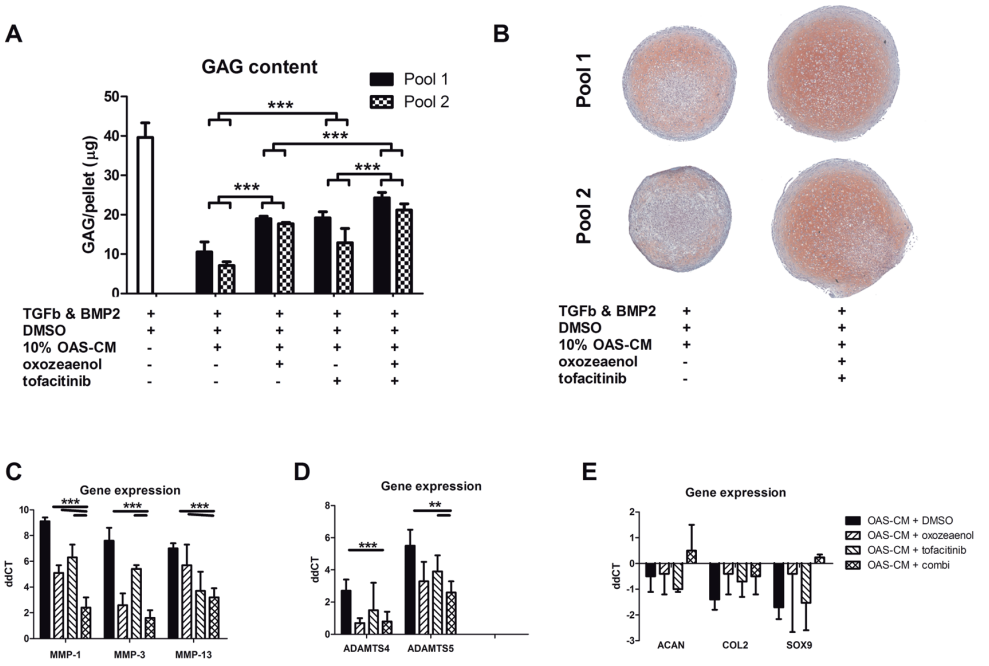



**Figure 4. TAK1 and JAK inhibitors are additive in counteraction of the effect of OAS-CM on fetal hMSCs.** Fetal hMSCs were cultured in chondrogenic medium and were exposed to 10% OAS-CM from two different pools and/or 1µg/ml oxozeaenol and/or 800ng/ml tofacitinib from day 3 after pelletation. (a) GAG content of pellets at day 7 after pelletation. Values represent mean +/- SD (n=3). (b) Histological sections of hMSCs from fetal origin in pellet culture, showing the counteraction of the effect of OAS-CM by the combination of 1µg/ml oxozeaenol and 800ng/ml tofacitinib. Sections were stained with Safranin O and Fast Green. Original magnification 50x. (c-e) mRNA expression (ddCT) was measured at 24h after addition of 10% OAS-CM +/- µg/ml oxozeaenol and/or 800ng/ml tofacitinib from day 3 after pelletation of hMSC. Values represent the mean +/- SD (n=6). Generalized estimated equations model with correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001.

equally well, as compared to the combination of both inhibitors. Overall (also at the later time-point), in this experiment tofacitinib had the strongest effect on MMP13 expression, while oxozeaenol blocked more efficiently MMP1 and MMP3 expression, as compared to tofacitinib. These data confirm the experiments with single inhibitors (Figures 2.C and 3.C) that also showed this relative difference in regulation of MMP1 and MMP3 versus MMP13. OAS-CM-induced mRNA expression of aggrecanases (Figure 4.D) was significantly counteracted by the combination of both inhibitors. Additive effects were not found here. In fact, oxozeaenol significantly counteracted OAS-CM-induced aggrecanase expression, while tofacitinib effects did not reach significance. This is in line with the experiments shown in figures 2D and 3D, where tofacitinib was less potent in down-regulation of aggrecanases as compared to oxozeaenol.

**Effects of protein kinase inhibitors on cartilage formation by adult MSC**

MSCs from two adult donors were cultured as cell pellets for 28 days in chondrogenic medium with TGFβ and BMP2. After 7,14 and 21 days, administration of 10% OAS-CM, oxozeaenol, tofacitinib or combinations of both was started and pellets were cultured for a total of 28 days. MSCs from both donors deposited significant amounts of GAGs in cell pellets from day 14 onward (Figure 5.A). The OA-like conditions simulated by the OAS-CM inhibited chondrogenesis of hMSCs strongly: when the addition of OAS-CM was started after 7 days of culture, hardly any GAGs were measured after 28 days of culture. Addition of OAS-CM from day 14 or 21 on not only inhibited further chondrogenesis, but also previously deposited GAGs were degraded, as indicated by GAG measurement in pellets that were harvested after 14 or 21 days. This indicates a strong effect of the OAS-CM. In both hMSC donors, administration of oxozeaenol started at day 7 or 14 did not result in prevention or counteraction of the effect of OAS-CM, of which the administration was started at the same time as the oxozeaenol. Administration of OAS-CM and oxozeaenol from day 21 on resulted in counteraction of the effect of OAS-CM. Administration of tofacitinib and OAS-CM from day 14 or 21 on significantly prevented the OAS-CM-induced loss of GAGs (Figure 5.B). This effect was strongest for hMSCs from donor 1.

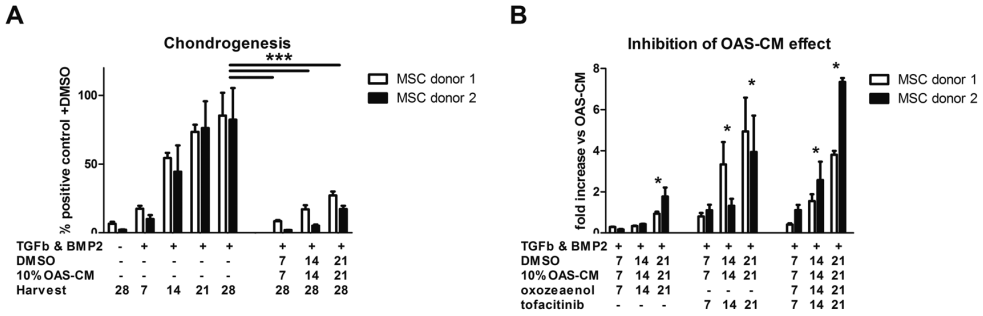



**Figure 5. The TAK1 inhibition and the combination of the TAK1 and JAK inhibitor partially counteract the effect of OAS-CM in adult hMSCs.**

Adult hMSCs from 2 different donors were cultured for 28 days in chondrogenic medium and exposed to 10% OAS-CM, and/or 1μg/ml oxozeaenol and/or 800ng/ml tofacitinib from day 7, 14 or 21. OAS-CM from the two different pools was pooled together for this experiment. (a) GAG content as a percentage of positive control pellets cultured in the presence of DMSO. Negative control pellets cultured for 28 days without TGF-β and BMP2, positive control pellets cultured with TGF-β and BMP2 for 7, 14, 21 or 28 days and pellets cultured for 28 days and treated with OAS-CM from day 7, 14 or 21 (n=3). Generalized estimated equations model with correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001. (b) GAG content as fold increase versus pellets cultured in the presence of OAS-CM and DMSO for 28 days. Treatment with OAS-CM and/ or oxozeaenol and/or tofacitinib was started after 7, 14 or 21 days (n=3). Generalized estimated equations model with correction for multiple testing. *p<0.05 versus pellets cultured in the presence of OAS-CM and DMSO.

In hMSCs from both donors, administration of OAS-CM and a combination of both inhibitors from day 14 or 21 on resulted in a significant rescue of chondrogenesis. In donor 1, oxozeaenol had no additional effect. In donor 2, the combination of OAS-CM and both inhibitors resulted in the deposition of more GAGs than when pellets were treated with OAS-CM and the single inhibitors. These results obtained in experiments with adult hMSCs confirmed those from the experiments with fetal hMSCs. When OAS-CM and inhibitors were added before chondrogenesis was initiated, no relevant levels of GAGs were detected. When OAS-CM and inhibitors were added after initiation of chondrogenesis, tofacitinib or the combination of both oxozeaenol and tofacitinib were able to partially rescue chondrogenesis.

## Discussion

Previously we have demonstrated that factors produced by OA synovium inhibit chondrogenic differentiation of hMSCs [211]. In the present study we investigated the rescue of chondrogenic differentiation in OA-like conditions by inhibition of protein kinases. Our studies indicate for the first time that both the JAK inhibitor tofacitinib and the TAK1 inhibitor oxozeaenol can improve chondrogenesis of both fetal and adult hMSCs in the presence of OAS-CM. Measurement of GAG content also showed that the effects of TAK1 and JAK inhibition on chondrogenesis were additive. At the mRNA level, both inhibitors counteracted the OAS-CM-induced downregulation of expression of the master gene of chondrogenesis, sox9, and of the cartilage-specific ECM molecules aggrecan and type II collagen. Moreover, both inhibitors counteracted OAS-CM- induced up-regulation of expression of MMPs.

Our findings in a model for chondrogenic differentiation of hMSCs under catabolic, OA-like, conditions are in line with studies in adult articular chondrocytes where MAP kinase activation has been associated with increased expression of MMPs and aggrecanases and with decreased expression of aggrecan/proteoglycans [229]. Although in both situations genes for ECM molecules are down-regulated and those for cartilage-degrading enzymes are up-regulated, this does not predict that the same pathways are used in hMSCs during early chondrogenesis and in adult articular chondrocytes .

Besides inflammatory cytokines, also the growth factor TGFβ can signal via TAK1. This growth factor is an important stimulator of cartilage-related ECM production. We found that the blocking of its intracellular signaling via the non-canonical, TAK1-dependent, pathway did not have much effect on proteoglycan content in cell pellets after chondrogenic differentiation in anabolic conditions (Figure 2.A). This is in line with studies in adult articular chondrocytes, indicating that ECM synthesis is mainly regulated via the activation of Smads [230]. These

two signaling routes, however, are not completely separate, since interactions between TAK1 and the Smad proteins have been described [231, 232].

In catabolic conditions (+ OAS-CM) we did find effects of inhibition of TAK1. Improvement of chondrogenesis by inhibition of TAK1 was found in these conditions. It has been suggested, from in vitro data, that BMP and TGFβ signaling via TAK1 can regulate chondrogenesis, hypertrophic differentiation and chondrocyte proliferation [233-238]. Moreover, deletion of TAK1 in chondrocytes resulted in cartilage defects during embryonic development [239]. There are indications that TAK1 is especially involved in the earliest phase of chondrogenesis [239]. In our experiments with fetal hMSCs we started TAK1 inhibition three days after pelletation, which may be after this critical phase. The results of the time-course experiment with adult hMSCs stress the importance of adequate timing of the inhibition, since an early start with OAS-CM and TAK1 inhibition did not result in significant levels of GAG production. Also, inhibition of the signaling via TAK1 of factors in the OAS-CM which impair chondrogenesis might outweigh possible negative effects of TAK1 inhibition on the same process.

It has been shown that in adult human articular chondrocytes MMP levels can be down-regulated by inhibiting JAK3 or TAK1 [240, 241]. In the present study we found the same in fetal hMSCs during early chondrogenic differentiation in OA-like circumstances. The involvement of both pathways, which are used by cytokines signaling via totally different receptors, indicates that multiple cytokines in OAS-CM jointly determine expression of MMPs. The effect of treatment with one of the two inhibitors would then be dependent on the relative contribution of these cytokines. In line with this, tofacitinib was more potent in down-regulating OA-induced MMP13 expression as compared to oxozeaenol, while it was equal or less potent in downregulation of MMP1 and MMP3 expression.

The effects of both inhibitors were additive in regulation of MMP1 but not in the regulation of the other MMPs, the aggrecanases and the cartilage ECM molecules. This suggests that in some aspects both inhibitors block the same pathways, and in other they do not. Interestingly, oxozeaenol significantly counteracted the OAS-CM-induced upregulation of mRNA expression of the aggrecanases ADAMTS4 and ADAMTS5, while tofacitinib was less potent in this respect. The effect of the TAK1 inhibitor is in agreement with the fact that aggrecanase expression is induced by IL-1 and TNFα, which signal intracellularly via TAK1 [242, 243]. On the other hand, JAK inhibition had no clear effect on aggrecanase levels, although aggrecanases can be induced by IL-6 and oncostatin M, that need JAKs for intracellular signaling [243, 244]. This could implicate that IL-6 and oncostatin M levels in OAS-CM are too low to induce aggrecanases, or that relevant receptors and co-receptors of these cytokines are expressed at a low level in hMSCs.

hMSC of two adult donors were used to verify the findings in hMSCs of a fetal donor. Several factors such as donor age are known to affect the chondrogenic differentiation capacity of adult hMSCs. As expected, these hMSCs showed much slower chondrogenic differentiation. Also in these experiments tofacitinib and to a lesser degree oxozeaenol partially blocked the effects of OAS-CM. The smaller effects of oxozeaenol on hMSCs of these two adult donors can reflect differences between donors, but also the OAS-CM caused much stronger inhibition of chondrogenic differentiation compared to the studies with cells of the fetal donor. This is in line with clinical findings that microfracture treatment, which is based on the formation of cartilaginous repair tissue by hMSCs, is known to have better clinical results for younger patients [57]. Since the experiments with adult hMSCs are more representative for the patient population presenting with cartilage lesions or OA, this validation of the results obtained with the fetal hMSCs is essential. Studies with hMSC from more donors and lower concentrations of the OAS-CM are needed to explore the therapeutic window of TAK1 and JAK inhibition.

In summary, we showed that both the JAK inhibitor tofacitinib and the TAK1 inhibitor oxozeaenol can improve chondrogenesis in terms of GAG content of cell pellets, which is impaired by the presence of OAS-CM. Inhibition of ECM synthesis and stimulation of ECM degradation by OAS-CM appeared to be counteracted by both inhibitors. Our findings could have major implications for therapies that are currently being applied or developed to heal cartilage damage. Many of these therapies depend in some manner on tissue repair and chondrogenesis by hMSCs, such as abrasion arthroplasty (in conjunction with debridement), Pridie drilling, and microfracture. These therapies in essence mimic a full thickness defect [57, 155, 245, 246]. These therapies depending on chondrogenesis of precursor cells are likely to fail in a diseased joint environment. Especially in these conditions JAK and TAK1 inhibitors can rescue chondrogenic differentiation and thereby promote cartilage regeneration by mesenchymal progenitor cells. The presence of an inflammatory milieu which impairs chondrogenic differentiation is not confined to the OA joint, but will also be present in joints with a cartilage defect, most times accompanied by ACL lesions or a torn meniscus. Therefore, blocking the deleterious effect of an inflammatory milieu on chondrogenesis by the use of protein kinase inhibitors might be an attractive option to boost cartilage repair in both joints with traumatic defects and joint diseases with a clear inflammatory component.

## Acknowledgement

The research leading to these results has received funding from the Netherlands Institute of Regenerative Medicine (NIRM) and from the European Union's 7$^{th}$ Framework Programme under grant agreement no. NMP3-SL-2010-245993.

# Chapter 4

## Synovial macrophage polarization state impacts chondrogenic differentiation of mesenchymal stem cells


N. Fahy, M.L. de Vries - van Melle, J. Lehmann, N. Grotenhuis, E. Farrell, P.M. van der Kraan, J.M. Murphy, Y.M. Bastiaansen - Jenniskens, G.J.V.M. van Osch

Submitted, November 2013

## Abstract

**Objective:** Mesenchymal stem cells (MSCs) are a promising cell type for the repair of damaged cartilage in osteoarthritis (OA). However, OA synovial fluid and factors secreted by synovium impede the chondrogenic differentiation of MSCs and the mechanism responsible for this effect remains unclear. In this study, we sought to investigate how M1 and M2 synovial macrophages contribute to the inhibition of chondrogenic differentiation of MSCs.
**Design:** The constitution of synovial macrophage subsets was analysed by immunohistochemical staining of human OA synovium sections for CD86 (M1 marker) and CD206 (M2 marker). To assess the effect of macrophage subsets on chondrogenesis, collagen type II (*COL2*) and aggrecan (*ACAN*) gene expression were compared between MSCs undergoing chondrogenic differentiation in medium conditioned (CM) by human OA synovial explants, isolated human synovial macrophages and fibroblasts, or primary human monocytes differentiated towards an M1 or M2 phenotype.
**Results:** OA synovium contained both M1 and M2 macrophages. In contrast to medium conditioned by a population of cells enriched in synovial fibroblasts, medium conditioned by a population of cells enriched in synovial macrophages down-regulated chondrogenic gene expression by MSCs. Additionally, CM of M1 polarised monocytes significantly decreased *COL2* and *ACAN* gene expression by MSCs, while this effect was not observed for treatment with CM of M2 polarised monocytes.
**Conclusion:** MSC chondrogenesis is inhibited by OA synovium CM through factors secreted by synovial macrophages and our findings suggest that M1 polarised subsets are key mediators of this anti-chondrogenic effect. Modulation of macrophage phenotype may serve as a beneficial strategy to maximise the potential of MSCs for efficient cartilage repair.

## Introduction

The repair of damaged articular cartilage resulting from trauma or degenerative joint disease poses a major challenge due to the limited capacity of cartilage for self renewal, attributable to its avascular nature. Mesenchymal stem cells (MSCs) are considered a promising cell type for the repair of damaged cartilage due to their chondrogenic differentiation potential [247, 248]. However, in order to achieve cartilage repair *in vivo,* chondrogenically differentiating MSCs are exposed to inflammatory mediators produced in response to injury or disease. Osteoarthritic synovial fluid and medium conditioned by synovium explants have been reported to inhibit the chondrogenic differentiation of MSCs [210, 211]. Therefore the presence of a destructive inflammatory environment, as found in osteoarthritis (OA), may impede the use of MSCs in cartilage repair strategies.

The synovial membrane is an area of high functional importance within the joint, responsible for the production of synovial fluid, which lubricates and nourishes chondrocytes. The membrane is composed of two cell types, synovial macrophages and fibroblasts. Synovial hyperplasia, increased vascular density and inflammatory cell infiltration are common features of OA [249]. Pro-inflammatory factors from the synovial fluid of injured and osteoarthritic joints stimulate cartilage degradation and inhibit matrix synthesis [36, 208, 250, 251]. Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and Oncostatin M (OSM) have been identified as some of the key players involved in synovial inflammation, with synovial macrophages considered to play a prominent role in the production of these mediators [35, 252-254].

Macrophages exhibit a high degree of plasticity, having the potential to change phenotype according to environmental cues. They can be categorised as classically activated (M1), representing pro-inflammatory phenotypes, or alternatively activated (M2) macrophages, encompassing wound healing and regulatory subsets with anti-inflammatory properties [255]. M1 macrophages are associated with high production of pro-inflammatory mediators such as TNF-α, IL-6 and Interleukin-12 (IL-12), and cell surface expression of the co-stimulatory molecule CD86, required for T cell activation and cytokine production [256, 257]. Macrophages of the M2 subtype produce immunoregulatory factors including Interleukin-10 and chemokine (C-C motif) ligand 18 (CCL18), and are associated with the surface expression of the mannose receptor (CD206) [258, 259]. The M1-associated cytokines IL-6, IL1β and TNF-α induce destructive processes in chondrocytes including down regulation of collagen type II and aggrecan synthesis, as well as up regulation of matrix metalloproteinase-9 and cyclooxygenase-2 expression [260-264].

We hypothesise that M1 polarised macrophages mediate the earlier shown anti-chondrogenic effects of OA synovium on MSC chondrogenesis [211]. Our findings highlight a role of synovial macrophages in inhibiting the chondrogenic differentiation of MSCs. Furthermore, we identify M1 polarised subsets as key mediators of this anti-chondrogenic effect.

## Materials and Methods

**Synovium conditioned medium preparation**

OA synovial tissue was isolated from 6 patients undergoing total knee replacement at Erasmus MC, University Medical Center, Rotterdam. Ethical approval was granted by the local ethical committee (number MEC 2004-322). The synovial tissue was separated from neighbouring tissues based upon morphological and physical characteristics. Isolated tissue was washed in physiological saline, and cut in to pieces varying between 1-3 $mm^2$. Synovial tissue (200 mg) was cultured in one well of a 24-well plate with 1 ml of culture medium composed of Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX TM (Invitrogen, Carlsbad, CA,USA) supplemented with 1.5 µg/ml fungizone (Gibco, Carlsbad, CA, USA), 50 µg/ml gentamycin (Gibco), 1% insulin-transferrin-selenium (BD Biosciences, Erembodegem, Belgium), 25 µg/ml ascorbic acid-2-phosphate (Sigma–Aldrich, St. Louis, MO, USA) 40 µg/ml L-Proline (Sigma–Aldrich) and 1 mM sodium pyruvate (Gibco), referred to as basic culture medium (BCM). Synovial tissue from each donor was cultured for 3 days, with synovium conditioned medium (SCM) harvested and culture medium refreshed every 24 hours. SCM was stored at -80°C, and was not subjected to more than one freeze thaw process. SCM harvested at day 3 of culture was used to assess the effect of SCM on MSC chondrogenesis.

**Isolation of synovial macrophages and fibroblasts**

OA synovial tissue was isolated from a patient undergoing total knee replacement at Erasmus MC, University Medical Centre, Rotterdam. The synovium was separated from neighbouring tendon and fat, washed in saline and cut in to 2 $mm^2$ pieces. The tissue was digested following a 45 minute incubation with 1 mg/ml collagenase B (Roche, Penzberg, Germany) at 37°C with gently agitation. Digested tissue was then strained by gently mincing the tissue through a 100 µm filter (Becton Dickinson, Franklin Lakes, NJ, USA) and washed twice with phosphate buffered saline (PBS). The resulting cell suspension was layered on top of 15 ml of Ficoll (Ficoll-Paque™ PLUS, GE Healthcare) and separated by density gradient centrifugation at 1,000 g for 15 minutes. The interphase layer was removed, washed in BCM and seeded at a density of 500,000 cells/$cm^2$ in 6-well plates. Cells were allowed to adhere at 37°C for 15 minutes for a macrophage enriched population (MEP). Following this incubation

period, un-adherent cells were removed, transferred to a new well and allowed to adhere for 120 minutes to generate a fibroblast enriched population (FEP) [265, 266]. Following this adherence incubation, cells were washed twice with PBS, and fresh medium was added. MEP and FEP conditioned medium (MEP CM and FEP CM) was harvested following 24 hours of culture.

Expression of the fibroblast marker 11-fibrau was assessed to confirm the cell specific enrichment of each fraction [267]. Isolated synoviocytes were cultured on glass slides and cells were allowed to adhere at 37°C for 60 minutes for a MEP fraction, and 12 hours for a FEP fraction. These incubation periods were previously identified as optimal for cell attachment to glass slides following pilot experiments. Cells were fixed in 70% ethanol for 1 hour and washed twice with PBS. Following blocking with 10% goat serum (Sigma-Aldrich), slides were incubated for 1 hour with monoclonal anti-human 11-fibrau (Imgen biosciences, MA, USA; 4.2 µg/ml). Slides were incubated for 30 minutes with a biotinylated anti-mouse link (Biogenex, fremount, CA, USA), diluted 1:50 with PBS/1% BSA/5% human serum (CLB), and thereafter with an alkaline phosphatise-conjugated streptavidin label (Biogenex) diluted 1:20 with PBS/1% BSA/5% human serum (CLB). Finally sections were incubated with Neu Fuchsin, $NaNO_2$, Naphtol AS-MX phosphate, Di-methylformamid with levamisole (all Sigma-Aldrich) inTRIS-HCl buffer (0.2M, pH 8.5) in the dark for 30 minutes for colour development. Sections were counterstained with haematoxylin and slides mounted utilising VectaMount (Vectorlabs, Peterborough, UK).

**Human monocyte isolation and differentiation to macrophages**

Buffy coats were obtained from 3 healthy male blood donors at the Sanquin blood bank Rotterdam, and peripheral blood monocytes were isolated by Ficoll density gradient centrifugation as described previously [268]. In brief, buffy coats were diluted (1:5) in PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich), and added to a Ficoll layer at a ratio of 2:1.The cells were separated by density gradient centrifugation at 1,000 g for 15 minutes with brake off, following which the interphase was removed, washed with 0.5% BSA/2mM EDTA in PBS, and resuspended in 100 µl of anti-CD14+ magnetic bead solution (CD14 microbeads human, Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes in the dark at 4°C. Monocytes were separated from peripheral blood mononuclear cells (PBMCs) by magnetic activated cell sorting (MACS, MACS Separation columns LS and MidiMACS™ Separator, Miltenyi Biotec).

Isolated monocytes were seeded at a cell density of 500,000 cells/$cm^2$ in 24-well plates and were cultured in xVivo 15 medium (Lonza) supplemented with 10% FCS, 50 µg/ml gentamycin and 1.5 µg/ml fungizone. Monocytes were stimulated with 10 ng/ml IFN-γ

(PeproTech, Rocky Hill, NJ, USA) & 100 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich) for 72 hours for differentiation towards an M1 phenotype or 10 ng/ml IL-4 (PeproTech) for 72 hours for differentiation towards an M2 phenotype. Cells were washed to remove stimuli and further cultured for 24 hours in BCM, after which M1 conditioned medium (M1 CM) or M2 conditioned medium (M2 CM) was harvested. CM was subjected to centrifugation at 400 g for 5 minutes, stored at -80$^{o}$C and was not subjected to more than one freeze thaw process. Differentiation of monocytes was assessed by analysing M1 and M2 characteristic gene expression and protein production profiles by differentiated cells.

**Cytokine assays**

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentration of IL-6 and CCL18 in harvested SCM, M1 CM and M2 CM as per the manufacturer's instructions (R&D systems, Minneapolis, MN, USA).

**Isolation and culture of human mesenchymal stem cells**

MSCs were isolated from heparinised bone marrow aspirates taken from the iliac crest of healthy volunteers with informed consent. All procedures for the collection of bone marrow from healthy donors have been approved by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland (reference: 2/08) and by the institutional National University of Ireland Galway Research Ethics Committee (reference: 08/May/14). MSCs were isolated by direct plating and cells were cultured for 12–15 days to deplete the non-adherent hematopoietic cell fraction. Adherent colonies were trypsinised and MSCs were expanded in expansion medium consisting of Minimum Essential Medium alpha (Invitrogen) supplemented with 10% FBS (Lonza, Verviers, Belgium), 1 ng/ml fibroblast growth factor 2 (R&D systems), 25 µg/ml ascorbic acid-2-phosphate (Sigma–Aldrich), 1.5 µg/ml fungizone (Gibco) and 50 µg/ml gentamicin (Gibco). Once the cells reached subconfluency, they were trypsinised and re-seeded at 2,300 cells/cm$^2$ in a T175 flask for further expansion. MSCs were used for chondrogenesis experiments at passage 3.

For chondrogenic differentiation MSCs were encapsulated in 1.2% low viscosity alginate powder (CP Kelco, Atlanta, GA, USA) dissolved in physiological saline at a cell density of 4 x 10$^6$ cells/ml. Alginate beads containing MSCs were formed by purging through a 23 gauge needle allowing droplets to fall into 102 mM $CaCl_2$ solution to form a gel. After rinsing, MSC beads were distributed to the wells of a 12-well plate with 13 beads per well and 100 µl medium per bead. Negative control MSC beads were cultured in incomplete chondrogenic medium (ICM) composed of BCM to which 0.1 µM dexamethasone (Sigma–Aldrich) was added. For induction of chondrogenesis, MSC beads were cultured in complete chondrogenic medium (CCM), which was composed of ICM with the addition of 10 ng/ml transforming

growth factor (TGF) β1 (R&D systems). Medium was refreshed 3 times per week. MSC alginate beads were cultured for 14 days, following which medium was supplemented with CM from all conditions. 5% SCM and 10% FEP and MEP CM were added to MSC beads, which were previously identified as optimal concentrations following pilot dose response experiments. A large variation in cell number was observed between monocytes that were differentiated with M1 or M2 stimuli. To account for this variability between M1 and M2 CM, the average cell number used to generate M1 and M2 CM across all donors, as determined by DNA quantification, was equivalent to a concentration of 20% CM. The cell number of all individual conditions was then normalised to this concentration to account for outliers (table 1). Culture was continued for a further 3 days after which mRNA was isolated, or 14 days after which samples were harvested for DNA and glycosaminoglycan (GAG) quantifications.

**Table 1. Calculation of the percentage of M1 and M2 conditioned medium (CM), based on macrophage cell number (mg DNA).**

| Donor | Condition | DNA (mg) | % CM |
|---|---|---|---|
| 1 | M1 | 1.64 | 19.95 |
| | M2 | 1.12 | 29.28 |
| 2 | M1 | 1.58 | 20.65 |
| | M2 | 0.79 | 41.43 |
| 3 | M1 | 3.00 | 10.88 |
| | M2 | 1.67 | 19.51 |

The average cell number used to generate M1 and M2 CM across all donors was equivalent to a concentration of 20% CM.

**Quantification of DNA content**

MSCs were released from alginate after 28 days of culture by incubation with 55 mM sodium citrate in 20 mM EDTA and digested overnight at 60°C with an equal volume of papain solution (250 µg/ml papain in buffer containing 50mM EDTA and 5mM L-cystein (Sigma-Aldrich) ). Macrophages were lysed with 500 µl TritonX-100 (Sigma-Aldrich) and sonicated on ice for 10 seconds. All samples were incubated with 12.5 µg/ml ribonuclease type 3 (Sigma-Aldrich) and 415 U/ml heparin solution (Leo Pharmaceuticals, Ballerup, Denmark) for 30 minutes at 37°C. A standard curve was prepared utilising deoxyribonucleic acid sodium salt from calf thymus (Sigma-Aldrich). Ethidium bromide (5 µg/ml, Gibco) was added to the samples and DNA quantified by spectrophotometric detection of ethidium bromide binding at 340 and 590 nm.

**Glycosaminoglycan measurement**

GAG content of digested MSC alginate samples was determined spectrophotometrically at 530 and 590 nm after reaction with dimethylmethylene blue (Polysciences, Valley Road,

Warrington, PA, USA); the pH was lowered to 1.75 for measurements in alginate samples using chondroitin sulphate from shark cartilage as the standard (Sigma-Aldrich)[269, 270].

**Gene expression analyses of MSCs and macrophages**

MSCs were released from alginate beads following 8 minutes incubation with ice-cold 55mM sodium citrate at 4°C with gentle agitation. The cell solution was centrifuged at 400 g or 8 minutes at 4°C and the pellet resuspended in 1 ml RNAbee (TelTest, Friendswood, TX, USA) per $1x10^6$ nuclei and stored at -80°C. Chloroform was added to all samples at a quantity of 200 µL/mL RNABee. Cell culture medium was aspirated from M1 and M2 monolayer cultures and 350 µl of buffer RLT (Qiagen, Hilden, Germany) was added to each well for direct cell lysis. M1 and M2 cell lysates were stored at -80 °C. RNA isolation was performed on MSCs and macrophage monolayers using a commercially available RNeasy Microkit (Qiagen) according to manufacturer's instructions. Nucleic acid content was determined spectrophotometrically using a NanoDrop ND1000 spectrophotometer (Isogen Life Science B.V., the Netherlands) at 260 and 280 nm. cDNA synthesis was performed using RevertAid First Strand cDNA synthesis Kit (MBI Fermentas, Germany) according to manufacturer's instructions. qRT-PCR was performed in 20 µL reactions on cDNA using the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) utilising either Taqman Universal PCR mastermix (Applied Biosystems, Foster City, CA, USA) or SybrGreen (Eurogentec, Seraing, Belgium). Gene expression of *IL-6* was assessed as a marker of M1 and *CCL18* and macrophage mannose receptor 1 (*MRC1)* were analysed as markers of M2 polarised macrophages [259, 271-273]. For MSCs, the expression of the cartilage-related genes collagen type II (*COL2*) and aggrecan (*ACAN*) were assessed. Primer pairs were used to determine the transcript levels of genes of interest using the settings previously described [79, 268, 274]. Gene expression levels were normalised to Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and relative expression was calculated using the $2^{-\Delta CT}$ method [275].

**Evaluation of macrophage subtypes in synovium**

5 µm sections were cut from paraffin embedded OA synovial tissue explants from 6 donors. Sections were stained for CD86 as a marker of M1 macrophages, or CD206 as a marker of the M2 subset [256, 259, 276]. Sections were deparaffinised, washed and heat-mediated antigen retrieval was performed. Following blocking with 10% goat serum (Sigma-Aldrich), sections were incubated for 1 hour with primary antibodies CD206 (Abcam, Cambridge UK; 2.5 mg/ml) or CD86 (Genetex, Irvine, CA, USA; 0.45 µg/ml). Sections were incubated for 30 minutes with a biotinylated anti-rabbit Ig link (Biogenex) diluted 1:50 with PBS/1%BSA, and thereafter with an alkaline phosphatase-conjugated streptavidin label (Biogenex) diluted 1:50 in PBS/1%BSA. Finally sections were incubated with Neu Fuchsin, $NaNO_2$, Naphtol

AS-MX phosphate, Di-methylformamid with levamisole (all Sigma-Aldrich) inTRIS-HCl buffer (0.2M, pH 8.5) in the dark for 30 minutes for colour development. Sections were counterstained with haematoxylin and slides mounted utilising VectaMount (Vectorlabs). Following microscopic analysis, all sections with a synovial initimal layer were evaluated. Sections were graded by two independent blinded observers according to the degree of positive staining evident in each section.

**Statistical analysis**

Normality was verified using Kolmogorov-Smirnov and Shapiro-Wilk normality tests using SPSS 15.0. Student's t-test was used to analyse paired data. For paired data that was not normally distributed, Kruskal-Wallis test was performed, followed by the Mann Whitney U test. False discovery rate was used to correct for multiple testing. For all statistical analyses, differences were considered statistically significant at $p<0.05$.

## Results

**Synovium conditioned medium negatively impacts chondrogenic differentiation of MSCs**

To confirm the negative effect of OA synovium on MSC chondrogenesis, the effect of SCM on chondrogenic gene expression in MSCs during differentiation was analysed initially.

MSCs encapsulated in alginate were chondrogenically primed for 14 days and treated for an additional 3 days with 5% SCM, prepared with synovium from 6 different donors. Gene expression levels of *COL2* were significantly lower ($p=0.002$) in MSCs following treatment with 5% SCM, compared to MSCs that were not exposed to SCM (Figure 1.A); this inhibitory effect was observed following treatment with SCM from all 6 donors.

Additionally, SCM from 4 of the 6 donors resulted in decreased gene expression levels of *ACAN* in SCM-treated beads compared to untreated MSCs ($p=0.27$) (Figure 1.A). Furthermore, SCM treatment significantly decreased ($p=0.005$) GAG content of MSC beads that were cultured for 28 days (Figure1B). Together, these findings confirm that OA synovium negatively impacts the chondrogenic differentiation of MSCs. Additionally, the levels of IL-6 and CCL18 in SCM were quantified to assess the release of M1 and M2-associated factors by synovium from each donor. All donors secreted high levels of IL-6 and lower levels of CCL18, albeit with high variability between donors (Figure 1.C).

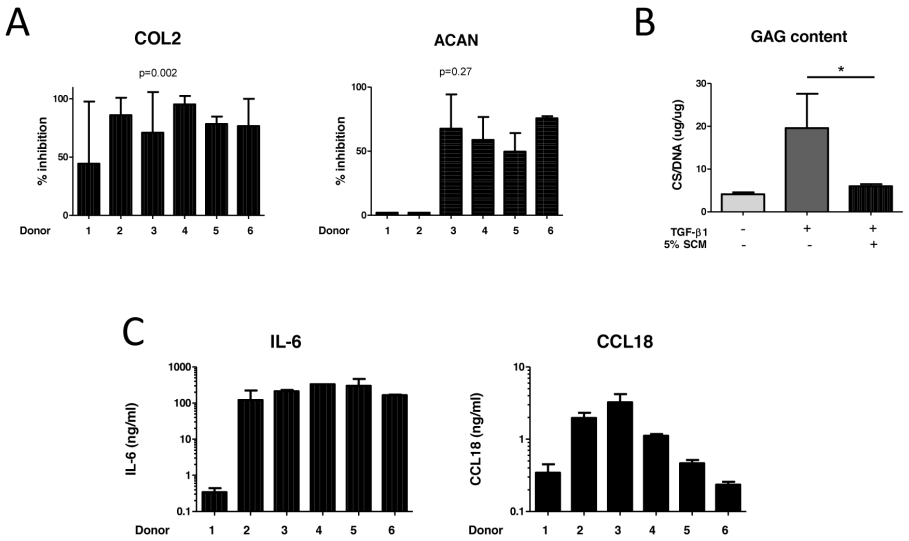



**Figure 1. Osteoarthritic SCM negatively affects chondrogenic differentiation of MSCs.**
MSC alginate beads were treated with 5% SCM generated from 6 OA synovium donors at day 14 of chondrogenesis. (A) Percentage inhibition in *COL2* and *ACAN* gene expression levels compared to positive control following 3 days of 5% SCM treatment. Values represent the mean ± SD of experimental triplicates. Statistical significance was determined by Kruskal-Wallis test followed by Mann-Whitney U test. The overall inhibitory effect on *COL2* was significant, (P=0.002). (B) GAG content of MSC alginate beads at day 28 of chondrogenesis, following 5% SCM treatment for 14 days. Values represent the mean ± SD of experimental triplicates with two synovium donors. Statistical significance was determined by Kruskal-Wallis test followed by Mann-Whitney U test. *=p<0.05. (C) ELISA analysis and quantification of IL-6 and CCL18 levels in SCM of 6 donors with OA.

## Synovial macrophages are responsible for the inhibitory effect of osteoarthritic synovium on MSCs

Having observed a detrimental effect of SCM on the chondrogenesis of MSCs, we hypothesised that synovial macrophages were responsible for this effect. Cells isolated from synovium were separated to generate fibroblast-enriched (FEP) and macrophage-enriched (MEP) fractions based on the rapid adhesion of macrophages to tissue culture plastic. Morphological differences between cells in the FEP and MEP fractions were clear; FEP contained more cells with characteristic fibroblast and MEP more with a macrophage morphology (Figure 2.A). Immunohistochemical stainings for the fibroblast membrane marker 11-Fibrau confirmed this (Figure 2.B) [267]. To compare the effect of MEP and FEP CM on the chondrogenic differentiation of MSCs, chondrogenically primed MSC alginate beads were treated with 10% MEP or FEP CM for 3 days. *COL2* gene expression levels in MSCs were reduced following treatment with MEP CM. FEP CM however, did not negatively impact *COL2* expression (Figure 2.C) supporting the hypothesis that synovial macrophages are responsible for the anti-chondrogenic effect of OA synovium.

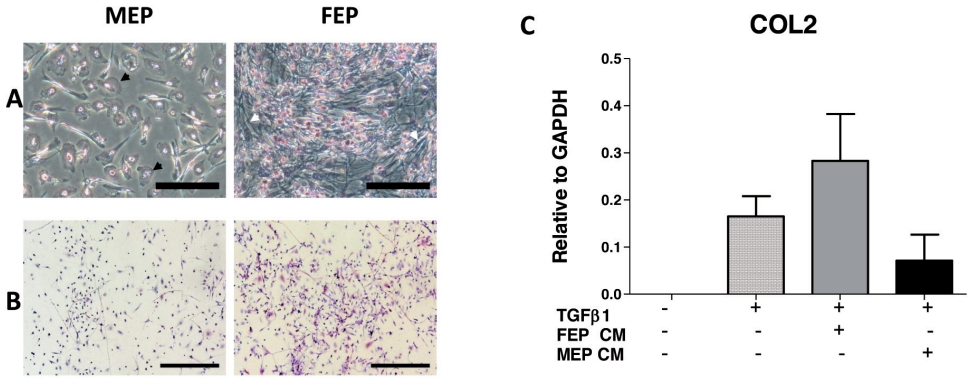



**Figure 2. Synovial macrophages inhibit collagen type 2 expression in MSCs.**
MSC alginate beads were treated with 10% CM generated from synovial macrophage enriched (MEP) or fibroblast enriched (FEP) populations. (A) Phase contrast images of Haematoxylin-eosin stained MEP and FEP. Black arrows indicate characteristic macrophage morphology, and white arrows indicate characteric fibroblast morphology of both fractions. Scale bar indicates 50 µm. (B) Staining of MEP and FEP for the fibroblast marker 11-fibrau. Scale bar indicates 150 µm. (C) Inhibition of COL2 gene expression in MSCs following stimulation with MEP CM. Values represent the mean ± SD of experimental triplicates of one synovium donor.

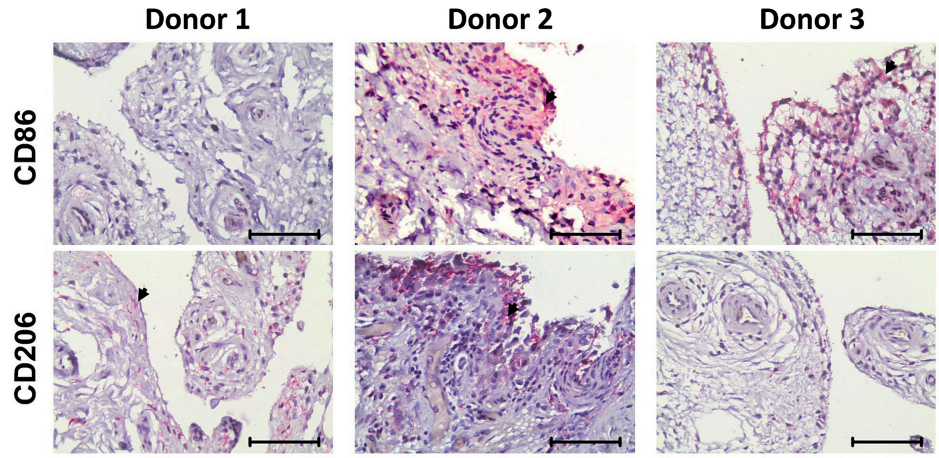



**Figure 3. M1 and M2 macrophages are present in OA synovium.**
Photomicrographs of OA synovium stained with anti-CD86 or anti-CD206. Images are representatives of paired sections from three donors. Black arrows indicate positive cells, scale bare indicates 100 µm.

### M1 and M2 macrophages are present in end-stage OA synovium

Having identified a role for synovial macrophages in the inhibition of chondrogenic gene expression in MSCs, we aimed to identify whether M1 or M2 macrophages in OA synovium could be responsible for this effect. Immunohistochemical analyses for CD86 as an M1 marker and CD206 as an M2 marker were performed on sections from OA synovium donors

(n=6). We detected the expression of both M1 and M2 markers in sections from all 6 donors, confirming the presence of both M1 and M2 polarised macrophage subsets. However, a large variation in the level of expression of each marker between donors was observed (Figure 3). We detected high expression of both markers in some donors, conversely other donors exhibited low expression of both, or high expression of one marker (Figure 3).

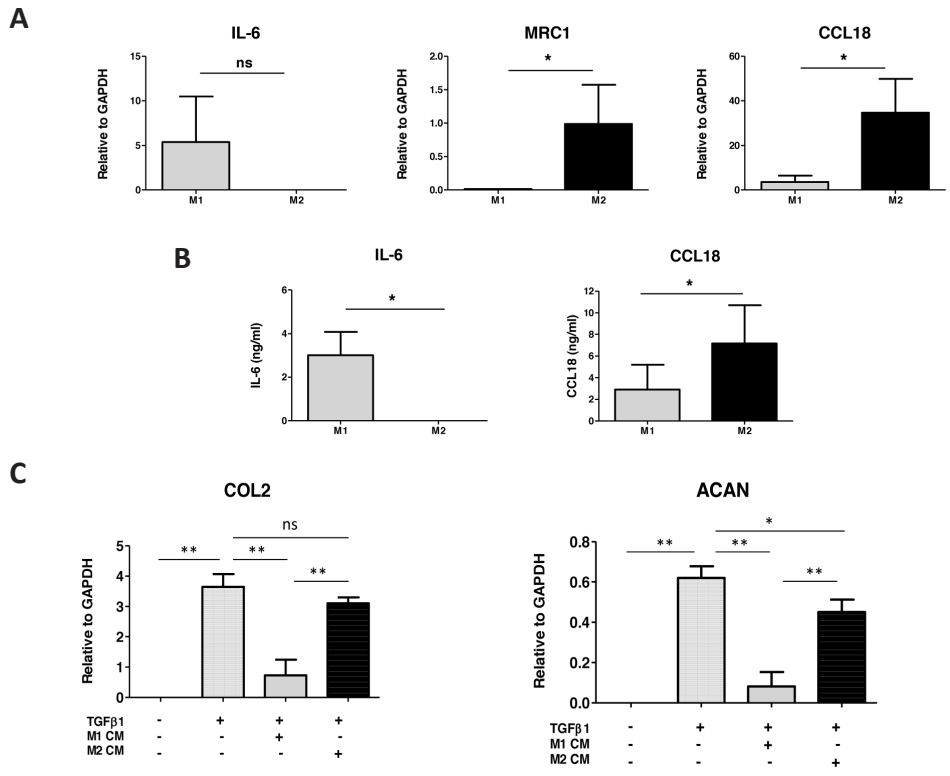



**Figure 4. M1, and not M2, macrophages inhibit chondrogenesis of MSCs.**
Primary monocytes from three donors were differentiated towards an M1 or M2 phenotype following stimulation with IFN-γ & LPS or IL-4 for 3 days. (A) Gene expression of the M1 marker *IL-6* and the M2 markers *CCL18* and *MRC1* in M1 or M2 stimulated macrophages. Values represent the mean ± SD, statistical significance was determined by a paired Student's t-test, *=p<0.05. (B) ELISA analysis and quantification of IL-6 and CCL18 levels in M1 and M2 macrophages. Values represent the mean ± SD, statistical significance was determined by a paired Student's t-test, *=p<0.05. (C) Gene expression of *COL2* and *ACAN* following stimulation of MSC beads with 20% M1 or M2 CM for 3 days. Values represent the mean ± SD of three macrophage donors. Statistical significance was determined by a Kruskal-Wallis test followed by Mann-Whitney U test, **=p<0.005, ***=p<0.001.

**M1 polarised macrophages negatively impact the chondrogenesis of MSCs**

Next, we aimed to address whether the negative effect of OA synovium on the chondrogenic differentiation of MSCs was associated with a specific macrophage subtype. Initially, we confirmed that we can differentiate primary human monocytes towards M1 and M2 subtypes. Medium conditioned by M1 differentiated monocytes contained significantly higher levels of the M1-associated cytokine IL-6 ($p=0.039$), but low levels of the M2-associated chemokine CCL18.

Conversely, medium conditioned by M2 differentiated monocytes did not contain IL-6, but CCL18 was significantly ($p=0.0278$) increased (Figure 4.A). Moreover, differentiated monocytes exhibited *IL6*, *CCL18* and *MRC1* gene expression profiles of M1 and M2 polarised cells (Figure 4.A). Treatment of chondrogenic MSC alginate beads with 20% M1 CM significantly inhibited *COL2* ($p<0.001$) and *ACAN* ($p<0.001$) gene expression levels compared to untreated chondrogenic MSCs (80% ± 14.1%/ 5 fold decrease and 86.8% ± 11.5%/ 7.6 fold change decrease, respectively) (Figure 4.B). We did not observe any significant difference in *COL2* gene expression levels between MSCs which were treated with 20% M2 CM and untreated chondrogenic MSCs ($p=0.124$).
However, treatment with 20% M2 CM did significantly reduce *ACAN* gene expression ($p=0.002$). These results suggest that M1 macrophages play a key role in inhibiting the chondrogenic differentiation of MSCs by suppressing chondrogenic gene expression.

## Discussion

The inhibitory effect of factors secreted by OA synovium on MSC chondrogenesis has been previously reported, however, the exact mechanism responsible for this effect has yet to be elucidated [210, 211]. In this study, we report that synovial macrophages isolated from OA synovium are the resident cell type responsible for the negative impact of OA synovium on MSC chondrogenesis. Furthermore, we show that M1 polarised subsets inhibit chondrogenic differentiation of MSCs. These findings may implicate M1 synovial macrophages as orchestrators of the anti-chondrogenic effect of OA synovium.

Increased inflammatory cell infiltration and pro-inflammatory mediator production by the synovial membrane are features of OA pathology, and are postulated to drive destructive events in neighbouring chondrocytes such as the inhibition of matrix synthesis [249, 251]. Synovial macrophages play a key role in the production of such inflammatory factors, however, little is known regarding the effect of macrophage subsets on MSC chondrogenesis. Considerable levels of the M1 marker IL-6 were measured in OA SCM of

all six donors. This finding is in line with reports that levels of IL-6 are significantly higher in the synovial fluid of osteoarthritis patients compared to healthy donors [277]. The presence of the M2-associated cytokine CCL18 in OA SCM from all six donors, indicates that OA synovium also contains anti-inflammatory M2 macrophages [268]. This was further confirmed by CD206 immunohistochemical staining on OA synovium sections. Remarkably, SCM from donor 1 contained less IL-6 and CCL18 than SCM from other donors, and failed to reduce *COL2* and *ACAN* gene expression in MSCs. These findings may suggest that the anti-chondrogenic effect of OA synovium is dependent on its inflammatory status. Interestingly, Gierman et al. have detected comparable expression levels of inflammatory mediators by end-stage OA and normal synovial tissue, and an effect of OA synovium on GAG release from cartilage explants was not observed in their study [278]. Beekhuizen et al. previously demonstrated that OA synovium did not affect GAG release but did inhibit GAG synthesis in cultured osteoarthritic cartilage explants [36]. We have observed an inhibitory effect of OA SCM on the expression of extracellular matrix genes *ACAN* and *COL2* in MSCs during chondrogenic differentiation, and significantly reduced GAG content compared to MSCs not exposed to SCM. In this system, as in cartilage explants, end stage OA synovium may not directly induce cartilage degradation and GAG release, but adversely affect GAG production. Furthermore, MEP CM was found to negatively impact MSC chondrogenesis, where this effect was not observed for FEP CM. Together, these findings suggest that synovial macrophages are responsible of the negative effect of OA SCM on MSC chondrogenesis The presence of IL6 and CCL18 in OA SCM already suggested the presence of both M1 and M2 macrophages in OA synovium, which was confirmed by CD86 and CD206 immunostainings on OA synovium sections. A mixed expression pattern of M1 and M2 macrophages has been previously detected in the synovium of patients with OA, and the macrophages localised to the synovial lining preferentially expressed markers of M2 polarised cells [279]. However, we detected similar levels of M1 and M2 markers localised to the lining layer in end-stage OA synovial tissue. Interestingly, end-stage OA synovium was recently reported to be refractory to additional in vitro pro-inflammatory stimuli, which may suggest a decreased plasticity of synovial macrophages in diseased synovium [278]. We have observed considerable secretion of IL-6 and CCL18 by OA synovial tissue following three days of culture, suggesting that synovial macrophages remained in an activated state during the entire culture period. However, we cannot exclude that macrophage activation and induction of inflammatory processes occurred in response to tissue injury induced by cutting the synovial tissue prior to culture, and this may be partly responsible for this observed effect.

M1 and M2 CM, prepared from human blood derived monocytes polarised into an M1 or M2 phenotype, were used as a model system to address the effect of both subsets on MSC chondrogenesis. The significant difference in protein production and differential gene

expression of IL6 and CCL18 between M1 and M2 stimulated cells validate the use of M1 and M2 CM as a model system for macrophage subtypes. Corresponding with OA SCM, M1 CM significantly decreased *COL2* and *ACAN* gene expression of MSCs, where inhibition of *COL2* was not observed following treatment with M2 CM. Although M2 CM treatment inhibited *ACAN* gene expression, this inhibitory effect was less than the effect observed following treatment with M1 CM. Interestingly, M1 polarised macrophages have been recently reported to inhibit the proliferation and viability of MSCs in vitro; this effect was not observed with cells and mediators of the M2 subset[280]. However, in our study we did not observe any difference in DNA content between M1 CM, M2 CM or untreated chondrogenic MSCs, suggesting that M1 polarised cells elicit an unfavourable effect in our system, by directly impacting chondrogenic gene expression.

We have shown that medium conditioned by synovial macrophages and M1 polarised monocytes inhibit the chondrogenic differentiation of MSCs, whereas synovial fibroblasts or M2 conditioned medium do not have this effect. These findings suggest that the negative effects of OA synovium on cartilage repair are mediated by M1 polarised macrophages. Therefore, modulation of macrophage phenotype or targeted inhibition of M1 polarisation factors, may maximise the potential of MSCs for efficient cartilage repair in a pro-inflammatory environment. In addition to their multipotent nature and ability to form cartilage, undifferentiated MSCs are known to have the potential to elicit immunomodulatory effects on host cells by paracrine signalling. MSCs have been reported to exert anti-inflammatory effects on osteoarthritic synovium [281]. Moreover, MSCs have the ability to polarise pro-inflammatory macrophages towards an M2 phenotype, characterised by decreased production of TNF-α and IL-12, high IL-10 release and surface antigen expression of CD206 [282-284]. MSCs have been reported to retain their immunomodulatory activity and capacity to inhibit lymphocyte proliferation following chondrogenic priming for one or two weeks, although this anti-proliferative effect was lower compared to adipogenically or osteogenically differentiated cells [285]. However, the ability of chondrogenically primed MSCs to modulate pro-inflammatory macrophages in inflamed synovium requires further exploration.

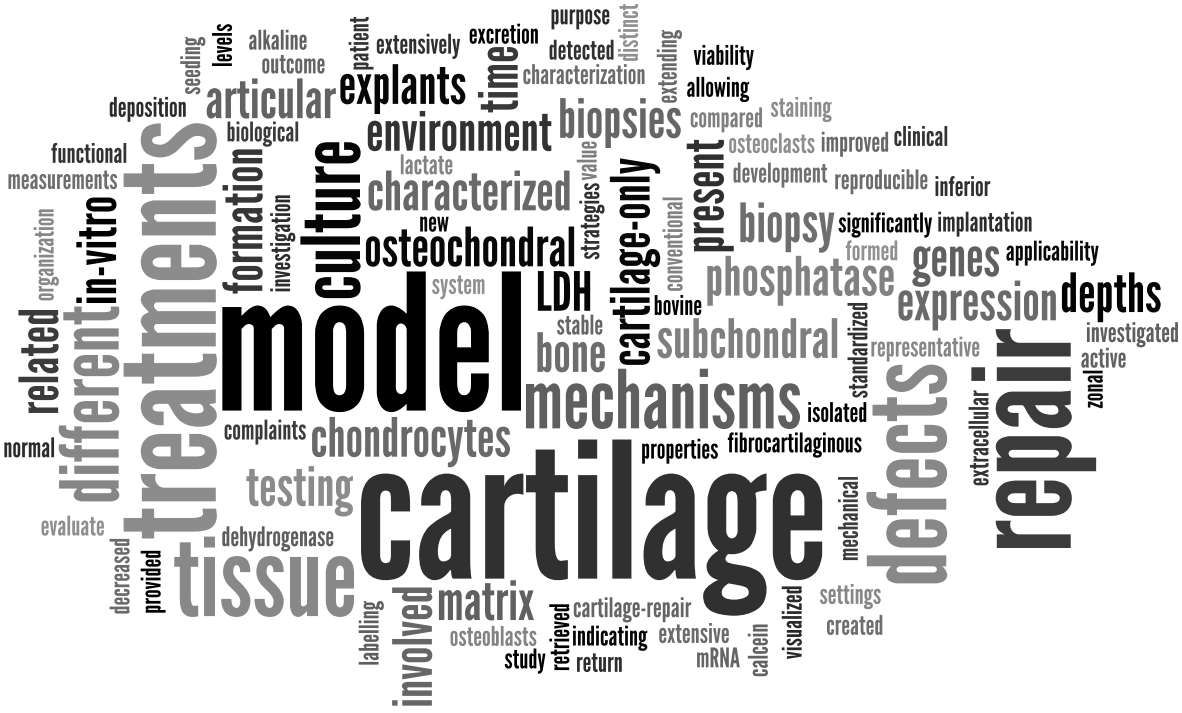
In summary, we have confirmed the inhibitory effect of OA SCM on the chondrogenic differentiation of MSCs and have identified M1 polarised macrophages as potential mediators of this anti-chondrogenic effect. We propose that modulation of synovial macrophage phenotype towards an anti-inflammatory state or attenuation of M1 mediators may be of vital importance for the development of efficient MSC-based cartilage regeneration strategies.

## Acknowledgement

The research leading to these results has received funding from the European Union's 7th Framework Programme under grant agreement nos. NMP3-SL-2010-245993 and HEALTH-2007-B-223298 and Science Foundation Ireland (grant number 09/SRC/B1794). Y.M. Bastiaansen-Jenniskens is supported by a VENI fellowship from the Dutch Technology foundation STW. This research was also facilitated by a National University of Ireland travelling studentship.

# Chapter 5

## An osteochondral culture model to study mechanisms involved in articular cartilage repair




M.L. de Vries – van Melle, E.W. Mandl, N. Kops, J.L.M. Koevoet,
J.A.N. Verhaar, G.J.V.M. van Osch

Tissue Engineering: Part C 2012. 18 (1)

## Abstract

Although several treatments for cartilage-repair have been developed and used in clinical practice the last 20 years, little is known about the mechanisms that are involved in the formation of repair tissue after these treatments. Often, these treatments result in the formation of fibrocartilaginous tissue rather than normal articular cartilage. Because the repair tissue is inferior to articular cartilage in terms of mechanical properties and zonal organization of the extracellular matrix, complaints of the patient may return. The biological and functional outcome of these treatments should thus be improved. For this purpose, an in-vitro model allowing investigation of the involved repair mechanisms can be of great value. We present the development of such a model.

We used bovine osteochondral biopsies and created a system in which cartilage defects of different depths can be studied. Firstly, our biopsy model was characterized extensively: we studied the viability by means of lactate dehydrogenase (LDH) excretion over time and we investigated expression of cartilage related genes in osteochondral biopsies and compared it with conventional cartilage-only explants. After 28 days of culture, LDH was detected at low levels and mRNA could be retrieved. The expression of cartilage related genes decreased over time. This was more evident in cartilage-only explants, indicating that the biopsy model provided a more stable environment. We also characterized the subchondral bone: osteoclasts and osteoblasts were active after 28 days of culture indicated by tartrate acid phosphatase staining and alkaline phosphatase measurements respectively and matrix deposition during culture was visualized using calcein labelling.

Secondly, the applicability of the model was further studied by testing two distinct settings: 1. implantation of chondrocytes in defects of different depths; 2. two different seeding strategies of chondrocytes. Differences were observed in terms of volume and integration of newly formed tissue in both settings, suggesting that our model can be used to model distinct conditions or even to mimic clinical treatments. After extensive characterization and testing of our model, we present a representative and reproducible in-vitro model that can be used to evaluate new cartilage repair treatments and study mechanisms in a controlled and standardized environment.

## Introduction

Due to the low intrinsic repair capacity of cartilage, untreated cartilage lesions are destined to progress into early osteoarthritis (OA). Several treatment options have been developed over the last 20 years, including autologous chondrocyte implantation (ACI). In ACI, cartilage is harvested arthroscopically from non-weight bearing areas of the joint and digested to isolate chondrocytes, which are expanded in culture. After obtaining a sufficient number, the cells are implanted back into the joint, covered by a periosteal flap [49]. Over the years, several variations of this treatment have been investigated and introduced, including the use of biomaterials to cover the defect or to seed the chondrocytes on before implantation [50, 51].

Other examples of treatments for cartilage lesions are marrow stimulation techniques, such as the microfracture procedure popularized by Steadman. In this single-surgery procedure, conical holes are punched through the subchondral plate, allowing a bone marrow clot to fill the defect [55, 140]. The formation of repair tissue is addressed to mesenchymal stem cell (MSC) differentiation and possibly migration of cells from the surroundings into the defect [56].

For both ACI-based treatments and the microfracture procedure, the formed repair tissue is often of a fibrocartilaginous nature, which does not have the desired zonal organization of the extracellular matrix (ECM) nor mechanical properties similar to those of the native articular cartilage. Also, return of complaints is often reported from 24 months post treatment [59, 157, 159].

Current articular cartilage-repair strategies require improvement in terms of biological and functional outcome, which would either be achieved by improving current treatments or developing new cartilage-repair strategies [286, 287]. To achieve this, insight is required in the working mechanisms behind the existing treatments. Knowledge on several topics involved in the repair process could provide these insights: identification of the optimal cell source, integration of formed repair tissue into the native tissue and the role of cells present in the native tissue. For example, removal of the calcified cartilage without damaging the subchondral plate is reported as critical step in the microfracture procedure, while the exact mechanistic reason is for this step being critical is unknown [135]. Altogether, the basic repair mechanisms involved in current treatments are still largely unknown [59, 99, 143]. To improve biological and functional outcome or even to prevent the development and progression of early OA, these mechanisms need to be elucidated. To study these mechanisms, we developed a model that can provide more complexity than cell culture, and less complexity and more standardization than animal models.

In this study, we present the development and testing of an osteochondral culture model. We first evaluated cartilage and bone metabolism over time in culture, particularly focussing on cartilage metabolism in osteochondral biopsies compared to the cartilage-only explant system that is regularly used. Then we demonstrated that defect depth can be controlled reproducibly and finally, we tested the applicability of our model to study cell-based cartilage repair strategies in vitro by seeding cells in created defects. We conclude that this multifunctional osteochondral culture model can be used to evaluate new methods to repair cartilage and to study the mechanisms involved in articular cartilage repair.

## Materials & methods

### Osteochondral biopsy obtainment and culture

The four proximal sesamoïd bones of fresh MCP joints from 3 to 8 months old calfs were used to create osteochondral biopsies using a Ø 8mm diamond coated trephine drill (Synthes, Oberdorf, Switzerland). Per MCP joint, four biopsies were obtained. Subsequently, biopsies were washed in Dulbecco's Modified Eagle's Medium-High Glucose (DMEM-HG, 4.5g/L glucose, Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Lonza, Verviers, Belgium), 50µg/mL gentamicine (Gibco) and 1.5µg/mL fungizone (Gibco), from now on referred to as "culture medium". Biopsies were cut to about 5mm in length and kept overnight in culture medium to verify sterility. Biopsies were placed in a 2% low-gelling agarose (gelling temperature 37 – 39°C, Eurogentec, Liege, Belgium) in physiological saline solution, such that the subchondral bone was surrounded by agarose and the cartilage was above the agarose surface to prevent outgrowth of cells from the subchondral bone. For comparison, cartilage explants without bone were obtained for from fresh bovine MCP joints using a Ø 6mm dermal biopsy punch (Stiefel Laboratories, Sligo, Ireland) and scalpel. Unless stated otherwise, all osteochondral biopsies and cartilage explants were cultured for 28 days in culture medium at 37°C and 5% $CO_2$. Medium was refreshed three times per week.

### Characterization of the model: viability and activity

Osteochondral biopsy viability and bone-matrix activity were studied. Lactate dehydrogenase (LDH), which is excreted by dying cells, was measured in medium at every refreshment as non-destructive method to indicate cell death during culture. ALP activity was measured in medium at every refreshment as a measure for bone formation activity. Medium was divided in two portions, one of which was subjected to LDH assay immediately; the other was stored at -80°C for alkaline phosphatase (ALP) assay.

The LDH assay (Roche Diagnostics) was performed according to the manufacturer's instructions. In short, 100µl used culture medium was incubated for 30 minutes in the dark with 100µl reagents consisting of 98% dye solution and 2% catalyst. Culture medium that was not used for culture was used for blank measurement. Absorbance was measured at 490nm using a Wallac 1420 victor2 spectrophotometer (Perkin-Elmer, Wellesley, MA, USA).

ALP activity was measured in medium by determining the release of paranitrophenol as described previously [288]. In short, medium was incubated for 10 minutes at 37°C with 20mM paranitrophenylphosphate in 1M diethanolamine buffer supplemented with 1mM $MgCl_2$ at pH 9.8. Subsequently, the reaction was stopped by adding 0.06M NaOH. Adsorption was measured at 405nm using a Wallac 1420 victor2 spectrophotometer (Perkin-Elmer, Wellesley, MA, USA).

To study the effect of culturing on bone and cartilage in our osteochondral model, osteochondral biopsies and cartilage explants were harvested directly after preparation, after 7 and 28 days of culture for RNA isolation and qPCR. Cartilage explants were snap-frozen in liquid nitrogen. For the osteochondral biopsies, cartilage and bone were separated using a scalpel and separately snap-frozen in liquid nitrogen. All samples were stored at -80° until further processing. To evaluate tissue morphology and to assess osteoclast activity, osteochondral biopsies were fixed in 4% formalin for at least 24 hours and subsequently processed for histology as described in the histology section.

**Characterization of the model: matrix deposition**

To study bone-matrix deposition, biopsies with osteochondral defects were labeled overnight after 14 days of culture to verify matrix deposition during culture using 2mg/mL calcein (Sigma-Aldrich, St. Louis, MO, USA) suspended in medium supplemented with 2mg/mL sodium carbonate. To allow matrix deposition, culture medium was supplemented with 0.1µM dexamethasone (Sigma-Aldrich) and 10mM β-glycerol phosphate (βGP, Sigma-Aldrich). Calcein-labeled biopsies were harvested for fluorescence microscopy and histology directly after overnight labeling and 14 days after labeling. Samples were fixed in 4% formalin for at least 24 hours and subsequently processed for MMA embedding as decribed in the histology section.

**Testing the applicability of the model for studying cell-based treatments**

To validate the osteochondral culture model, two settings were tested: 1. seeding of chondrocytes in defects of three different depths: shallow cartilage defects, full-thickness cartilage defects and osteochondral defects; 2. Applying two different seeding strategies in osteochondral defects: direct seeding of chondrocytes versus chondrocytes seeded on membranes prior to placement in the defect.

To evaluate the feasibility of creating a model to study the effect of different depths of defects in the osteochondral biopsies, we used a Ø 6mm dermal biopsy punch (Stiefel Laboratories) and scalpel to create one defect per biopsy of chondral (shallow, figure 1A), subchondral (intermediate, figure 1B) or osteochondral (deep, figure 1C) nature. Defect depth was controlled by the extent of removal of tissue. To obtain chondral defects, a thin layer of cartilage was removed carefully. For subchondral defects, cartilage was removed down to the calcified cartilage layer, which remained intact. To obtain osteochondral defects, the calcified cartilage layer was removed and parts of the subchondral bone were removed by scraping the surface using a scalpel. To investigate the effect of defects of different depths on repair by seeded cells, bovine chondrocytes were isolated from cartilage explants from MCP joints. Explants were incubated for 90 minutes in physiological saline supplemented with 0.2% protease (Sigma-Aldrich). Subsequently, explants were digested overnight in culture medium supplemented with 0.15% collagenase B (Roche Diagnostics, Mannheim, Germany). The cell suspension was filtered and washed in physiological saline. Cell numbers were determined using a haemocytometer. P0 bovine chondrocytes were seeded into the defects of different depths at a concentration of $4*10^6$ cells per defect in 100µl culture medium, incubated for 1 hour at 37°C, centrifuged at 1300rpm for 30 seconds and subsequently covered with a Ø 5mm ChondroGide membrane (Geistlich Biomaterials, Wolhusen, Switzerland) and sealed with TissueCol fibrin glue (Baxter, Utrecht, the Netherlands) (figure 1E.1). Biopsies were harvested for histology after 28 days of culture.

To test a second setting, defects of osteochondral depth were created as described. Bovine chondrocytes were either seeded into the defects as described above or seeded onto a Ø 5mm ChondroGide membrane at a density of $4*10^6$ cells per membrane, pre cultured for three days and subsequently placed in osteochondral defects and sealed with TissueCol fibrin glue (figure 1E.2). Biopsies were harvested for histology after 28 days of culture.

**Histology**

Upon harvesting for histology, biopsies were fixed in 4% formalin for at least 24 hours. Biopsies were either embedded in methylmethacrylate (MMA), or decalcified in 10% formic acid in PBS and embedded in paraffin, and sectioned in 5µm sections. For staining of MMA sections, slides were deplastified by incubation in a 1:1 mixture of xylene and chloroform for 60 minutes and subsequently hydrated. For staining of paraffin sections, slides were deparaffinized using xylene and subsequently hydrated.

Haematoxylin and eosin (H&E) staining was performed to study general cell morphology and safranin-O staining was performed to visualize glycosaminoglycans in the ECM. Stainings were performed on MMA or paraffin sections. For safranin-O staining, slides were first stained with

0.1% light green in distilled water for 5 minutes, subsequently washed in 1% acetic acid and stained with 0.1% safranin-O (Fluka, Buchs, Switzerland).

MMA sections were stained using tartrate resistant acid phosphatase (TRAP) to verify osteoclast activity as described previously [289, 290]. In short, slides were incubated for 20 minutes in 0.2M acetate buffer containing 50mM L(+)tartaric acid (Acros Organics), whose pH was set at 5. Slides were then incubated in 0.2M acetate buffer containing 0.5mg/mL naphtol AS-MX phosphate (Sigma-Aldrich) and 1.1mg/mL fast red TR salt (Sigma-Aldrich) for 1 to 4 hours, during which the progress of the staining was monitored. Slides were counterstained with haematoxylin.

**Quantification of repair tissue**

In the experiments performed to test the applicability of the osteochondral culture model for studying cartilage repair mechanisms in vitro, repair tissue formed by seeded chondrocytes was defined as safranin-O positive stained tissue other than the native cartilage, which was visually discriminated. We used ImageJ software to score both the extent of newly formed repair tissue attached to or directly lying alongside the bottom of the created cartilage defects and the volume of the newly formed repair tissue. Volume of repair tissue was defined as the amount of $mm^2$ safranin-O positive repair tissue; integration was defined as percentage of the bottom of the defect covered with safranin-O positive repair tissue. Three sections were scored per biopsy.

**RNA isolation and qPCR**

Deep-frozen samples were pulverized at 1,000rpm using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany). Pulverized samples were rapidly covered with 1.8mL/g RNABee (TEL-TEST, Friendswood, TX, USA). Chloroform was added at a quantity of 200µl per mL RNABee. Further RNA isolation was performed using the RNeasy Microkit (Qiagen, Hilden, Germany) according to manufacturer's instructions, including on-column DNAse treatment. RNA concentration and quality was measured using a spectrophotometer (NanoDrop ND1000 UV-VIS, Isogen Life Science B.V., the Netherlands). cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to manufacturer's instructions. qPCR was performed in 20µL reactions on a ABPrism 7000 system (Applied Biosystems, Foster City, CA, USA) using either Taqman Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec). Expression of collagen type 2 (Fw: CCGGTATGTTTCGTGCAGCCATCCT; Rv: GGCAATAGCAGGTTCACGTACA [291]), collagen type 1 (Fw:CAGCCGCTTCACCTACAGC; Rv:TTTTGTATTCAATCACTGTCTTGCC; probe:Fam-CGGTGTGACTCGTGCAGCCATC-Tamra), aggrecan (Fw:AATTACCAGCTACCCTTCACCTGTA; Rv:TCCGAAGATTCTGGCATGCT [291]), collagen type X

(Fw:ACTTCTCTTACCACATACACG; Rv:CCAGGTAGCCCTTGATGTACT), matrix metalloproteinase 13 (MMP13, Fw:TCTTGTTGCTGCCCATGAGT; Rv:GGCTTTTGCCAGTGTAGGTGTA [291]) and of the aggrecanases ADAMTS4 (Fw:GAAGCAATGCACTGGTCTGA; Rv:CCGAAGCCATTGTCTAGGAA) and ADAMTS5 (Fw:GCAGTATGACAAATGTGGCG; Rv:TTTATGTGAGTCGCCCCTTC) was assessed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Fw:GTCAACGGATTTGGTCGTATTGGG; Rv:TGCCATGGGTGGAATCATATTGG; probe:Fam-TGGCGCCCCAACCAGCC-Tamra [292]) and β-Actin (Fw:TTACAACGAGCTGCGTGTGG; Rv:TGGCAGGAGTGTTGAACGTC;) were tested as reference genes. Relative gene expression was calculated using the $2^{-\Delta CT}$ method [293].

**Statistics**

The results of histological scoring and of qPCR data were statistically tested using the Student T-test. Differences were considered statistically significant for $p<0.05$.

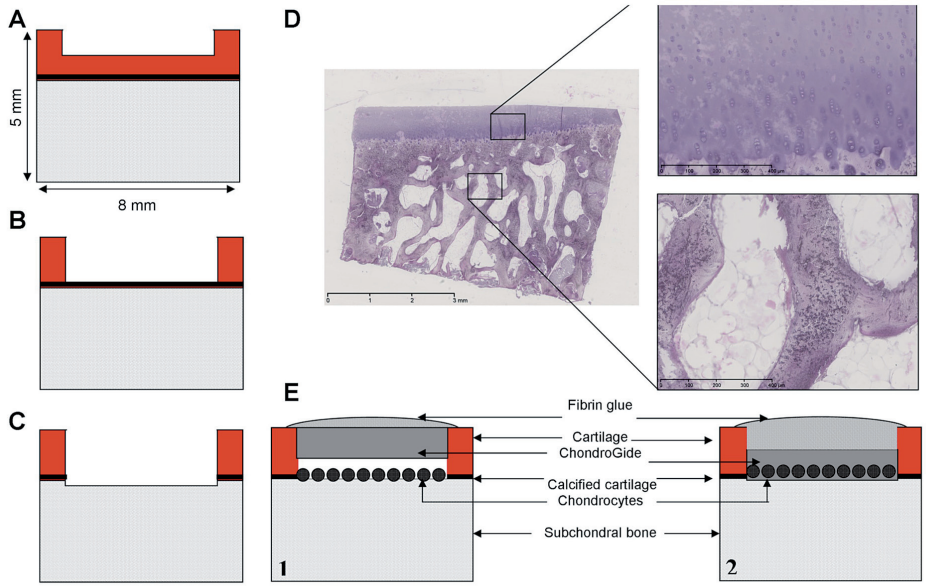



**Figure 1.**
A. Schematic representation of the biopsy model with a chondral defect; B. With a subchondral defect; C. With an osteochondral defect; D. H&E stained osteochondral biopsy after 28 days of culture; scale bar indicates 3mm. Insets: detail images of cartilage and bone respectively, scale bars indicate 400um; E. Schematic representation of validation settings of the osteochondral biopsy model: 1.osteochondral defect seeded with bovine chondrocytes covered with a ChondroGide membrane and fibrin glue; 2. osteochondral defect with a ChondroGide membrane, pre-seeded with bovine chondrocytes covered with fibrin glue.

## Results

### Characterization of the model

To verify the viability of the cells present in the osteochondral biopsies, LDH, which is secreted by dying cells, was measured in the culture medium three times per week. LDH secretion of osteochondral biopsies was compared to that of cartilage-only explants (figure 2.A). As was expected, during the first days in culture relatively high levels of LDH were measured caused by explantation and drilling of the tissue. From day 7 on, the LDH levels were much lower and became relatively stable for the remaining days of culture. In the cartilage-only explants, overall lower amounts of LDH were observed indicating less dying cells, which can be explained by the fact that less cells were present in the cartilage-only explants than in the osteochondral biopsies.

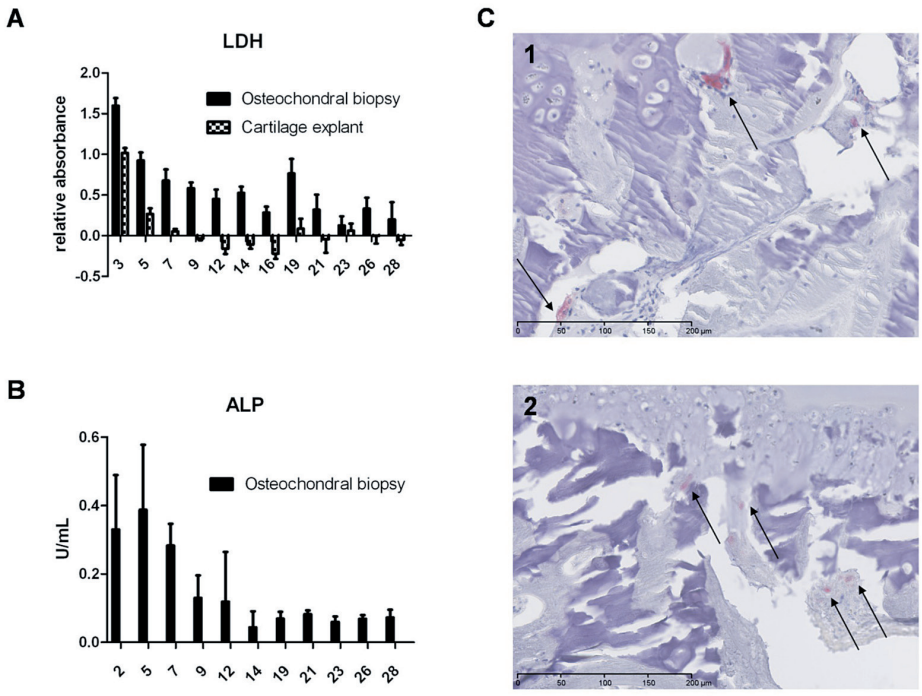



**Figure 2.**
A. LDH secretion over time detected in medium of osteochondral biopsies and cartilage-only explants; B. ALP activity in time detected in medium of osteochondral biopsies; C. TRAP stained osteochondral biopsy sections before culture (C1) and after 28 days of culture (C2), arrows indicate TRAP positive cells, scale bars indicate 200um;

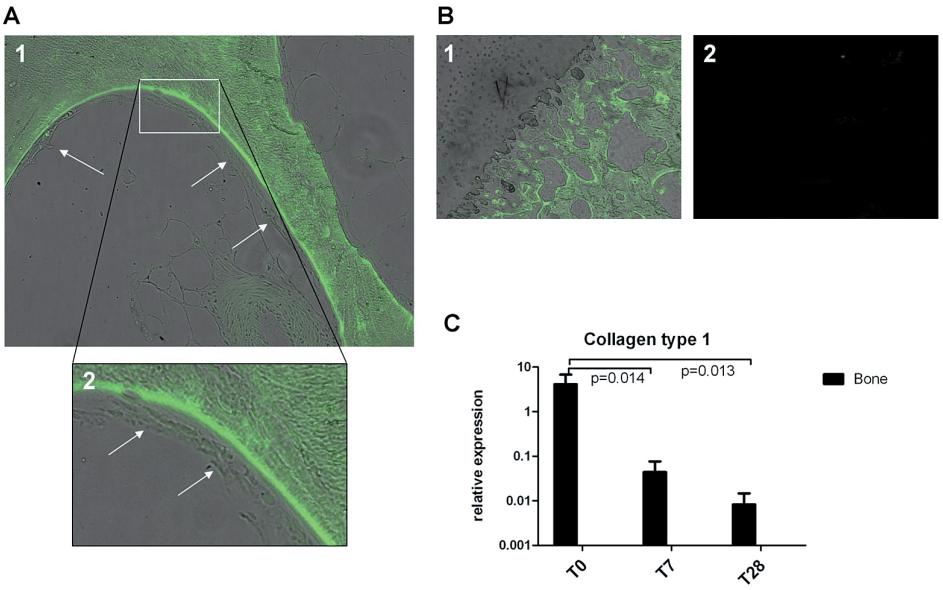



**Figure 3.**
A. Osteochondral biopsies labeled with calcein after 14 days of culture and harvested after 28 days of culture. Arrows indicate matrix and/or cells deposited during culture after calcein labeling; magnification 20x; B. Calcein labeling was specific for bone matrix; 1.cartilage was not labeled; 2. no autofluorescence of unlabeled osteochondral biopsies was detected; magnification 20x; C. Gene expression of collagen type 1 in bone of the osteochondral biopsies after 0, 7 and 28 days of culture (n=6).

TRAP stained active osteoclasts in both biopsies harvested directly after isolation and biopsies cultured for 28 days, which indicated that the bone was still actively remodeling after 28 days of culture (figure 2.C). As an indication of osteoblast activity, ALP activity was initially high, decreasing to a relatively stable level after one week of culture (figure 2.B). Calcein labeling showed that bone matrix was deposited during culture, visualized as green staining covered by matrix deposited after labeling, which is another indication for active remodeling of the bone during culture (figure 3.A and B).

RNA was isolated from the separated bone and cartilage of the osteochondral biopsies and cartilage-only explants. On the cartilage, qPCR for collagen type 2, aggrecan, collagen type X, MMP13 and the aggrecanases ADAMTS4 and ADAMTS5 was performed (figure 4, n=6). On the bone, qPCR for collagen type 1 was performed (figure 3.C). GAPDH and β-Actin were tested as housekeeper genes. Both housekeepers gave comparable results (data not shown), thus we decided to perform all calculations using the GAPDH data. The expression of collagen type 2 and aggrecan decreased over time in biopsy cartilage and for cartilage-only explants, which was more evident for the cartilage-only explants (figure 4.A). The gene expression of the aggrecanases ADAMTS4 and ADAMTS5 remained relatively stable

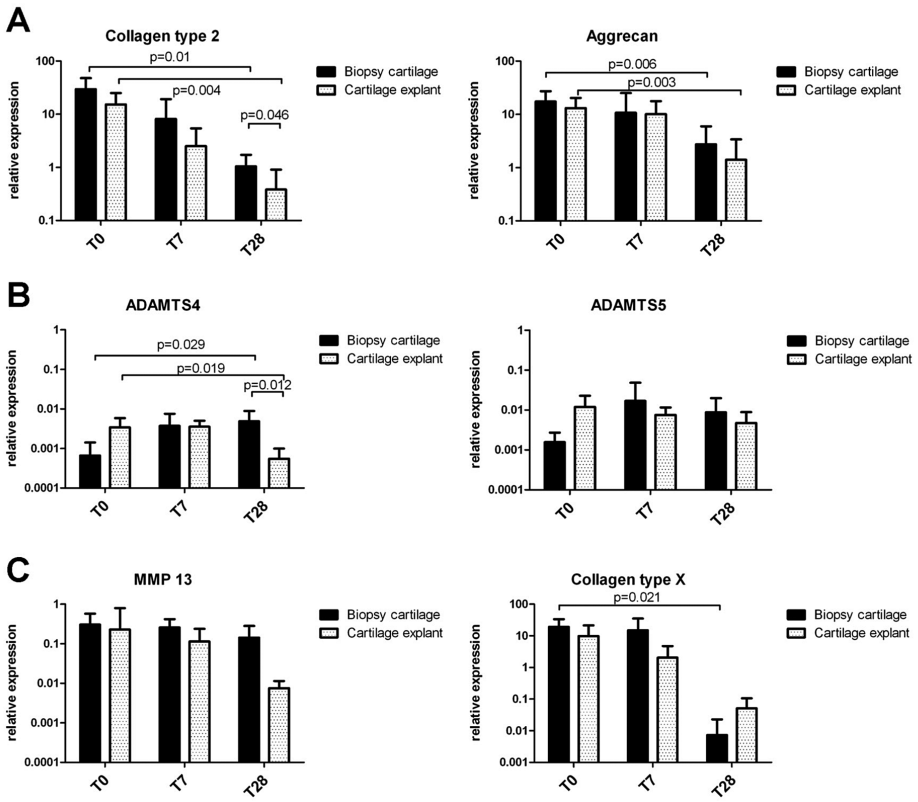



**Figure 4.**
Mean relative gene expressions (n=6) with standard deviations of osteochondral biopsy-cartilage and cartilage-only explants during 28 days of culture. A. The cartilage related genes collagen type 2 and aggrecan; B. The aggrecanases ADAMTS4 and ADAMTS5; C. The hypertrophy related genes MMP 13 and collagen type X;Gene expressions are calculated relative to GAPDH.

over time in both biopsy cartilage and explants, indicating that our culture conditions did not specifically favored aggrecanase induced cartilage-ECM deterioration (figure 4.B). The expression of the hypertrophic markers MMP13 and collagen type X decreased over time in cartilage from the osteochondral biopsies as well as in cartilage-only explants (figure 4.C). No significant differences were observed in MMP13 en collagen type X gene expression between osteochondral biopsy cartilage and conventional cartilage explants. This indicates that our culture system did not stimulate hypertrophy. Collagen type 1 expression in osteochondral biopsy bone decreased over time (figure 3.C).

**Testing the applicability of the model for studying cell-based treatments**

General morphology of osteochondral biopsies was visualized using H&E staining (figure 1.D). Defects of three different depths were created successfully and reproducibly (figure 5.A,

n=9). Production of glycosaminoglycans, visualized using safranin-O staining, was observed after direct seeding of chondrocytes in defects of different depths and in osteochondral defects with direct seeded chondrocytes and membrane seeded chondrocytes. Differences were detected between chondrocyte seeded defects of different depths in terms of quality and quantity of safranin-O positive repair tissue (figure 5). Significantly more repair tissue was found in osteochondral defects compared to chondral defects (figure 5.C, n=9, p=0.01). In terms of integration of newly formed repair tissue, no significant differences were observed between the three different depths of defects (figure 5.C). Differences were mainly observed in non-quantified terms: the repair tissue is more coherent and more positive for safranin-O for deeper defects (figure 5.B). Direct seeding of chondrocytes in an osteochondral defect resulted in significantly larger volume of safranin-O positive tissue as well as a better integration to the bottom of the defect than chondrocytes that were seeded on the membrane before applying in the defect (figure 5.E).

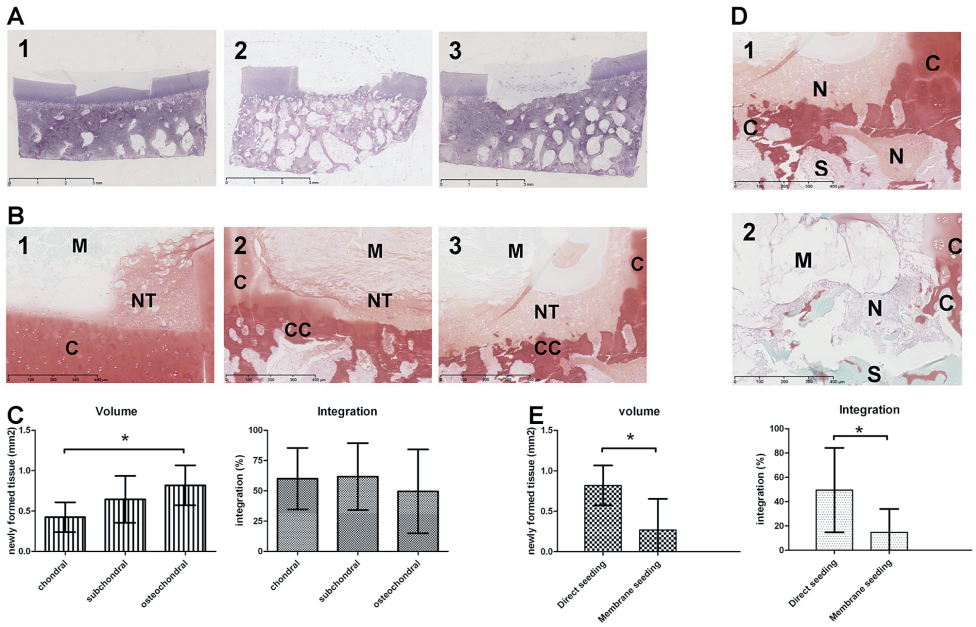



**Figure 5. The two validation settings of the osteochondral culture model.**
A. Validation setting 1: H&E stained osteochondral biopsies with defects of chondral (1), subchondral (2) and osteochondral (3) depth; scale bars indicate 3mm; B. Validation setting 1: Safranin O stained detail images of tissue formed by direct seeded chondrocytes in defects of three different depths; scale bars indicate 400µm; C. Validation setting 1: Scoring of volume and integration of newly formed tissue on safranin O stained sections; mean with standard deviation (n=9); p=0.01; D. Validation setting 2: Direct seeding (1) versus membrane seeding (2) of bovine chondrocytes in osteochondral defects; scale bars indicate 400µm; E. Validation setting 2: Scoring of volume (p=0.01) and integration (p=0.03) of newly formed tissue on safranin O stained sections; mean with standard deviation (n=9); newly formed tissue = NT, native cartilage = C, calcified cartilage remains = CC, chondrogide membrane = M;

## Discussion

Since multiple mechanisms involved in cartilage-repair are still unknown, we believe that a well-characterized in vitro model is necessary to elucidate the factors that determine cartilage repair. We have shown that our osteochondral culture model was viable during 28 days of culture based on three grounds: 1. LDH secretion by the osteochondral biopsies was high shortly after creation of the biopsies but decreased to steady low levels after 7 days; 2. mRNA was retrievable after 28 days in culture and 3. the subchondral bone was characterized: TRAP positive cells were present after 28 days in culture, calcein labeling showed matrix deposition and the ALP assay indicated that the bone was actively remodeling during the culture period. Furthermore we have demonstrated that different treatments can be evaluated using our model by quantification of the amount of newly formed tissue filling the defect and the integration of this tissue with the defect environment.

The first described osteochondral models were not intended for in vitro use and were directly implanted in vivo without in vitro characterization [294, 295]. We have shown that it was possible to create defects of different depths. Earlier described models either use one type of defect [296] or do not describe the depth of their defects [297] or do not characterize their model in vitro [294, 295]. Control over defect depth is of critical importance in modeling and studying cartilage defects and involved repair mechanisms, reflected by the differences we have found in terms of quantity and integration of newly formed repair tissue in defects of different depths. To our knowledge, none of the osteochondral models so far described in literature involved evaluation of the subchondral bone during or after culture. In our model, this was studied by means of TRAP staining, calcein labeling and ALP activity measurements, which indicated that the subchondral bone remained active during culture.

It is evident that the osteochondral biopsies were not equal after 28 days of culture to the situation before culturing. This is for example reflected by the fact that collagen type 2 and aggrecan gene expression in osteochondral biopsy cartilage were lower when cultured for 28 days than it was in native articular cartilage. However, collagen type 2 expression was significantly higher in osteochondral biopsy cartilage than in cartilage-only explants after 28 days of culture indicating that the osteochondral biopsy provides a more representative culture system. Also, the hypertrophic markers MMP13 and collagen type X expression decreases over time for both osteochondral biopsy cartilage and cartilage-only explants, indicating that our culture conditions did not induce hypertrophy of the cultured tissue.

Although our model still differs from the native situation, there are multiple mechanistic factors that make our osteochondral biopsy model more physiologically relevant than

the conventional cartilage-only explants due to the presence of the subchondral bone. Subchondral bone and cartilage are closely related anatomically but also influence each other in disease processes [26, 298-302]. Subchondral bone is also identified as a critical success factor of the microfracture procedure [134, 135]. These previously reported findings indicate that subchondral bone plays a critical role in repair tissue formation after cartilage-repair treatments. The results of gene expression analysis of biopsy-cartilage and cartilage-only explants supported this: the presence of subchondral bone resulted in a different expression pattern of cartilage related genes, supporting that our osteochondral biopsy model is more representative to the native situation than cartilage-only explants.

In vivo cartilage is dependent on diffusion of nutrients and oxygen. Chondrocytes residing in healthy articular cartilage are exposed to a gradient of oxygen and nutrient supply [303]. In cartilage of the osteochondral biopsies the exposure to oxygen and nutrients of the deep cartilage zone was likely lower than of that of the deep zone in cartilage-only explants. In the osteochondral biopsies nutrients and oxygen need to diffuse through the superficial and middle zone of the cartilage or through the subchondral bone and calcified cartilage to reach the deep zone cartilage cells. The deep zone of cartilage-only explants is directly exposed to nutrients and oxygen in the culture medium.This makes our osteochondral culture model more similar to the in vivo situation than the conventional cartilage-only explant cultures.

Another advantage of the osteochondral model can be that the cartilage is more intact than it is in cartilage-only explants. Cartilage that is explanted or otherwise damaged, shows chondrocyte death in wound edges [191, 304, 305]. Our osteochondral biopsy model contains not only damage in terms of the created cartilage defects, but also the outer edges of the biopsy itself should be considered as wound area due to the drilling procedure to create the biopsies out of the MCP joints. In the cartilage-only explants, the cartilage wound surface was even relatively larger, since these explants were cut off the subchondral bone, which makes the bottom of the explants a wound surface as well.

A difference between the model and the physiological situation is the absence of mechanical loading during culture. This may be an explanation for the strong decrease observed in the expression of collagen type 1 in the osteochondral biopsy bone. It is well-known that bone is a continuously remodeling tissue that has the capability to respond to the mechanical circumstances it is experiencing: bone needs mechanical stimulation to prevent demineralization and to maintain its mechanical strength [306]. The extension of our model with mechanical loading would provide the opportunity to model healing or regeneration processes of articular cartilage in a more joint-like environment, especially when various mechanical loading patterns can be applied. Furthermore, the presence of synovial fluid

in the culture system would also be possible to even better mimic the joint environment. These adaptations to the model can be made in future.

Possible applications of our model are numerous. We have demonstrated two possible approaches: creation of defects of different depths to simulate cartilage damage and two different seeding strategies. Differences were observed in terms of volume and integration of newly formed tissue, indicating that our model can be used to study various cartilage repair mechanisms and features in vitro. We observed that the placement of the membrane in the defect was a critical factor for the success of both approaches: if the membrane did not remain in place, poor attachment of seeded cells onto the defect was found, which corresponds with clinical findings: ACI or ACI-derived treatments fail when the periosteal flap or membrane does not stay in place [307]. Cartilage repair treatments could be simulated in vitro. ACI and its derivatives could be modeled by applying different cell seeding strategies and membranes to cover the defect or to seed cells on. One could also imagine studying other approaches such as the use of different cell sources and biomaterials for cartilage-repair purposes. For example MSCs, which may provide an attractive cell source for cartilage-regeneration strategies based on their rapid expansion in vitro and their chondrogenic differentiation potential [72, 286]. Our model could be used for example to study the effects of expansion conditions, exposure to growth factors, oxygen tension or genetic modification of cells by over expression or knock-down of factors upon or before use on cartilage formation capacity in our model. Another possible application of our model could be the screening and comparison of various biomaterials for example for their capacity to fill cartilage defects, to serve as a scaffold for implantation of cells, to study the effect of incorporation of bioactive factors or to study integration of materials or cells into the defect environment.

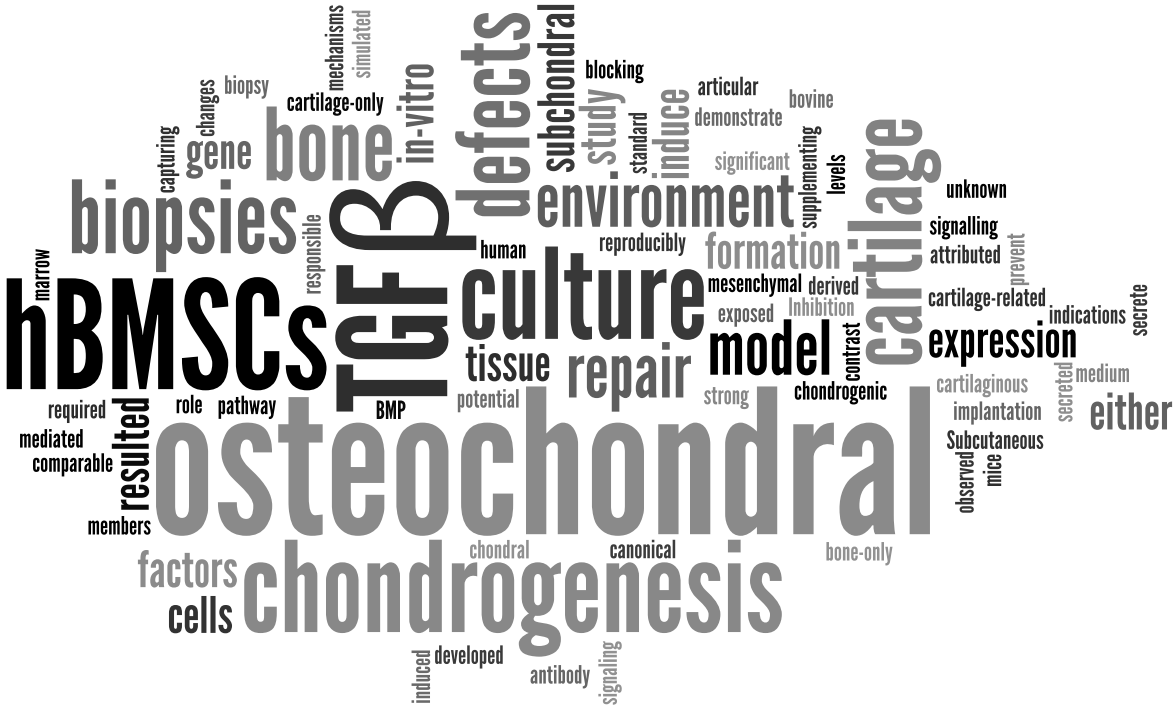
Overall, we have developed and validated a reproducible model that can be used in multiple experiments to study many cartilage repair mechanisms in vitro both for current treatments for cartilage defects as well as in development of new repair strategies.

## Acknowledgement

The research leading to these results has received funding from the Dutch Arthritis Association and the European Union's 7$^{th}$ Framework Programme under grant agreement no. NMP3-SL-2010-245993.

# Chapter 6

## Chondrogenesis of mesenchymal stem cells in an osteochondral environment is mediated by the subchondral bone




M.L. de Vries – van Melle, R. Narcisi, N. Kops, J.L.M. Koevoet, P.K. Bos, J.M. Murphy, J.A.N. Verhaar, P.M. van der Kraan, G.J.V.M. van Osch

Tissue Engineering: Part A 2014. 20(1)

## Abstract

In articular cartilage repair, cells that will be responsible for the formation of repair tissue are often exposed to an osteochondral environment. To study cartilage repair mechanisms in-vitro, we have recently developed a bovine osteochondral biopsy culture model in which cartilage defects can be simulated reproducibly. Using this model, we now aimed to study the chondrogenic potential of human bone marrow derived mesenchymal stem cells (hBMSCs) in an osteochondral environment. In contrast to standard in-vitro chondrogenesis, it was found that supplementing TGFβ to culture medium was not required to induce chondrogenesis of hBMSCs in an osteochondral environment. hBMSC culture in defects created in osteochondral biopsies or in bone-only biopsies resulted in comparable levels of cartilage-related gene expression, whereas culture in cartilage-only biopsies did not induce chondrogenesis. Subcutaneous implantation in nude mice of osteochondral biopsies containing hBMSCs in osteochondral defects resulted in the formation of more cartilaginous tissue than hBMSCs in chondral defects. The subchondral bone did secrete TGFβ, however the observed results could not be attributed to TGFβ, since either capturing TGFβ with an antibody or blocking the canonical TGFβ signaling pathway did not result in significant changes in cartilage related gene expression of hBMSCs in the osteochondral culture model. Inhibition of BMP signalling did not prevent chondrogenesis either. In conclusion, we demonstrate that chondrogenesis of hBMSCs is induced by factors secreted from the bone. We have strong indications that this is not solely mediated by members of the TGFβ family but other, yet unknown, factors originating from the subchondral bone appeared to play a key role.

## Introduction

Human bone marrow derived mesenchymal stem cells (hBMSCs) are widely used for tissue engineering approaches because of their multi-lineage differentiation potential and expandability in-vitro [71, 308]. For cartilage tissue engineering, hBMSCs can provide a more favorable cell source then articular chondrocytes, since the availability and in-vitro expandability of chondrocytes are limited [68, 162]. In cartilage repair strategies based on either autologous chondrocytes or hBMSCs, the repair tissue formed is often of a fibro-cartilaginous nature, having inferior mechanical properties than native articular cartilage [47, 129, 158]. Therefore, tissue engineering strategies could provide a more successful solution for the regeneration of damaged cartilage [309]. To eventually achieve this, more insight is required in the complex mechanisms involved in chondrogenesis of hBMSCs.

TGFβ is generally recognized as key regulator of in-vitro chondrogenesis of hBMSCs: without supplementing TGFβ to the specific differentiation media, hBMSCs will not differentiate towards cartilage [72, 73, 310]. Environmental factors such as oxygen concentration, mechanical stimulation or co-culture with other cell types, like chondrocytes, have been recognized to affect chondrogenesis of hBMSCs [311-313]. Also, differential activation of signaling pathways in hBMSCs affects the quality of the generated cartilaginous tissue in-vitro [314]. Thus, the micro-environment in which hBMSCs reside influences the chondrogenic potential of the cells.

Several in-vivo studies involving orthotopic cartilage defects have demonstrated that implantation of hBMSCs without treatment of chondrogenesis-related growth factors prior to implantation results in the formation of cartilaginous tissue [315-318]. Contrastingly, upon ectopic implantation of hBMSCs, bone formation is reported, even when hBMSCs were stimulated to differentiate chondrogenically prior to implantation [74, 319]. These distinct responses of hBMSCs placed in different environments demonstrate that the micro-environment plays an important role in the induction and direction of differentiation of hBMSCs both in-vitro and in-vivo.

In an orthotopic cartilage defect, the surrounding cartilage, the subchondral bone as well as the synovial fluid affect the local micro-environment. However, the specific effects that each joint tissue can have on regeneration of cartilage are currently still unknown. We aimed to investigate mechanisms involved in chondrogenesis of human hBMSCs in a simulated joint-like environment in-vitro. Therefore we used a bovine osteochondral biopsy model that we have recently developed and validated [320]. This allows us to study cartilage repair mechanisms in a well characterized osteochondral environment in-vitro.

In sharp contrast to the general hypothesis that TGFβ is essential for chondrogenesis of hBMSCs, we found that chondrogenesis of hBMSCs in this osteochondral environment was not dependent on the addition of TGFβ to the culture system. We identified the subchondral bone as main source of secreted factors for chondrogenesis of hBMSCs. Subcutaneous implantation in nude mice of osteochondral biopsies with hBMSCs resulted in more newly formed cartilaginous tissue in osteochondral defects than in chondral defects, confirming the importance of the subchondral bone. Since TGFβ was our main candidate to induce chondrogenesis, we measured the presence of TGFβ in the culture media. Subsequently, we captured TGFβ secreted by subchondral bone using an antibody against TGFβ and blocked the canonical TGFβ signaling pathway by prevention of Smad2/3 phosphorylation. Neither of these strategies resulted in inhibition of chondrogenesis of hBMSCs. Altogether, our findings demonstrate that chondrogenesis of hBMSCs is stimulated by the bone and this is not solely mediated by TGFβ.

## Materials & methods

### hBMSC isolation and expansion

Bone marrow aspirates from healthy donors and patients undergoing total hip replacement surgery after informed consent was obtained: for the healthy donors, all procedures for the collection of marrow have been approved by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland (Ref: 2/08) and by the institutional National University of Ireland Galway Research Ethics Committee (reference: 08/May/14); for the donors undergoing total hip replacement all procedures have been approved by the local ethical committee of the Erasmus MC, University Medical Center Rotterdam (MEC 2004-142).

Heparinized bone marrow aspirates were seeded at a density of 2-5*$10^5$ cells/$cm^2$ in Minimum Essential Medium – alpha (MEM-α, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FCS, Lonza, Verviers, Belgium), 50μg/mL gentamicine (Gibco) and 1.5μg/mL fungizone (Gibco), 1ng/mL fibroblast growth factor 2 (FGF2, AbD Serotec, Kidlington, UK) and 25μg/mL ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA). Non-adherent cells were washed off after 24 hours and adherent cells were further expanded. At subconfluence, hBMSCs were trypsinized and replated at a density of 2,300 cells/$cm^2$. Medium was refreshed twice per week. Passage 3 or 4 hBMSCs were used for experiments.

**Osteochondral culture model**

Cartilage defects were created in bovine osteochondral biopsies as described previously [320]. In short, osteochondral biopsies of 8mm in diameter and 5mm in length were created using a hollow drill (Synthes, Oberdorf, Switzerland) from the four proximal sesamoïd bones of fresh metacarpal phalangeal joints of 3 to 8 months old calves (figure 1.A). Biopsies were incubated overnight in Dulbecco's Modified Eagle's Medium with Glutamax (DMEM-HG, Gibco) supplemented with 10% FBS, 50µg/mL gentamicine and 1.5µg/mL fungizone. Using a 6mm in diameter dermal biopsy punch (Stiefel Laboratories, Durham, NC, USA) and scalpel, cartilage defects were created of chondral, subchondral and osteochondral nature as described previously [320]. Biopsies were placed in 2% low-gelling agarose (gelling temperature 37-39°C, Eurogentec, Li§ege, Belgium) in such way that the cartilage was above the agarose surface. By cutting the cartilage from the bone, we created cartilage-only explants and bone-only explants. To exclude cartilage remnants on the bone-only explants, about 1mm of the subchondral bone was removed. In the bone-only and cartilage-only explants, also 6mm defects were created.

**hBMSCs in alginate in the osteochondral culture model**

To culture hBMSCs in alginate inside the simulated subchondral cartilage defects in the osteochondral culture model, hBMSCs were resuspended in 1.2% low viscosity alginate (Keltone, San Diego, CA, USA) in physiological saline at a density of $10*10^6$ cells/mL. Simultaneously, 50µL of alginate cell-suspension and 50µL 102mM $CaCl_2$ were added into the simulated cartilage defects, allowing in-situ gelation. Equal amounts of alginate cell-suspension were used in all conditions. To study the roles of bone and cartilage, hBMSCs in alginate were cultured in defects in osteochondral biopsies, bone-only explants or cartilage-only explants (figure 2.A). To determine whether either living cells present in the osteochondral biopsies or factors released from the bone matrix affect the behavior of hBMSCs, biopsies were snap frozen in liquid nitrogen and stored at -80°C. Subsequently, six freeze-thaw cycles were performed, using a 60°C water bath and liquid nitrogen. hBMSCs in alginate were cultured in defects in these devitalized osteochondral biopsies. Unless stated otherwise, samples were cultured for 28 days at 37°C and 5%$CO_2$ in 1.5mL incomplete chondrogenic medium (ICM) per biopsy, consisting of DMEM-HG supplemented with insulin, transferrin and selenium (ITS+1, B&D Bioscience, Bedford, MA, USA), 40µg/mL L-proline (Sigma-Aldrich), 1mM sodium pyruvate (Gibco), 1.5 µg/mL fungizone, 50µg/mL gentamicin, 25µg/mL ascorbic acid-2-phosphate, $10^{-7}$M dexamethasone (Sigma-Aldrich). When 10ng/mL TGFβ1 (R&D Systems, Minneapolis, MA, USA) was added to this medium, it is referred to as complete chondrogenic medium (CCM). To evaluate whether induction of chondrogenesis in ICM was due to endogenously produced TGFβ, 1.5µg/mL pan specific anti-TGFβ1,2,3 (anti-TGFβ, MAB1835, R&D systems) was used to capture produced TGFβ. This

dosage was determined based on previous measurements of TGFβ1 levels in used culture medium of osteochondral biopsies, combined with manufacturer's instructions to use 0.25 - 1.25 µg/mL to neutralize 1ng/mL TGFβ. To prevent phosphorylation of Smad2/3 (pSmad2/3) and activation of the canonical TGFβ signaling pathway, 10ng/mL SB-505124 (Sigma-Aldrich) was added to ICM. 10ng/mL dorsomorphin (Biomol International, Exeter, UK) was added to ICM to prevent phosphorylation of Smad1/5/8 (pSmad1/5/8) and activation of the BMP-associated signaling pathway. The dosage of SB-505124 and dorsomorphin was based on previous research [314]. Medium was refreshed three times per week. Used medium was stored once per week at -80°C for later analysis of the concentration of TGFβ1 using an ELISA kit for human TGFβ1 (R&D systems) according to manufacturers instructions. Samples were cultured for 28 days before harvesting the hBMSCs for mRNA isolation or biochemical assays. For western blot, samples were harvested 1.5 hours after refreshing the medium after four days of culture.

**hBMSCs in alginate beads as controls**

As controls for hBMSCs cultured in the osteochondral biopsy model, hBMSCs were cultured in alginate beads. hBMSCs were resuspended in alginate at a density of $10*10^6$ cells/ml. The alginate-cell suspension was pressed through a 22 gauge needle in 102mM $CaCl_2$. Beads were washed twice in physiological saline and once in ICM. 100µL medium was used per alginate bead, 10 to 12 beads were cultured per well in 24-well plates. hBMSCs in alginate beads were cultured in CCM or ICM. Anti-TGFβ, SB-505124 or dorsomorphin was added to CCM as controls for the hBMSCs cultured in alginate in the osteochondral biopsy system. Medium was refreshed three times per week. Samples were cultured for 28 days before harvesting for mRNA isolation or biochemical assays.

**mRNA isolation and qRT-PCR**

After 28 days of culture, alginate beads were dissolved using 55mM sodium citrate (150µL/bead, Sigma-Aldrich) in 20mM ethylene diamintetraacetate (EDTA, Sigma-Aldrich). hBMSCs in alginate cultured in the osteochondral model, bone-only and cartilage-only explants were removed using a spatula and dissolved in 450µL sodium citrate in EDTA. All samples were incubated at 4°C while rotating and subsequently centrifuged for 8 minutes at 1,200 rpm. The supernatant was removed and the samples were resuspended in 150µL/bead or 500µL/sample RNABee (TEL-TEST, Friendswood, TX, USA). Chloroform (Sigma-Aldrich) was added at a quantity of 200µL/mL RNABee. Further RNA isolation was performed using the RNeasy Microkit (Qiagen, Hilden, Germany) according to manufacturer's instructions, including on-column DNAse treatment. RNA concentration and quality was measured using a NanoDrop ND1000 UV-VIS spectrophotometer (Isogen Life Science, de Meern, the Netherlands). cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-

Rot, Germany) according to manufacturer's instructions. qRT-PCR was performed in 20µL reactions on a ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA) using either Taqman Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec). The expression of the cartilage-related genes collagen type 2 and aggrecan and the hypertrophy-related gene collagen type X was determined [79, 274]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH [79]) was selected as reference gene after comparison with two other housekeeping genes (data not shown). Relative gene expression was calculated using the $2^{-\Delta CT}$ method [275].

**Glycosaminoglycan content**

After 28 days of culture, alginate was digested overnight at 56°C in 200µg/mL papain in 50mM EDTA supplemented with 5mM L-cystein (Sigma-Aldrich). The amount of GAGs was determined using dimethylmethylene blue (DMB) assay; the protocol was modified for measurements in alginate as reported previously: the pH of the DMB reagent was lowered to 1.75 using formic acid [269, 270]. A spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, USA) was used to measure the metachromatic reaction of GAGs with DMB at 540 and 595nm. Chondroitin sulfate C (Sigma-Aldrich) was used as a standard. The DNA content in papain-digested samples was determined after RNAse (Sigma) treatment using ethidium bromide (Gibco). Using a spectrofluorometer (Wallac 1420 Victor 2, Perkin-Elmer, Wellesley, MA, USA), the extinction and emission were measured at 340nm and 590nm respectively. Calf thymus DNA (Sigma-Aldrich) was used as a standard.

**Western blot**

After four days of pre-culture to allow stabilization of culture conditions, hBMSCs that were cultured in alginate beads were stimulated with TGFβ1 alone, TGFβ1 and anti-TGFβ or TGFβ1 and SB-505124. Alternatively, hBMSCs cultured in alginate in the osteochondral biopsy model were stimulated with TGFβ1, anti-TGFβ, SB-505124 or left untreated as control. After 1.5 hours of stimulation, alginate was dissolved and removed using cold sodium citrate and centrifugation as described in the mRNA isolation section. M-PER Protein extraction reagent (Thermo Scientific, Rockford, IL, USA) with 1% protease inhibitor (Roche Diagnostics, Basel, Switzerland) was added. Total protein content was determined using a bicinchoninic acid assay kit (BCA assay, Pierce, Rockford, IL, USA). Per sample 10µg of total protein lysate was subjected to gel electrophoresis using a 10% sodium dodecyl sulphate polyacryl amide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were treated for 2.5 hours with a blocking buffer consisting of 0.1% Tris/Tween (TBS-T) supplemented with 5% dried milk powder, washed in TBS-T and incubated overnight at 4°C with the primary antibody anti-α-tubulin (1:1000, Cell Signaling Technology, Danvers, MA, USA) or the primary antibody anti-pSmad2 (1:1000, Cell Signaling Technology). Subsequently, membranes were

incubated for 1.5 hours at room temperature with anti-horseradish peroxidase conjugated secondary antibody (1:1000, Cell Signaling). Blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Durham, NC, USA) according to manufacturer's instructions. For anti-α-tubulin, exposure time was 1-5 seconds and for anti-pSmad2, exposure time was 2-4 minutes.

**In-vivo implantation of osteochondral biopsies with hBMSCs in alginate**

Passage 3 hBMSCs from three healthy donors were resuspended in 1.2% alginate, which was solidified in simulated chondral or osteochondral defects in osteochondral biopsies (n=3 per donor per defect type) as described above for the in-vitro experiments. Equal amounts of alginate cell-suspension were used in all conditions: 50µL of alginate per defect containing $10*10^6$ hBMSCs/mL. Alginate without cells was solidified in both defect types (n=3 per defect type) as a control. Biopsies were cultured overnight to allow stabilization of the system. Simultaneously, osteochondral biopsies with hBMSCs from all three donors in both defect types in alginate were cultured in-vitro as controls (n=3 per donor per defect type) as described above and harvested for mRNA isolation after 28 days. Four osteochondral biopsies were implanted subcutaneously per female NMRI nu/nu mouse (Charles River, Wilmington, MA, USA) under isoflurane anesthesia. The osteochondral biopsies were covered using a 8mm in diameter Neuro-Patch membrane (Braun, Melsungen, Germany) to prevent in-growth of host tissue. Before surgery and 6-10 hours after surgery, mice received 0.05mg/kg bodyweight of Temgesic (Reckitt Benckiser, Slough, UK). During surgery, mice received 9mg/kg bodyweight of Ampi-dry (Dopharma, Raamsdonksveer, The Netherlands). After 12 weeks, mice were euthanized by cervical dislocation. Osteochondral biopsies were explanted and fixed in 4% formalin. After at least 1 week of fixation, biopsies were decalcified using 10% formic acid (Sigma-Aldrich) for three weeks. Subsequently, biopsies were embedded in paraffin, sectioned in 6mm sections and subjected to histology. Animal experiments were conducted with approval of the animal ethical committee (EMC2353, protocol number 116-11-06).

**Histology & quantification**

For safranin-O staining, paraffin sections were first stained with 0.1% light green for 8 minutes, subsequently washed in 1% acetic acid and stained with 0.1% safranin-O (Fluka, St. Gallen, Switzerland) for 12 minutes. Newly formed tissue was discriminated visually from native tissue. The surface area of the simulated cartilage defect was measured and the surface area of newly formed safranin-O positive tissue was determined using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

**Data analysis**

Unpaired data was analyzed using Student's t-test. Normality of paired data was verified with Kolmogorov-Smirnov and Shapiro-Willk normality tests using SPSS 15.0. When necessary, logarithmic transformation was performed to obtain normal distribution of the data. For paired data that was normally distributed, a generalized estimated equations model was used. Correction for multiple testing was performed using the false discovery rate. If paired data was not normally distributed, a Kruskal-Wallis test was performed followed by the Mann Whitney U test. For all statistical analyses, differences were considered statistically significant for $p<0.05$.

## Results

**hBMSCs differentiate in the osteochondral model without the addition of TGFβ**

After 28 days of culture in CCM (with TGFβ) or ICM (without TGFβ), chondrogenesis of hBMSCs in alginate in simulated subchondral defects was assessed. Interestingly, chondrogenesis of hBMSCs in subchondral defects was observed after culture in ICM. Both after 7 and 28 days of culture, no significant differences were observed between hBMSCs in alginate in simulated subchondral defects cultured in CCM or ICM in terms of collagen type 2 and aggrecan gene expression as well as in GAG production (figure 1.B&D). Throughout culture, TGFβ1 secretion by the osteochondral biopsies was measured in culture medium and it was found that about 600pg/mL TGFβ1 was secreted by the biopsies in 72 hours (figure 1.C). For hBMSCs from five different donors, no significant differences were observed in collagen type 2 and aggrecan gene expression between culture in alginate beads in CCM and culture in alginate in simulated subchondral defects in ICM (figure 1.E). Collagen type X gene expression was significantly lower in hBMSCs cultured in alginate in simulated subchondral defects than in hBMSCs cultured in alginate beads (figure 1.E). Based on these findings, for further experiments ICM was used for hBMSCs cultured in alginate in osteochondral biopsies.

**Subchondral bone and not cartilage stimulates hBMSC chondrogenesis**

To assess the specific roles of bone and cartilage in the osteochondral biopsy model, bone-only and cartilage-only explants were prepared. hBMSCs were cultured for 28 days in alginate in simulated defects in osteochondral biopsies, bone-only or cartilage-only explants (figure 2.A). Collagen type 2 and aggrecan gene expression levels in hBMSCs cultured in bone-only explants or osteochondral biopsies were found to be comparable, where the levels in hBMSCs cultured in cartilage-only explants were significantly lower ($p=<0.001$, figure 2.B). Significantly more TGFβ1 was produced by osteochondral biopsies and bone-only biopsies versus cartilage-only explants in 72 hours after 28 days of culture ($p=0.03$, figure 2.C).

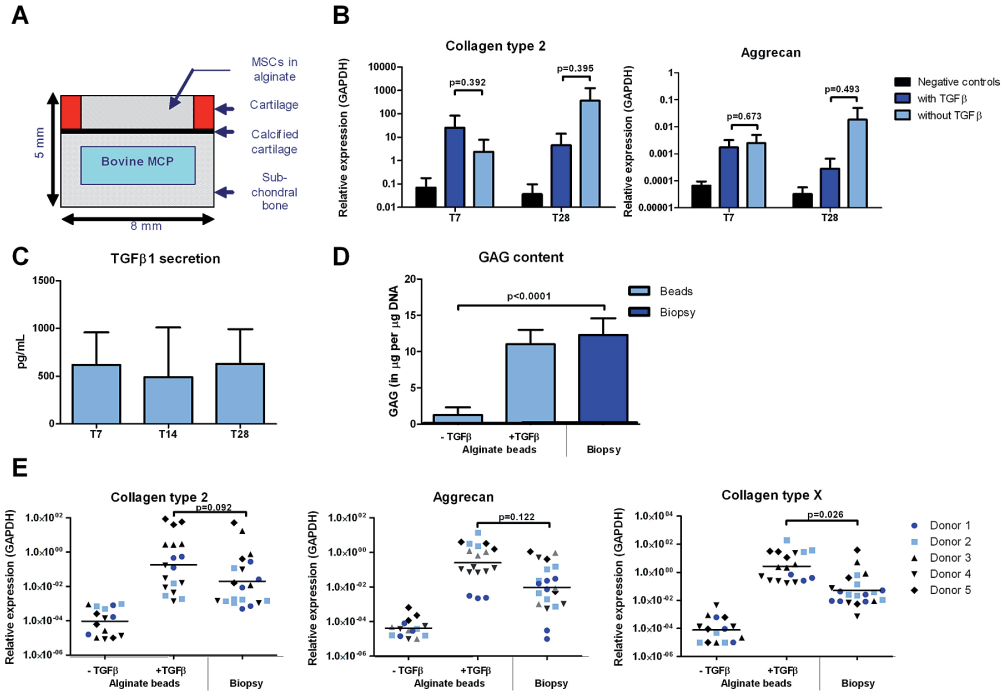



**Figure 1.**
A. Schematic representation of the bovine osteochondral biopsy model with a simulated subchondral defect containing HBMSCs in alginate; B. Collagen type 2 and aggrecan gene expression relative to GAPDH after 7 or 28 days of culture of HBMSCs from one donor in alginate in simulated subchondral defects with or without supplementing TGFβ (n=6, mean with standard deviation, student's t-test); C. TGFβ1 secretion measured in culture medium used for 72 hours of osteochondral biopsies (n=3, mean with standard deviation) after 7, 14 or 21 days in culture to which no exogenous TGFβ was supplemented; D. µg of GAGs normalized to µg of DNA measured either in HBMSCs from one donor cultured for 28 days in alginate beads with or without TGFβ or in alginate in simulated subchondral defects without TGFβ (n=6, student's t-test); E. Collagen type 2, aggrecan and collagen type X gene expression of HBMSCs from five different donors (n=6 for MSC donor 1 and 2, n=3 for MSC donors 3, 4 and 5, generalized estimated equations model with correction for multiple testing);

The stimulating role of subchondral bone on chondrogenesis of hBMSCs was confirmed by the results of the in-vivo experiment: subcutaneous implantation of osteochondral biopsies with hBMSCs from three different donors in alginate in chondral defects resulted in the formation of significantly less cartilaginous repair tissue than hBMSCs in osteochondral defects (p=0.004, figure 3.B-E). The newly formed tissue by hBMSCs in osteochondral defects appeared more cartilage-like in terms of intensity of safranin-O staining as well as in cell morphology than the newly formed tissue in chondral defects (figure 3.B&C). This was also reflected in gene expression levels of accompanying in-vitro controls: the expression of collagen type 2 ($p<0.001$) and aggrecan ($p<0.001$) was significantly higher in hBMSCs cultured in alginate in osteochondral defects than in hBMSCs cultured in alginate in chondral defects (figure 3.A).

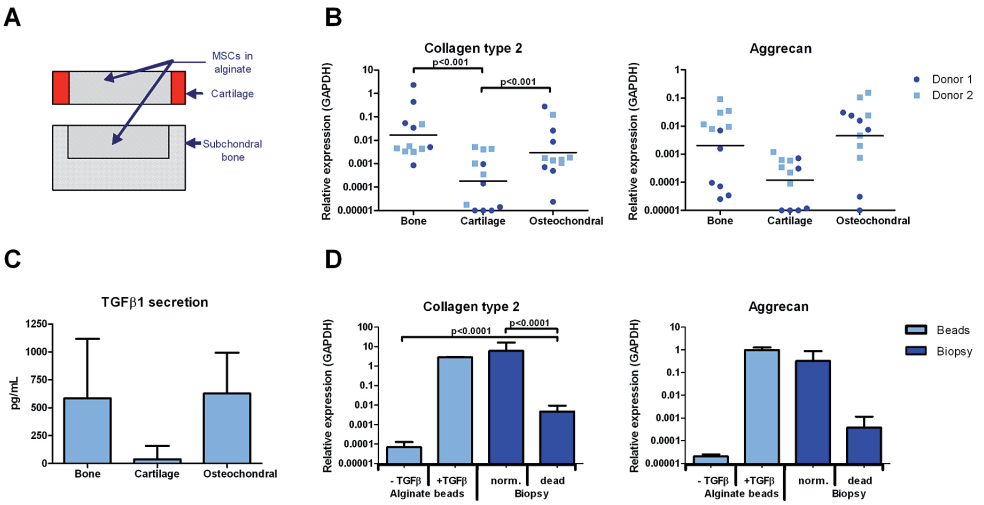



**Figure 2.**
A. Schematic representation of bovine cartilage-only and bone-only biopsies with a simulated defect containing HBMSCs in alginate; B. Collagen type 2 and aggrecan gene expression relative to GAPDH of HBMSCs from two different donors in alginate cultured in either bone-only biopsies, cartilage-only biopsies or simulated subchondral defects in osteochondral biopsies (n=6 per MSC donor, generalized estimated equations model with correction for multiple testing); C. TGFβ1 secretion of bone-only, cartilage-only or osteochondral biopsies measured in culture medium used for 72 hours after 28 days of culture in total (n=3, mean with standard deviation); D. Collagen type 2 and aggrecan gene expression of HBMSCs from one donor cultured for 28 days in alginate beads with or without supplementing TGFβ or in alginate in simulated subchondral defects in osteochondral biopsies (norm.) or in devitalized osteochondral biopsies (dead) (n=6, mean with standard deviation, student's t-test);

After 12 weeks in vivo, the native bovine cartilage of the osteochondral biopsies stained negative for safranin O (figure 3B-D). This indicates that GAGs have been degraded during the experiment, which was most likely due to the lack of mechanical loading during the experiment, since the osteochondral biopsies were implanted subcutaneously.

**Chondrogenesis is partly caused by actively produced factors**

When hBMSCs were cultured in alginate in osteochondral biopsies that were devitalized by subjecting them to six repeated freeze-thaw cycles, chondrogenesis was partly inhibited (figure 2.D): collagen type 2 gene expression was significantly higher than in hBMSCs cultured in alginate beads without supplementing TGFβ and significantly lower than in hBMSCs cultured in alginate in normal osteochondral biopsies. This implies that part of the factors that were responsible for the chondrogenesis of hBMSCs in the osteochondral biopsies were released from the subchondral bone matrix and that part of it was actively produced or activated by cells in the osteochondral biopsies.

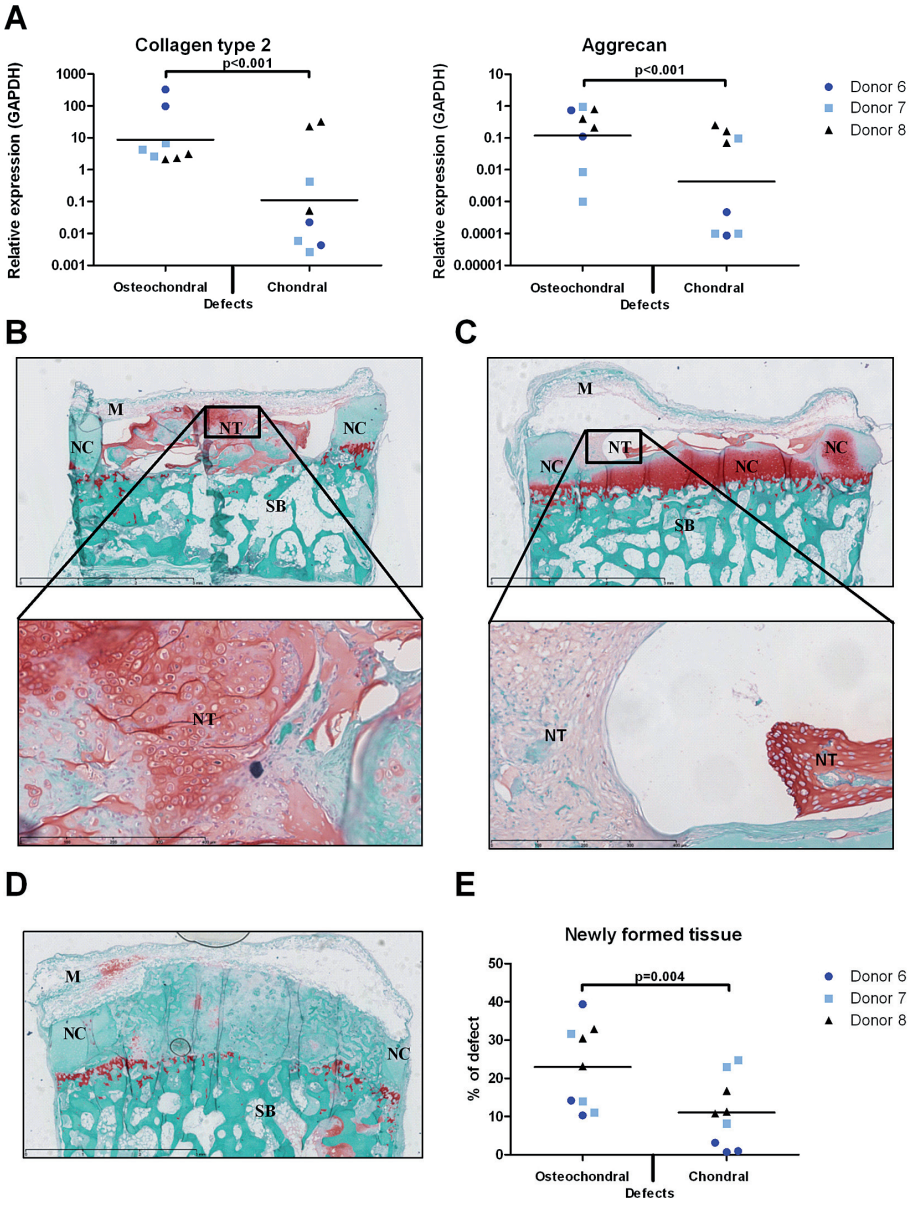



**Figure 3. Results from in vivo experiments and accompanying in vitro controls.**
A. Collagen type 2 and aggrecan gene expression relative to GAPDH of hBMSCs cultured in vitro in alginate beads in complete chondrogenic medium or in alginate in osteochondral biopsies with simulated chondral or osteochondral defects in incomplete chondrogenic medium for 28 days (hBMSCs from 3 donors, n=3 per donor, generalized estimated equations model with correction for multiple testing); B,C&D. Osteochondral biopsies with hBMSCs in alginate in simulated osteochondral (B) or chondral (C) defects or controls with osteochondral defects filled with alginate without cells (D) were implanted subcutaneously in nude mice for 12 weeks. Representative safranin-O stained sections,

*Figure 3. Continued*
scale bars in upper pictures represent 3mm, scale bars in magnified pictures represent 400um. M: NeuroPatch membrane; NC: native bovine cartilage; NT: newly formed tissue; SB: subchondral bone; E. Quantification of newly formed safranin-O positive tissue relative to the defect area (hBMSCs from 3 donors, n=3 per donor, generalized estimated equations model with correction for multiple testing);

**Inhibition of TGFβ signaling does not block chondrogenesis**

Anti-TGFβ was used to capture TGFβ secreted by the osteochondral tissue. Strikingly, even though the anti-TGFβ captured about 80% of the produced TGFβ (figure 4.B), no significant differences were observed in collagen type 2 and aggrecan gene expression as well as GAG production of hBMSCs cultured in alginate in the osteochondral biopsy model (figure 4.A&E). Controls in which hBMSCs in alginate beads were cultured in the presence of both anti-TGFβ and TGFβ demonstrated the effectiveness of the antibody: collagen type 2 ($p<0.0001$) and aggrecan ($p<0.0001$) gene expression and GAG production ($p=0.01$) were significantly lower when the antibody was used (figure 4.A&E). Western blot for pSmad2 was in line with these results: a decrease in pSmad2 was observed when hBMSCs in alginate beads were stimulated with both anti-TGFβ and TGFβ (figure 4.F). When the canonical TGFβ signaling pathway was blocked by prevention of Smad2/3 phosphorylation using SB-505124, chondrogenesis of hBMSCs cultured in alginate in the osteochondral biopsies remained unchanged (figure 4.C&E). This was confirmed by western blot for pSmad2: when hBMSCs in alginate in the osteochondral biopsies were treated with SB-505124, no pSmad2 was detected, indicating that the canonical TGFβ pathway was successfully blocked (figure 4.F). Controls in alginate beads cultured in CCM reflected the effectiveness of SB-505124 by significantly decreasing collagen type 2 ($p=0.04$) and aggrecan ($p<0.001$) gene expression as well as GAG production ($p<0.001$).

Apart from TGFβ, bone is known to contain high levels of other members of the TGFβ family, BMPs. To verify whether signaling of BMPs played a role in chondrogenesis of hBMSCs in an osteochondral environment, the BMP associated signaling pathway was blocked via prevention of pSmad1/5/8 using dorsomorphin. This did not affect chondrogenesis of hBMSCs in alginate in osteochondral biopsies (figure 4.C&E). The secretion of TGFβ increased when dorsomorphin was added to the ICM of the osteochondral biopsies (figure 4.D).

## Discussion

Cartilage defects heal poorly due to the low intrinsic repair capacity of articular cartilage. Therefore solutions that stimulate repair and help to prevent the development of OA are a major research topic in orthopaedics. As a part of this process, hBMSCs are exhaustively investigated as a potential cell source for cartilage tissue engineering purposes based on

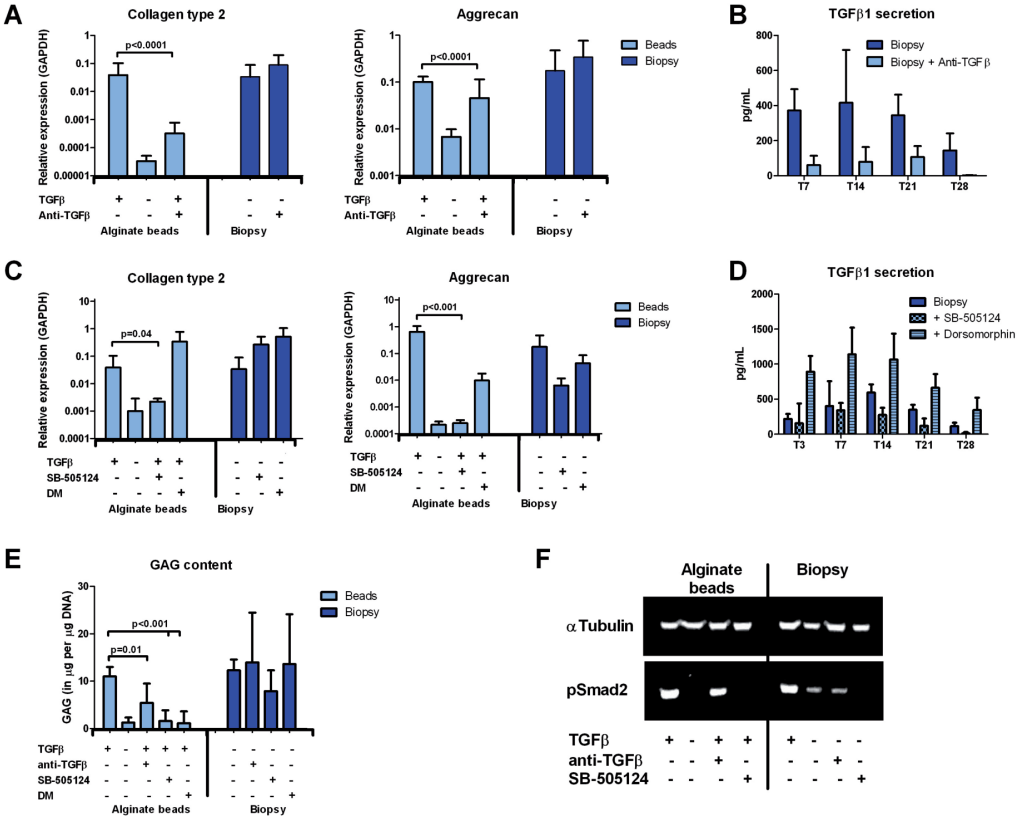



**Figure 4.**
A. Collagen type 2 and aggrecan gene expression relative to GAPDH of HBMSCs from two different donors cultured for 28 days in alginate beads with or without TGFβ and anti- TGFβ or in simulated subchondral defects with or without anti-TGFβ (n=6 per MSC donor, mean with standard deviation, Kruskal-Wallis test followed by the Mann Whitney U test); B. TGFβ secretion of osteochondral biopsies cultured with or without anti-TGFb measured in medium used for 72 hours (n=3, mean with standard deviation); C. Collagen type 2 and aggrecan gene expression of HBMSCs from two different donors cultured for 28 days in alginate beads with or without TGFβ or in simulated subchondral defects without TGFβ. Samples were cultured with the pSmad2/3 inhibitor SB-505124 or the pSmad1/5/8 inhibitor dorsomorphin (DM) (n=6 per MSC donor, mean with standard deviation, Kruskal-Wallis test followed by the Mann Whitney U test); D. TGFβ secretion of osteochondral biopsies measured in medium used for 72 hours with or without the pSmad2/3 inhibitor SB-505124 or the pSmad1/5/8 inhibitor dorsomorphin (n=3, mean with standard deviation); E. GAG production relative to DNA concentration of HBMSCs from one donor cultured in alginate beads with or without TGFβ or in alginate in simulated subchondral defects without TGFβ. Samples were cultured with anti- TGFβ, SB-505124 or dorsomorphin (n=6, mean with standard deviation, student's t-test); F. Western blot for αTubulin and phosphorylated Smad 2 (pSmad2) on HBMSCs cultured for four days in alginate beads or in alginate in simulated subchondral defects harvested after 1,5 hours of stimulations with or without TGFβ, anti-TGFβ and/or SB-505124;

their multipotency, expandability in-vitro and the possibility to use autologous cells. When hBMSCs are cultured in a 3D in-vitro setting, for example in pellets or in alginate beads, TGFβ is essential to induce chondrogenesis [72, 73, 310, 314]. In the present study, we have found that when hBMSCs were cultured in alginate in an osteochondral environment in-vitro, chondrogenesis is induced independent of TGFβ.

We initiated this study by culturing hBMSCs in alginate in simulated subchondral defects in a bovine osteochondral biopsy model with and without the addition of TGFβ to the culture media, hypothesizing that TGFβ would be required to induce chondrogenesis. Strikingly, in the osteochondral culture system chondrogenesis of hBMSCs was present even when no TGFβ was supplemented. The osteochondral culture model appeared to provide a favourable micro-environment for chondrogenesis of hBMSCs. This finding corresponds with clinical outcomes of the microfracture procedure: a bone marrow clot fills a cartilage defect and hBMSCs, either present or recruited, are held responsible for the spontaneous generation of cartilaginous repair tissue, without the supplementation of any exogenous factors [55, 59]. This led to our renewed hypothesis: TGFβ secreted by the osteochondral biopsies themselves was responsible for the induction of chondrogenesis in the hBMSCs. This was supported by measurements of significant TGFβ1 levels in the culture media throughout culture.

To study whether the induction of chondrogenesis could be addressed to a specific part of the osteochondral culture system, the separate roles of bone and cartilage were studied by culturing hBMSCs in alginate in simulated defects in bone-only or cartilage-only biopsies. The findings that the subchondral bone played the most important role and also produced the majority of the TGFβ1, fitted the hypothesis. Again, this corresponds with the clinical outcomes of the microfracture procedure, since it is known that careful removal of the calcified cartilage layer before puncturing the subchondral plate is required [135]. This implies that both in our culture system as well as in a clinical setting, the subchondral bone plays an important role in the generation of repair tissue.

To validate our findings, osteochondral biopsies containing hBMSCs in alginate in either osteochondral or chondral simulated defects were implanted subcutaneously in nude mice. More newly formed cartilage-like tissue was observed in osteochondral defects than in chondral defects. This validates our culture model and it also confirms the in-vitro finding that subchondral bone plays a critical role in the stimulation of chondrogenesis of hBMSCs in an osteochondral environment. These findings correspond with recent clinical and animal studies in which autologous hBMSCs were injected in the knee joints of patients or animals with OA. These studies showed a decrease in size of cartilage lesions and enhanced regeneration of the cartilage using hBMSCs with exposure to growth factors before or after

implantation [318, 321, 322]. This is in contrast with other studies, suggesting that treatment with growth factors before or following implantation is required to achieve regeneration of osteochondral defects by hBMSCs [323, 324]. Despite the fact that hBMSCs were shown to undergo chondrogenic differentiation, we can not exclude that other cells might have contributed to the formation of repair tissue. It is possible that cells from the subchondral bone of the osteochondral biopsy or from the murine host contributed to the formed repair tissue, since it is known that hBMSCs can secrete trophic factors that can recruit other cells [325]. Eitherway, the presence of hBMSCs was crucial for the formation of cartilaginous repair tissue, since in control defects with alginate without hBMSCs, no formation of safranin O positive repair tissue was observed.

To study whether the factor(s) that induce chondrogenesis in the osteochondral culture system are actively produced by cells or secreted from the matrix of the subchondral bone during culture, hBMSCs in alginate were cultured in devitalized osteochondral biopsies. The results from this experiment suggest that part of the factor or factors involved were secreted by the bone matrix, but also that they were at least partly, actively produced by the cells present in the subchondral bone of the osteochondral biopsies.

After these observations, we aimed to confirm that TGFβ was actually the key player behind the observed effect, since among the possible factors that can be released by bone TGFβ is the most likely candidate to induce chondrogenesis, whereas BMPs and VEGF, for example, are more associated with bone formation [326]. To achieve this, an antibody against TGFβ was used, which resulted in the majority of the secreted TGFβ being captured. Surprisingly, chondrogenesis of hBMSCs in alginate in simulated subchondral defects remained unchanged, which was in sharp contrast to our hypothesis. Even though levels were low, the possibility remained that the residual TGFβ was responsible for the observed chondrogenesis. To rule out this option, we aimed to inhibit TGFβ signalling.

Previously, we demonstrated that when canonical TGFβ signalling is blocked by prevention of Smad2/3 phosphorylation using the inhibitor SB-505124 in hBMSCs in pellet culture in the presence of TGFβ, chondrogenesis was inhibited [314]. The use of an antibody against TGFβ to capture the majority of the produced TGFβ did not prevent chondrogenesis of bMSCs in osteochondral defects. However, the use of the antibody against TGFβ resulted in some remaining Smad2 phosphorylation indicating that we might not have blocked TGFβ signaling pathways sufficiently. Therefore we used SB-505124, which resulted in a complete prevention of Smad2 phosphorylation, where chondrogenesis remained uninhibited, thus indicating that chondrogenesis was mediated by factors other than TGFβ. When the canonical BMP signaling was inhibited via prevention of Smad1/5/8 phosphorylation by dorsomorphin in

our culture system, the situation remained the same as observed before: chondrogenesis of hBMSCs was uninhibited. These findings suggest that chondrogenesis of hBMSCs in our osteochondral culture system is not solely mediated by TGFβ and that the effect is also not addressable to BMPs.

It is known that apart from TGFβ and BMPs, other growth factors reside in the extracellular bone matrix, such as insulin like growth factor (IGF), and that various growth factors, such as FGF, IGF, platelet derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) can be produced by different cell types residing in the bone [327, 328]. During healing of bone fractures, it is known that a variety of growth factors are released by the extracellular bone matrix or produced by cells residing in the bone: for example as an acute response to a fracture, interleukin (IL)-1, IL-6, TGFβ and PDGF are released [329, 330]. In the homeostasis of bone, many of these and other factors are required to be present in a delicate balance, since bone is continuously remodeled. It is known for example that sclerostin, an inhibitor of Wnt signaling, is important in generating bone tissue, where its production by osteocytes can be inhibited by oncostatin M [331]. Since all of these factors are likely to be present in our osteochondral culture system, the observed chondrogenesis might be attributed to one of these factors or a combination of multiple factors.

The bovine biopsies used in the osteochondral culture system originate from three to eight months old calves. It is obvious that this young and healthy material is likely to provide a different environment than old and/or diseased material, such as osteoarthritic biopsies. Apart from degeneration of the cartilage, in osteoarthritis (OA), the subchondral bone undergoes major changes. The effects of these changes on cartilage repair mediated by bMSCs are difficult to predict and will require further studies.

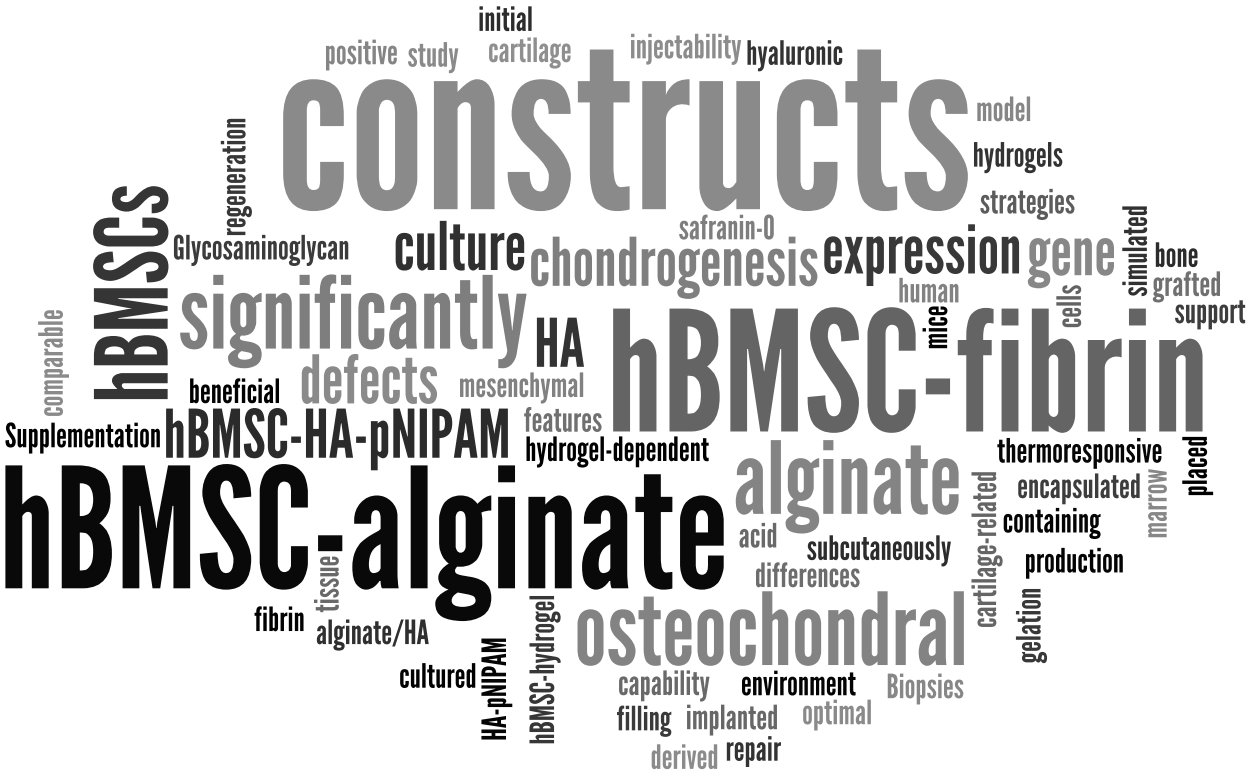
Altogether our study stresses that chondrogenesis of hBMSCs in an osteochondral environment is a complex process. Conventional culture systems like pellet culture or alginate beads might not be sufficiently representative to truly reach understanding of the complexity of the differentiation process. Using an osteochondral culture model, we have identified the subchondral bone as a key player in cartilage regeneration by hBMSCs, but that the commonly recognized in-vitro regulator of chondrogenesis TGFβ is not solely responsible for the observed effects. It is evident that more studies are required to truly identify the now unidentified key players in the induction of chondrogenesis of hBMSCs in a healthy, but also in a diseased osteochondral environment, like for example in OA.

## Acknowledgement

The research leading to these results has received funding from the European Union's 7th Framework Programme under grant agreement no. NMP3-SL-2010-245993 the Dutch Arthritis Association (LLP11), the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant No. FES0908) and Science Foundation Ireland (grant number 09/SRC/B1794). The research in this article was performed within the framework of the Erasmus Postgraduate School Molecular Medicine. The authors would like to thank Stefan Sandker and René van der Bel for conducting pilot experiments for the current study during their internships as part of the master technical medicine of the University of Twente, Enschede, the Netherlands.

# Chapter 7

## Chondrogenic differentiation of human bone marrow derived mesenchymal stem cells in a simulated osteochondral environment is hydrogel dependent




M.L. de Vries – van Melle, M.S. Tihaya, N. Kops, J.L.M. Koevoet, J.M. Murphy, J.A.N. Verhaar, M. Alini, D. Eglin, G.J.V.M. van Osch

European Cells and Materials 2014. 27

## Abstract

Hydrogels pose interesting features for cartilage regeneration strategies, such as the option for injectability and in situ gelation resulting in optimal filling of defects. We aimed to study different hydrogels for their capability to support chondrogenesis of human bone marrow derived mesenchymal stem cells (hBMSCs). hBMSCs were encapsulated in alginate, alginate with hyaluronic acid (alginate/HA), fibrin or thermoresponsive HA grafted with poly(N-isopropyl acrylamide) side-chains (HA-pNIPAM). Glycosaminoglycan production and cartilage-related gene expression were significantly higher in hBMSC-alginate and hBMSC-fibrin constructs than in the other constructs. Supplementation of alginate with HA was not beneficial. hBMSC-alginate, hBMSC-fibrin and hBMSC-HA-pNIPAM constructs were placed in simulated defects in osteochondral biopsies and cultured *in vitro* for 28 days. Biopsies containing hBMSC-alginate and hBMSC-fibrin were implanted subcutaneously in nude mice for 12 weeks. hBMSC-alginate constructs had significantly higher cartilage-related gene expression after 28 days of culture as well as significantly more safranin-O positive repair tissue after 12 weeks *in vivo* than hBMSC-fibrin constructs. Although initial experiments with hBMSC-hydrogel constructs suggested comparable results of hBMSC-alginate, hBMSC-fibrin and hBMSC-HA-pNIPAM constructs, culture in the osteochondral biopsy model *in vitro* as well as *in vivo* revealed differences, suggests that chondrogenesis of hBMSCs in an osteochondral environment is hydrogel-dependent.

## Introduction

The complexity of articular cartilage and its lack of self-repair capacity are widely recognized and these features have resulted in an on-going quest to identify the optimal cell sources and biomaterials that can be used for cartilage regeneration purposes. Human bone marrow derived mesenchymal stem cells (hBMSCs) are one of the potential cell sources based on their multipotency, their expandability *in vitro* and the possibility to use autologous cells to treat patients [71].

One of the prerequisites for hBMSCs to differentiate towards the chondrogenic lineage is that a 3D environment should be provided [72, 73, 248]. Pellet culture is widely used as an *in vitro* study model for chondrogenesis of hBMSCs. However, for translation towards treatment of patients, pellet cultures may not suffice to produce relevantly-sized repair tissue to fill cartilage defects.

A large variety of biomaterials have been developed, among which are various hydrogels that function as cell carriers. A major advantage of hydrogels over more solid biomaterials is that they generally can be shaped to fit the defect, and in some cases even allow in situ gelation, which can result in optimal filling of a cartilage defect [332, 333].

The different properties of various hydrogels will influence cell behaviour. In this study, we have compared different hydrogels as carriers for hBMSCs in terms of viability of the cells and their capacity to enable chondrogenic differentiation: alginate, fibrin and hyaluronan-poly(N)-isopropylacrylamide (HA-pNIPAM), each having their specific favourable characteristics. Alginate is a biological hydrogel that is widely used as a cell carrier in *in vitro* studies [92, 334]. From previous studies, it is known that alginate is a suitable carrier for stem cells, allowing chondrogenesis under appropriate culture conditions [335]. Apart from this, alginate is already approved for clinical use for various purposes, which would make development of a treatment for cartilage defects involving alginate as a cell carrier a realistic option [336-340]. Fibrin is well known for its applications for regeneration of various tissues, among which are repair strategies for cartilage and bone [341-344]. As it allows vascular ingrowth [345, 346], fibrin may represent a suitable cell carrier for the repair of osteochondral defects, that also require the repair of bone as well as cartilage. Another favourable feature of fibrin is that there is the possibility of autologous use with isolation of both fibrinogen and thrombin from blood.

HA-pNIPAM contains hyaluronic acid (HA), which is the natural backbone of proteoglycans in articular cartilage. HA is also present in the synovial fluid, where it has a lubrication

function. It has been shown that intra-articular injections of hyaluronic acid (HA) can provide relief of symptoms in patients with knee osteoarthritis [347, 348]. Interestingly, HA plays an important rule during early chondrogenesis in embryonic development; after initial deposition HA is degraded before further chondrogenesis takes place [349]. This makes HA-based gels interesting candidates for cartilage tissue engineering purposes. HA-pNIPAM is an engineered thermo-reversible gel that behaves in a non-Newtonian way: at temperatures below 32°C it is liquid and it gels when the temperature rises above 32°C. For this specific gel, azide pNIPAM side chains were grafted onto a propargyl derivative HA backbone using "click" chemistry, causing its thermo-reversible characteristics: at temperatures lower than 32°C, the pNIPAM sidechains are in their extended, hydrophilic state, where they are in a coiled, hydrophobic state when the temperature rises above 32°C [95, 350]. The association of the hydrophobic domains causes the actual gelation of the material. This specific gel has been developed for future minimally invasive treatments allowing injection of the material followed by in situ gelation. So far, the HA-pNIPAM gel has been studied for possible use in nucleus pulposus tissue regeneration [351, 352].

In the present study, hBMSC-hydrogel constructs were formed with alginate, fibrin and HA-pNIPAM. To study the possible beneficial effect of HA, hBMSC-alginate constructs were formed in which the alginate was enriched with additional high molecular weight HA. After verifying cell survival during culture in the four different hBMSC-hydrogel constructs, chondrogenic differentiation of hBMSCs in the constructs was assessed. The addition of HA to alginate appeared not to have a favourable effect on chondrogenesis of hBMSCs. hBMSC-alginate, hBMSC-fibrin and hBMSC-HA-pNIPAM constructs were cultured in an osteochondral biopsy model that can be used to mimic a joint-like environment *in vitro*. In this culture system, the addition of TGFβ to chondrogenic differentiation medium is not required, since factors released from the system itself are able to induce chondrogenesis of hBMSCs [353]. We observed that culture of all hBMSC-hydrogel constructs in this osteochondral environment resulted in chondrogenesis in terms of cartilage-related gene expression. Due to the experimental nature of the HA-pNIPAM production process, total removal of copper catalyst remnants cannot yet be ensured. Therefore, hBMSC-alginate and hBMSC-fibrin constructs were selected for an *in vivo* experiment to validate *in vitro* results. These constructs were placed in the simulated cartilage defects in osteochondral biopsies, which were then placed subcutaneously in nude mice for 12 weeks. It was found that hBMSC-alginate constructs resulted in significantly more cartilaginous repair tissue than hBMSC-fibrin constructs.

## Materials & methods

### hBMSC isolation and expansion

All procedures for the collection of bone marrow from three healthy male donors of 22, 20 and 22 years of age have been approved by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland (Ref: 2/08) and by the institutional National University of Ireland Galway Research Ethics Committee (reference: 08/May/14). hBMSCs were isolated based on their plastic adherence. Heparinized bone marrow aspirates were seeded at a density of 2-5*$10^5$ cells/$cm^2$ in hBMSC expansion medium consisting of Minimum Essential Medium – alpha (MEM-α, Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FCS, Lonza, Verviers, Belgium), 50µg/mL gentamicine (Gibco) and 1.5µg/mL fungizone (Gibco), 1ng/mL fibroblast growth factor 2 (FGF2, AbD Serotec, Kidlington, UK) and 25µg/mL ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA). To remove non-adherent cells, medium was refreshed after 3 days and cells were washed after 5 days and adherent cells were further cultured. At subconfluence, hBMSCs were trypsinized and replated at a density of 2,300 cells/$cm^2$. hBMSC expansion medium was refreshed twice per week. Passage 3 or 4 hBMSCs were used for experiments.

### hBMSC-hydrogel constructs

To create hBMSC-alginate constructs or hBMSC-alginate constructs enriched with HA, from now on referred to as hBMSC-alginate/HA constructs, hBMSCs were resuspended in 1.2% low viscosity alginate (Keltone, San Diego, CA, USA) or in 1.2% low viscosity alginate supplemented with 1% high molecular weight HA (1.5MDa, Contipro Biotech s.r.o. Czech Republic) in physiological saline at a density of 10*$10^6$ cells/mL. The alginate-cell suspension or alginate/HA cell suspension was pressed through a 22 gauge needle into 102mM $CaCl_2$. Constructs were washed twice in physiological saline and once in incomplete chondrogenic differentiation medium (ICM) consisting of Dulbecco's Modified Eagle Medium with glutamax (DMEM, Gibco) supplemented with insulin, transferrin and selenium, serum replacement (ITS+1, B&D Bioscience, Bedford, MA, USA), 40µg/mL L-proline (Sigma-Aldrich), 1mM sodium pyruvate (Gibco), 1.5 µg/mL fungizone (Gibco), 50µg/mL gentamicin (Gibco), 25µg/mL ascorbic acid-2-phosphate and $10^{-7}$M dexamethasone (Sigma-Aldrich). When 10ng/mL TGFβ1 (R&D Systems, Minneapolis, MA, USA) was added, the medium is referred to as complete chondrogenic medium (CCM). 100µL medium was used per construct, 10 to 12 constructs were cultured per well in 24-well plates.

To obtain a concentration of 10*$10^6$ cells/mL in hBMSC-fibrin constructs, hBMSCs were suspended at a density of 20*$10^6$ cells/mL in 40mg/mL fibrinogen from human plasma (Sigma-Aldrich) to which 60U/mL aprotinin (Sigma-Aldrich) was added to prevent early

degradation of fibrin. hBMSC-fibrin constructs were created by pipetting 25µL of fibrinogen-cell suspension onto Teflon coated hydrophobic glass slides with wells 7mm in diameter (Nutacon, Leimuiden, the Netherlands). An equal volume of 10U/mL thrombin from human plasma (Sigma-Aldrich) in 40mM $CaCl_2$ was added to the fibrinogen-cell suspension. hBMSC-fibrin constructs were incubated for 15 minutes at 37°C and 5% $CO_2$ after which medium was added and constructs were incubated for 30 minutes. Subsequently, constructs were carefully removed from the glass slide using a spatula and 2 constructs were cultured in 1.5mL chondrogenic or culture medium in 24-well plates.

hBMSCs were resuspended at a density of $10*10^6$ cells/mL in cold 17% HA-pNIPAM in PBS to create hBMSC-HA-pNIPAM constructs. HA-pNIPAM synthesis was reported elsewhere [95]. The HA-pNIPAM cell suspension was pipetted onto Teflon coated hydrophobic glass slides that where heated to 42°C and incubated at 42°C for 5 minutes. Glass slides with hBMSC-HA-pNIPAM constructs were transferred to PBS that was pre-heated to 37°C and incubated at 37°C and 5% $CO_2$ for 10 minutes. Subsequently, hBMSC-HA-pNIPAM constructs were carefully removed from the glass slide using a pre-heated spatula and 2 constructs were cultured in 1.5mL pre-heated CCM in 24 wells plates.

Unless stated otherwise, hBMSC-hydrogel constructs were cultured for 28 days at 37°C and 5% $CO_2$ in CCM. hBMSC-alginate constructs in ICM served as negative controls. Medium was refreshed three times per week. After 28 days, hBMSC-hydrogel constructs were harvested for mRNA isolation or biochemical assays.

**Osteochondral culture model**

Cartilage defects were simulated in bovine osteochondral biopsies as described previously [320]. In short, osteochondral biopsies of 8mm in diameter were created using a hollow drill (Synthes, Oberdorf, Switzerland) from the four proximal sesamoïd bones of fresh metacarpal phalangeal joints of 3 to 8 months old calves. After washing, the biopsies were cut to about 5mm in length and sterility was verified by overnight incubation in DMEM supplemented with 10% FBS, 50µg/mL gentamicin and 1.5µg/mL fungizone. Using a 6mm in diameter dermal biopsy punch (Stiefel Laboratories) and scalpel, cartilage was removed as well as the calcified cartilage layer and part of the subchondral plate, resulting in simulated osteochondral defects. To prevent outgrowth of cells from the subchondral bone, biopsies were placed in 2% low-gelling agarose (gelling temperature 37-39°C, Eurogentec, Liege, Belgium) in physiological saline in such way that the subchondral bone was surrounded by agarose and the cartilage was above the agarose surface.

To create hBMSCs-alginate construct in the simulated osteochondral cartilage defects in the osteochondral culture model, hBMSCs were resuspended in 1.2% low viscosity alginate or in 1.2% low viscosity alginate supplemented with 1% high molecular weight HA in physiological saline at a density of $10*10^6$ cells/mL. Simultaneously, 50µL of alginate cell suspension and 50µL 102mM $CaCl_2$ were pipetted into the simulated cartilage defects of subchondral nature, allowing the alginate to solidify inside the defects. To create hBMSC-fibrin or hBMSC-HA-pNIPAM constructs in simulated osteochondral defects, constructs were created as described above. After removal from the Teflon coated glass slides, constructs were press-fitted into simulated osteochondral defects. Unless stated otherwise, osteochondral biopsies with hBMSC-hydrogel constructs were cultured for 28 days at 37°C and 5%$CO_2$ in 1.5mL ICM per biopsy. Medium was refreshed three times per week. Used medium was stored once per week at -80°C for later analysis. After 28 days, hydrogel constructs were harvested for mRNA isolation or biochemical assays.

**mRNA isolation and qRT-PCR**

After 28 days of culture, hBMSC-alginate and hBMSC-alginate/HA constructs were dissolved using 150µL/construct 55mM sodium citrate in 20mM ethylene diamintetraacetate (EDTA, Sigma). hBMSC-alginate constructs cultured in the osteochondral model were removed using a spatula and dissolved in 450µL sodium citrate in EDTA. Samples were incubated at 4°C while rotating and subsequently centrifuged for 8 minutes at 1,200 rpm to remove all alginate remains. The supernatant was removed and the samples were resuspended in 150µL/construct or 500µL/sample RNABee (TEL-TEST, Friendswood, TX, USA). hBMSC-fibrin constructs were either transferred from 24-well plates or removed from simulated osteochondral defects, into 500µL RNABee and crushed manually. hBMSC-HA-pNIPAM constructs were transferred from 24-well plates using a pre-heated spatula and were dissolved in cold PBS, centrifuged at 1,200rpm for 8 minutes and the resulting cell pellet was resuspended in 500µL RNABee. Chloroform was added to all samples at a quantity of 200µL/mL RNABee. Further RNA isolation was performed using the RNeasy Microkit (Qiagen, Hilden, Germany) according to manufacturer's instructions, including on-column DNAse treatment. RNA concentration and quality was measured using a NanoDrop ND1000 UV-VIS spectrophotometer (Isogen Life Science B.V., the Netherlands).

cDNA was prepared using RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to manufacturer's instructions. qRT-PCR was performed in 20uL reactions on a ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA) using either Taqman Universal PCR mastermix (Applied Biosystems) or SybrGreen (Eurogentec). The expression of the cartilage-related genes collagen type 2 (*Col2*) and aggrecan (*ACAN*) and the hypertrophy-related genes collagen type X (*ColX*) and alkaline phosphatase (*ALPL*) was

determined [79, 274]. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was selected as reference gene after comparison with two other housekeeping genes (data not shown) [79]. Relative gene expression was calculated using the $2^{-\Delta CT}$ method [275].

**Glycosaminoglycan content and DNA assays**

After 28 days of culture, hBMSC-alginate, hBMSC-alginate/HA and hBMSC-HA-pNIPAM constructs were dissolved in 55mM sodium citrate in 20mM EDTA. hBMSC-fibrin constructs were crushed manually in the same solution. Subsequently, all samples were digested overnight at 56°C in 200μg/mL papain in 50mM EDTA supplemented with 5mM L-cystein (Sigma-Aldrich). The amount of sulfated glycosaminoglycans (GAGs) was determined using dimethylmethylene blue (DMB) assay of which the protocol was modified for measurement in alginate: the pH of the DMB reagent was lowered to 1.75 using formic acid [269, 270]. A spectrophotometer (VersaMax, Molecular Devices, Sunnyvale, USA) was used to measure the metachromatic reaction of GAGs with DMB at 540 and 595nm. Chondroitin sulfate C (Sigma-Aldrich) was used as a standard. The DNA content in papain-digested samples was determined after RNAse (Sigma-Aldrich) treatment using ethidium bromide (Gibco). Using a spectrofluorometer (Wallac 1420 Victor 2, Perkin-Elmer, Wellesley, MA, USA), the extinction and emission were measured at 340nm and 590nm respectively. Calf thymus DNA (Sigma-Aldrich) was used as a standard.

***In vivo* implantation of osteochondral biopsies**

hBMSC-alginate and hBMSC-fibrin constructs were created in simulated osteochondral defects as described above using passage 3 hBMSCs from three different donors. The hBMSC-hydrogel constructs in the simulated defects were cultured overnight to allow stabilization of the system and to verify sterility. Four osteochondral biopsies per mouse were implanted subcutaneously in female NMRI nu/nu mice (Charles River, Wilmington, MA, USA) under isoflurane anesthesia. The simulated defects in the osteochondral biopsies were covered using an 8mm diameter Neuro-Patch membrane (Braun, Melsungen, Germany) to prevent ingrowth of host tissue. Before and 6-10 hours after surgery, mice received 0.05mg/kg bodyweight of Temgesic (Reckitt Bensicker, Slough, UK). During surgery, mice received 9mg/kg bodyweight of Ampi-dry (Dopharma, Raamsdonksveer, the Netherlands). After 12 weeks, mice were euthanized by cervical dislocation. Osteochondral biopsies with hBMSC-hydrogel constructs were carefully explanted and fixed in 4% formalin. After at least 1 week of fixation, samples were decalcified using 10% formic acid (Sigma-Aldrich) for three weeks. Subsequently, biopsies were embedded in paraffin, sectioned in 6mm sections and subjected to histology. All animal experiments were conducted with approval of the local animal ethical committee (EMC2353, protocol number 116-11-06).

**Histology & quantification**

After sections were deparaffinized and rehydrated, safranin-O staining was performed to visualize glycosaminoglycans in the extracellular matrix and haematoxylin and eosin staining was performed to visualize general morphology. For safranin-O staining, slides were first stained with 0.1% light green for 8 minutes, subsequently washed in 1% acetic acid and stained with 0.1% safranin-O (Fluka) for 12 minutes. The cross-sectional area of the simulated cartilage defect was measured and the cross-sectional area of newly formed safranin-O positive tissue was determined using ImageJ software (National Institutes of Health, Bethesda, MA, USA). These measurements were performed on three sections of all osteochondral biopsies that were implanted subcutaneously. Newly formed tissue was discriminated visually from native cartilage. The presence or absence of bone formation in simulated defects was scored based on tissue morphology.

**Immunohistochemistry**

Immunohistochemical staining was performed for collagen type 2. To allow the use of mouse monoclonal antibodies, the primary and secondary antibodies were coupled before use. The primary antibody for collagen type 2 (II-II6B3, 0.4µg/mL, Developmental Studies Hybridoma Bank) was coupled overnight with goat-anti-mouse biotin (Jackson Laboratories, Bar Harbor, USA) followed by 2 hours incubation in 0.1% normal mouse serum (CLB, Amsterdam, the Netherlands). After sections were deparaffinized and rehydrated, antigen retrieval was performed by incubation for 30 minutes at 37°C in 0.1% pronase (Sigma-Aldrich) in PBS. Subsequently, slides were incubated for 30 minutes at 37°C in 1% hyaluronidase (Sigma-Aldrich) in PBS. Blocking for non-specific binding was performed using 10% goat serum (Sigma-Aldrich) in PBS. Slides were incubated overnight at 4°C with the coupled primary and secondary antibody or the negative mouse IgG control antibody (Serotec Ltd, Oxford, UK) in PBS containing 1% bovine serum albumin (PBS/BSA). Slides were incubated with enzyme-streptavidin conjugate (Label, HK-321-UK, Biogenex) diluted 1:100 in PBS/BSA and subsequently incubated with Neu Fuchsin substrate (Chroma, Kongen, Germany)

**Statistical analysis**

Normality was verified using Kolmogorov-Smirnov and Shapiro-Wilk normality tests using SPSS 15.0. When necessary, logarithmic transformation was performed to obtain normal distribution of the data. Student's t-test was used to analyze unpaired data. A generalized estimated equations model was used for paired data that was normally distributed. For paired data that was not normally distributed, Kruskal-Wallis test was performed, followed by the Mann Whitney U test. False discovery rate was used to correct for multiple testing. For all statistical analyses, differences were considered statistically significant at $p<0.05$.

## Results

### All four hydrogels support chondrogenesis of hBMSCs

hBMSC-hydrogel constructs were cultured up to 28 days in CCM and DNA content was determined at various time points as a measure of hBMSC survival. In alginate and alginate/HA constructs, DNA content decreased in time, where in fibrin and HA-pNIPAM this was not as evident (figure 1.a). hBMSC-alginate/HA constructs cultured for 28 days contained significantly less DNA than hBMSC-alginate (*p=0.006*), hBMSC-fibrin (*p=0.03*) or hBMSC-HA-pNIPAM (*p=0.009*) constructs. No differences were observed in terms of DNA content after 28 days of culture between hBMSC-alginate, hBMSC-fibrin and hBMSC-HA-pNIPAM constructs.

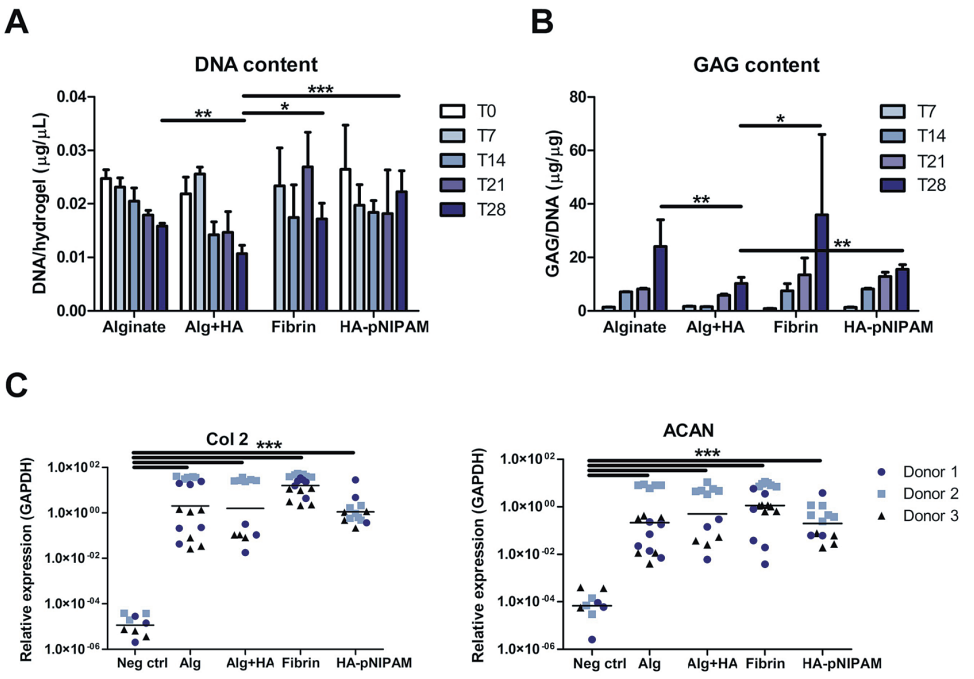



**Figure 1. Alginate, fibrin and HA-pNIPAM support chondrogenesis of hBMSCs.**
hBMSCs were cultured in complete chondrogenic medium up to 28 days. hBMSC-alginate constructs cultured in incomplete chondrogenic medium served as negative controls a. DNA quantification of hBMSC-hydrogel constructs cultured up to 28 days. hBMSCs from 1 donor, *n=6*; b. GAG quantification per DNA of hBMSC-hydrogel constructs cultured up to 28 days. hBMSCs from 1 donor, *n=6*; c. *Col2* and *ACAN* gene expression relative to *GAPDH* of hBMSC-hydrogel constructs cultured for 28 days. hBMSCs from 3 different donors, *n=6* per donor, negative controls *n=3* per donor. Means are represented with standard deviations. Student's t-test (a and b) or a generalized estimated equations model on log transformed data to obtain normally distributed data (c) was used. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Chondrogenic differentiation of hBMSC-hydrogel constructs cultured for 28 days in CCM was assessed by means of GAG content (figure 1.b) and cartilage related gene expression (figure 1.c). GAG content normalized to DNA content was determined in hBMSC-hydrogel constructs after 28 days of culture. In hBMSC-alginate/HA constructs, significantly less GAGs per DNA were observed than in hBMSC-alginate ($p=0.008$), hBMSC-fibrin ($p=0.044$) or hBMSC-HA-pNIPAM ($p=0.001$) constructs (figure 1.b). To verify whether deposited GAGs were potentially released from the hBMSC-hydrogel constructs into the medium, the GAG content of medium in which hBMSC-hydrogel constructs were cultured for three days was measured at various time points during the experiment. No GAGs were detected in used culture medium of all hydrogels (data not shown).

Chondrogenic differentiation, evaluated by cartilage related gene expression was observed in hBMSCs from three different donors in constructs formed with all four hydrogels cultured for 28 days in CCM. Chondrogenic differentiation was not observed in negative controls consisting of hBMSC-alginate constructs cultured in ICM (figure 1.c). The same was observed for the hBMSC-hydrogel constructs of the other hydrogels cultured in ICM (data not shown). No significant differences were observed between constructs from the different hydrogels cultured in CCM in terms of *Col2* and *ACAN* gene expression. Since no beneficial effect of the addition of HA to alginate was observed on GAG production by hBMSCs, hBMSC-alginate/HA constructs were not used for further experiments.

**Alginate favours hBMSC chondrogenesis in an osteochondral environment in vivo**

hBMSC-hydrogel constructs formed with alginate, fibrin or HA-pNIPAM were cultured in simulated defects in osteochondral biopsies for 28 days in ICM (figure 2.a). Significantly higher levels of *Col2* gene expression were measured in hBMSC-alginate constructs than were measured in the other hBMSC-hydrogel constructs ($p<0.001$). Due to the required temperature for creating and handling hBMSC-HA-pNIPAM construcs, we only managed to perform the experiment with hBMSC-HA-pNIPAM constructs with hBMSCs from one donor. For this reason and the fact that the use of HA-pNIPAM is not yet clinically relevant in its current form since total removal of copper catalyst remnants cannot yet be ensured, hBMSC-alginate and hBMSC-fibrin constructs were selected for the *in vivo* experiment.

After 12 weeks of subcutaneous implantation in nude mice, hBMSC-alginate and hBMSC-fibrin constructs in simulated defects in osteochondral biopsies were explanted and subjected to histology. hBMSC-alginate constructs formed substantial amounts of tissue positive for safranin-O staining, indicating the presence of GAGs in the newly formed tissue (figure 2.b). In hBMSC-fibrin constructs in simulated defects, hardly any safranin-O positive tissue was observed (figure 2.c).

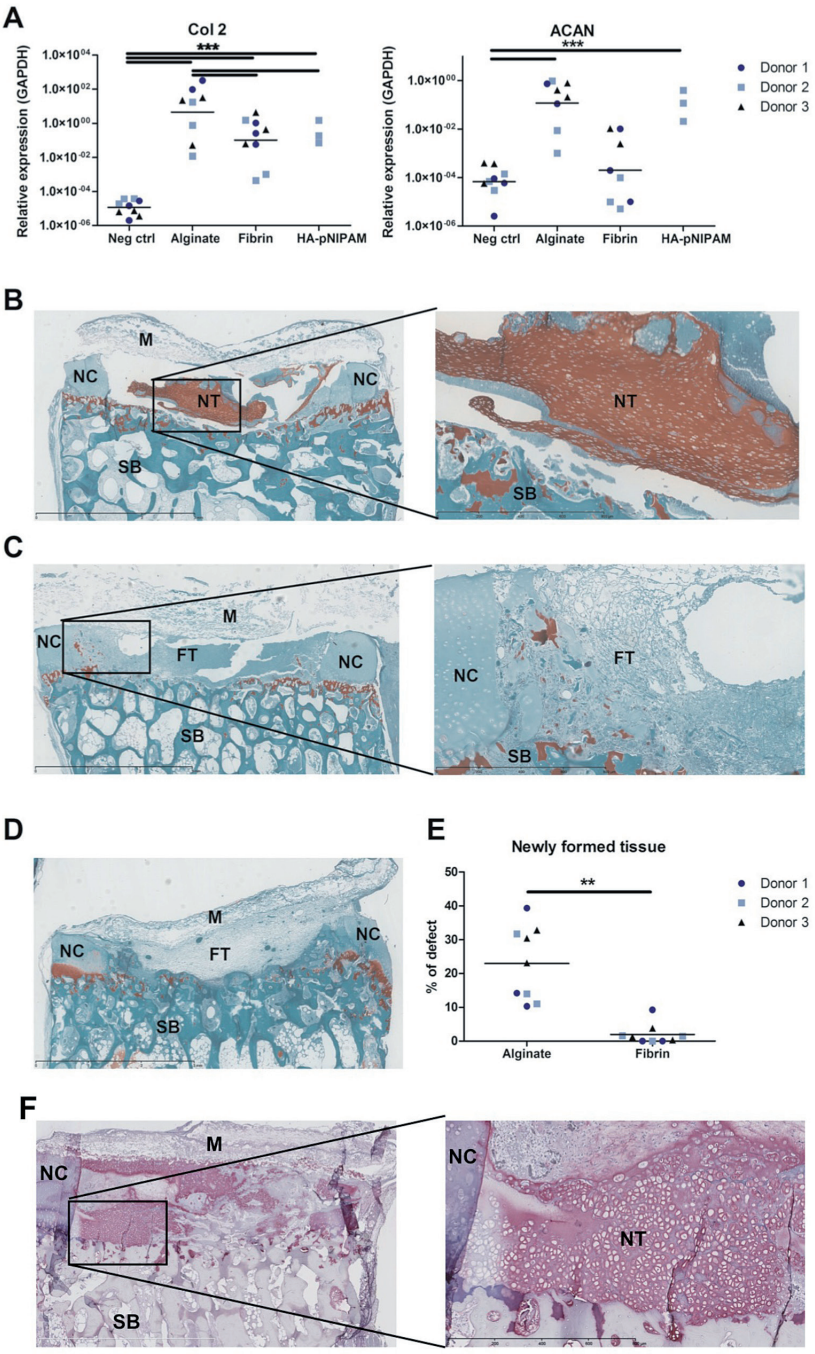


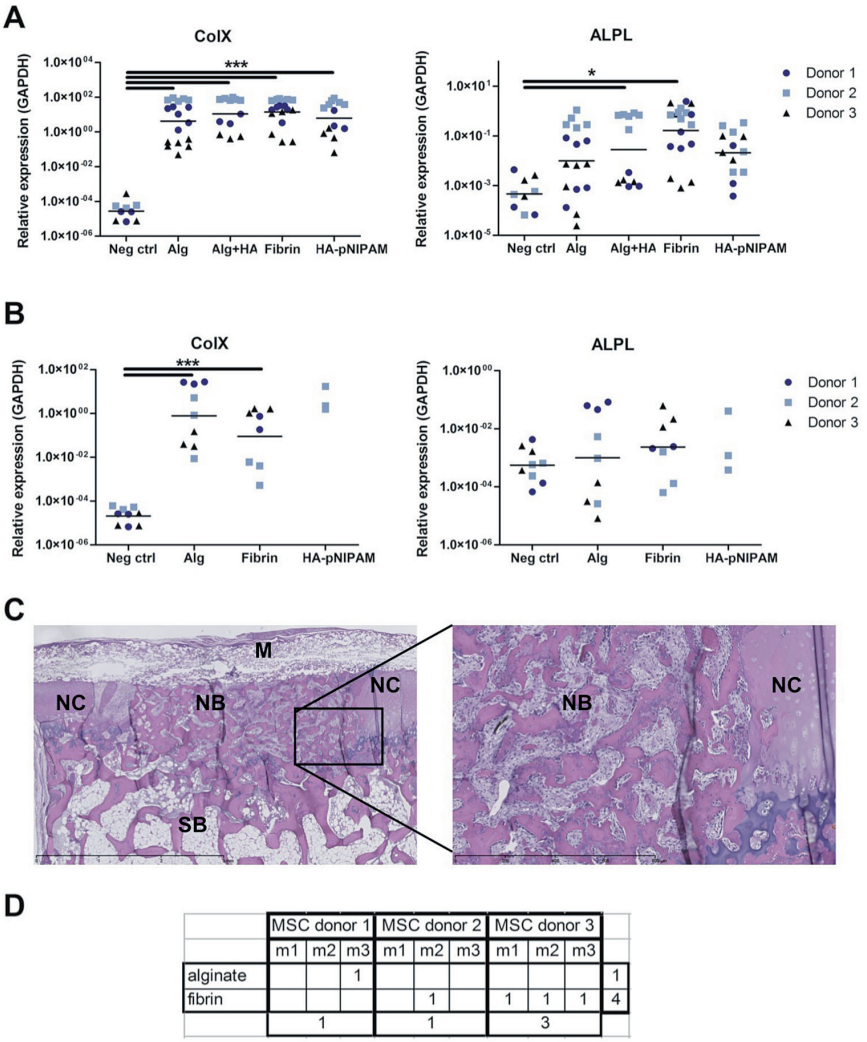

**Figure 2. Alginate, and not fibrin, favours *Col2* expression in vitro and chondrogenesis of hBMSCs in an osteochondral environment *in vivo*.**

*Figure 2. Continued*
a. Gene expression of *Col2* and *ACAN* relative to *GAPDH* of hBMSC-hydrogel constructs cultured in incomplete chondrogenic medium in simulated cartilage defects in an osteochondral biopsy model for 28 days. Negative controls: hBMSC-alginate constructs in incomplete chondrogenic medium without osteochondral biopsy. For alginate and fibrin: hBMSCs from 3 donors, *n=3* per donor; for HA-pNIPAM: hBMSCs from 1 donor, *n=3*; b,c,d. Representative safranin-O stained sections of hBMSC-alginate (b.), hBMSC-fibrin (c.) constructs in simulated cartilage defects in an osteochondral biopsy model that were implanted subcutaneously in nude mice for 12 weeks. hBMSCs from 3 donors, *n=3* per donor. d. control alginate construct without hBMSCs. Scale bars indicate 3mm (left images) or 800µm (insets); e. Quantification of subcutaneously implanted hBMSC-alginate and hBMSC-fibrin constructs in simulated cartilage defects in an osteochondral biopsy model. hBMSCs from 3 donors, *n=3* per donor. Generalized estimated equations model on log transformed data to obtain normally distributed data. **p<0.05, **p<0.01, ***p<0.001.*; f. Representative collagen type 2 immunostained sections of hBMSC-alginate constructs in simulated cartilage defects in an osteochondral biopsy model that was implanted subcutaneously in nude mice for 12 weeks. Scale bars indicate 3mm (left image) or 800µm (inset); SB: subchondral bone, NC: native cartilage, M: Neuro-Patch membrane, NT: newly formed tissue, FT: fibrous tissue.

This was reflected upon quantification of safranin-O positive repair tissue: significantly more safranin-O positive repair tissue was formed in hBMSC-alginate constructs in simulated defects (figure 2.e). Immunostaining demonstrated corresponding deposition of collagen type 2 in hBMSC-alginate constructs in simulated defects (figure 2.f.), where hardly any collagen type 2 positive tissue was observed in hBMSC-fibrin constructs (data not shown). In control defects, in which hydrogel constructs without hBMSCs were implanted, no safranin-O positive tissue was observed (figure 2.d).

**Occasional bone formation in vivo was found in hBMSC-fibrin constructs**

A known characteristic of hBMSCs is that upon *in vitro* chondrogenesis, these cells tend to become hypertrophic, which ultimately leads to terminal differentiation characterized by mineralization *in vitro* and bone formation *in vivo* [74, 354]. Gene expression of the hypertrophy-related genes *ColX* and *ALPL* were measured in hBMSC-hydrogel constructs cultured in CCM (figure 3.a) or in simulated cartilage defects in the osteochondral biopsy model in ICM (figure 3.b). No significant differences were observed in *ColX* or *ALPL* gene expression between groups, apart from the negative controls consisting of hBMSC-alginate constructs cultured in ICM. Despite the expression of hypertrophic genes *in vitro*, bone formation was only observed occasionally after 12 weeks of subcutaneous implantation of hBMSC-hydrogel constructs in the osteochondral biopsy model (figure 3.c). The presence of observed bone formation was scored (figure 3.d). Bone formation was observed in one out of nine hBMSC-alginate constructs, where it was observed in four out of nine hBMSC-fibrin constructs. In all samples in which bone formation was observed, the newly formed bone filled the entire defect. The occasional bone formation was mainly observed in hBMSC-fibrin constructs involving hBMSCs from one specific donor. No bone formation was observed in control defects, i.e. alginate or fibrin without hBMSCs in simulated defects implanted subcutaneously in nude mice for 12 weeks.




D

| | MSC donor 1 | | | MSC donor 2 | | | MSC donor 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | m1 | m2 | m3 | m1 | m2 | m3 | m1 | m2 | m3 | |
| alginate | | | 1 | | | | | | | 1 |
| fibrin | | | | | 1 | | 1 | 1 | 1 | 4 |
| | 1 | | | 1 | | | 3 | | | |

**Figure 3. Bone formation *in vivo* appears less frequent in alginate than in fibrin.**
a,b. *ColX* and *ALPL* gene expression relative to *GAPDH* of hBMSC-hydrogel constructs cultured in complete chondrogenic medium (a.) or in incomplete chondrogenic medium in simulated cartilage defects in osteochondral biopsies (b.). hBMSCs from 3 different donors, *n=6* (a.) or *n=3* (b.) per donor. Negative controls: hBMSC-alginate constructs in incomplete chondrogenic medium without osteochondral biopsies. Generalized estimated equations model on log transformed data to obtain normally distributed data. **p<0.05, **p<0.01, ***p<0.001*; c. Representative haematoxylin and eosin stained sections of hBMSC-fibrin constructs in simulated cartilage defects implanted subcutaneously in nude mice for 12 weeks in which bone formation was observed. SB: subchondral bone, NC: native cartilage, M: Neuro-Patch membrane, NT: newly formed tissue, FT: fibrous tissue. Scale bars indicate 3mm (left image) or 800µm (inset); d. Scoring of the presence of bone formation by hBMSC-alginate or hBMSC-fibrin constructs in simulated cartilage defects implanted subcutaneously in nude mice for 12 weeks. hBMSCs from 3 donors*, n=3* per donor. m# indicate the individual mice;

## Discussion

Alginate, fibrin and multiple HA-based biomaterials have been described for cartilage regeneration purposes in various settings and combinations [92, 355-358]. We compared these materials directly for their capacity to support chondrogenesis of hBMSCs in an osteochondral environment for the first time. Although results of hBMSC-hydrogel constructs after conventional *in vitro* culture were comparable, chondrogenesis of hBMSCs in an osteochondral environment both *in vitro* and *in vivo* appeared to be hydrogel dependent.

The viability of hBMSCs encapsulated in the different hydrogels was not compromised and in conventional *in vitro* experiments, chondrogenesis was supported by all four hydrogels. Upon culture in the osteochondral biopsy model, hBMSC-alginate constructs cultured in ICM in the osteochondral model resulted in significantly higher *Col2* and *ACAN* gene expression than hBMSC-fibrin constructs. These results were supported by the *in vivo* experiment: implantation of osteochondral biopsies with hBMSC-alginate constructs resulted in significantly more safranin-O positive repair tissue after 12 weeks than was found in biopsies with hBMSC-fibrin constructs. Our findings are in line with findings in literature: it was reported that upon blending fibrin and alginate, higher fibrin content was more favorable for hBMSC proliferation, where higher alginate content supported chondrogenesis [359]. However, no significant differences in DNA content were observed after 28 days of culture. These findings suggest that conventional *in vitro* culture might not be sufficiently representative to study the applicability of in various hydrogels with hBMSC for cartilage repair.

A possible explanation for the differences between hBMSC-alginate and hBMSC-fibrin constructs after *in vivo* implantation can be that fibrin allows binding of cells and growth factors to its 3D architecture, where alginate does not interact with cells and only encapsulates them [360]. In previous research, we have found that factors other than TGFβ secreted by the osteochondral biopsies are capable of inducing chondrogenesis of hBMSCs [353]. It is possible that upon culture in CCM, the high dosage of TGFβ circumvented this effect in conventional culture without the osteochondral biopsies.

A point of concern is the integration of newly formed tissue onto the native cartilage and subchondral bone during the 12 weeks of subcutaneous implantation. It is possible that the relatively poor integration was an artefact of processing for histology; the removal of possible hydrogel remnants may have caused slight shrinkage of the newly formed tissue, thereby damaging the fragile attachment onto the native bovine tissue. Another option is that integration was not optimal. Presently there is no way to distinguish these possible explanations.

Interestingly, no beneficial effects on chondrogenesis were observed in hBMSC-alginate/HA constructs. High molecular weight HA is very viscous, therefore the viscosity of alginate/HA was also higher than that of alginate only. It is possible that the higher viscosity of alginate/HA has compromised the diffusion of nutrients and growth factors into the hBMSC-alginate/HA constructs, thereby decreasing the effectiveness of GAG deposition. Diffusion of nutrients is known to affect the effectiveness of hBMSC chondrogenesis [361]. Also, increasing density of HA hydrogels has been shown to negatively affect matrix deposition by hBMSCs [362]. Still chondrogenesis took place in alginate/HA, indicated by comparable levels of *Col2* and *ACAN* gene expression after 28 days of culture in chondrogenic medium for all four hydrogels. Since positive findings were reported for the use of HA both clinically and in laboratory settings [363-369], we still believe that HA can be beneficial for cartilage tissue engineering purposes, albeit not in the current combination of high molecular weight HA with alginate. Therefore, besides alginate and fibrin, HA-pNIPAM was used to prepare hBMSC-hydrogel constructs in the osteochondral biopsy model. Due to the thermo-responsive nature of HA-pNIPAM, this was found to be very challenging. As mentioned before, HA-pNIPAM is a non-Newtonian liquid that gels for T>32°C and is liquid for T<32°C. This poses some practical requirements, such as adequate pre-warming of media and tools that come in contact with hBMSC-HA-pNIPAM constructs. For culture in the osteochondral biopsy system, these requirements were hard to meet, resulting in a successful experiment with hBMSCs from only one donor. Based on this experience, we decided not to use hBMSC-HA-pNIPAM constructs for the *in vivo* experiment. Apart from this, it is very likely that upon subcutaneous implantation the temperature inside the simulated defects would drop below the required 32°C, thereby losing the gel-state of the hBMSC-HA-pNIPAM constructs. Given the positive effects on cell viability and chondrogenesis, we believe that HA-pNIPAM could be an interesting for cartilage regeneration strategies, despite the technical challenges with its use in our culture systems. Modification of this material making its gelation non-reversible while maintaining its in situ gelation capacity would make the use of this material in our culture system more feasible. Another option would be to study HA-pNIPAM in orthotopic *in vivo* models, ensuring the required temperature.

In vitro chondrogenesis of hBMSCs is known to be accompanied by hypertrophy [68, 74, 314]. In strategies aiming for cartilage regeneration, terminal differentiation leading to mineralization and ultimately to bone formation is undesirable [79, 319, 354]. Relatively high levels of *ColX* and *ALPL* gene expression were found in all hydrogels both in normal *in vitro* culture of hBMSC-hydrogel constructs and in the osteochondral biopsy model. No significant differences between the different hydrogels were observed. Histology for safranin O and immunohistochemical staining for collagen type 2 after in vitro experiments could provide more information on potential differences in terms of the quality of the formed cartilaginous

tissue. However, this is unfortunately not possible since decalcification of osteochondral biopsies is required prior to (immuno)histology. For example, removal of calcium removes the cross-links of alginate, which causes the relatively small amounts of newly formed tissue after the in vitro experiments to fall apart. *In vivo* bone formation was associated primarily with hBMSC-fibrin constructs suggesting that alginate prevented the progression of hypertrophy towards terminal differentiation of hBMSCs. Since no bone formation was observed in control defects containing alginate or fibrin without hBMSCs, we can conclude that the observed bone formation in hBMSC-fibrin constructs in simulated defects *in vivo* was formed or induced by hBMSCs. However, it is possible that growth factor binding by fibrin in the osteochondral biopsies was responsible for slower progression of chondrogenesis than in hBMSC-alginate constructs, implying a delay in expression of hypertrophic genes rather than lower expression.

## Conclusion

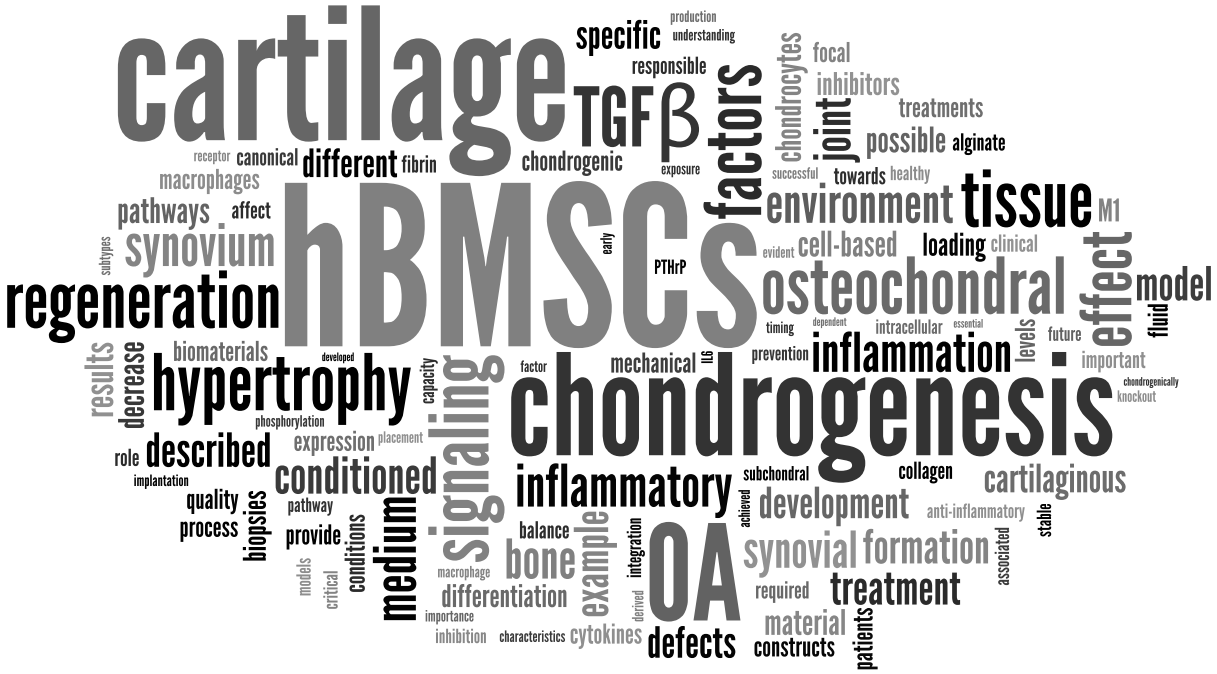
The present study revealed important clues for the future development of clinical hBMSC-based cartilage regeneration strategies. This study demonstrates that conventional culture of hydrogel constructs might not be sufficiently representative to study chondrogenesis of hBMSCs in detail. Culture of hBMSC-hydrogel constructs in the more representative joint-like environment provided by the osteochondral biopsies revealed differences in chondrogenic potential of hBMSCs encapsulated in different hydrogels that were confirmed *in vivo*, indicating that chondrogenesis of hBMSCs is strongly hydrogel-dependent. Models providing a representative joint-like environment such as the osteochondral model are of vital importance to gain insight in hBMSC chondrogenesis for the future development of clinical hBMSC-based cartilage regeneration

## Acknowledgement

The research leading to these results has received funding from the European Union's 7$^{th}$ Framework Programme under grant agreement nos. NMP3-SL-2010-245993 and HEALTH-2007-B-223298, and Science Foundation Ireland (grant number 09/SRC/B1794). The research in this article was performed within the framework of the Erasmus Postgraduate School Molecular Medicine. The authors would like to thank Lizette Utomo for conducting pilot experiments for the present study during her internship as part of the master technical medicine of the University of Twente, Enschede, the Netherlands and Matteo d'Este of the AO Research Institute in Davos for his contribution to the HA-pNIPAM hydrogel data.

# Chapter 8

## General discussion, Summary, Nederlandse samenvatting



## General discussion

The incidence of OA, as leading cause of disability in the aging population, is increasing in western society. This poses high burden on society in terms of wellbeing and quality of life of people as well as in terms of costs for health care [370]. Since the development of focal cartilage lesion towards OA is a process that may take decades, evaluation of treatments that aim to prevent the development of OA is a tedious process. The complex architecture of articular cartilage increases the difficulty of regeneration. This is the case for both the microfracture technique as well as for autologous chondrocyte implantation and its derivatives [59, 157, 159]. As reviewed in **chapter 2**, it is known that after initial successful treatment with one of these techniques complaints return, because of inferior quality of the cartilaginous tissue or by incomplete or total lack of integration into surrounding tissue. This implicates that, although these treatments may potentially delay the onset of OA, prevention of early development of OA is not guaranteed. Therefore, the development of cell-based cartilage regeneration strategies is of vital importance for sufficient repair of cartilage defects. The use of autologous chondrocytes has several disadvantages; defects need to be created in order to obtain chondrocytes, their in vitro expandability is limited and chondrocytes tend to dedifferentiate upon in vitro culture [68]. Because of these disadvantages of chondrocytes, this thesis focuses on the use of hBMSCs for this purpose.

**Inflammation**

In OA and in otherwise injured joints, inflammation plays an important role. Synovial inflammation causes pain and swelling of the joint [371]. Inflammatory factors produced by OA synovium, such as IL1 and TNFα are known to negatively affect chondrogenesis of hBMSCs [211]. In **chapter 3 and 4**, we confirmed this effect using OA synovium conditioned medium as a model for synovial inflammation. Several inflammatory cytokines IL6 signal via JAK signaling, where others such as TNFα and IL1α signal via TAK1 [212-222]. In **chapter 3** it is reported that inhibition of these signaling pathways can to some extent counteract the negative effect of OA synovium conditioned medium on hBMSC chondrogenesis. Simultaneous inhibition of both pathways has an additive effect. However, it was found that timing is critical in this set-up. Counteraction of the effect of OA synovium conditioned medium by the inhibitors was only successful when the administration of the medium and the inhibitors was started after the induction of chondrogenesis in hBMSCs. When chondrogenesis was impaired by OA synovium conditioned medium without prior induction of chondrogenesis, no counteraction by the inhibitors was achieved. In embryonic endochondral ossification phosphorylation of Stat1, a transcription factor that is involved in JAK signaling, functions as inhibitor of chondrocyte proliferation prior to hypertrophy and it is also involved in ECM remodeling [372].

Early inhibition of TAK1 could alter signaling in such way that the balance of signaling pathways essential for chondrogenesis is compromised.

TAK1 is known to be involved in intracellular signal transduction of different stimuli that exert their effects via distinct pathways. These pathways have different downstream targets and thereby result in the expression of different genes. TAK1 is mainly involved in the innate immune response and pro-inflammatory signaling [416]. It can for example be activated by IL-1, TNFα or TLR agonists, resulting in activation of NF-kB, JNK or p38 respectively. Different stimuli result in distinct downstream signaling cascades, for example TAK1 activation by TLR agonists leads to TNF-receptor associated factor 6 (TRAF6) signaling, where activation by IL1 leads to TRAF2 signaling [417-420]. Subsequently, TAK1 forms heterotrimer complexes with TAK1-binding protein 1 (TAB1) [417,418] and TAB2 or TAB3 [419,420]. In order for TAB2 or TAB3 to be able to form these complexes with TAK1 and TAB1, binding of K63 polyubiquitin is required [417,421]. The exact binding mechanism of K63 polyubiquitin to TAB2 or TAB3 is unknown, but it is known that the binding affinity increases with chain length of polyubiquitin [421,422]. It is possible that this chain length plays a role in determining further downstream signal transduction after TAK1 activation.

Apart from the innate immune system, TAK1 can also be involved in non-canonical signaling pathways of TGFβ and Wnt [213, 215][423,424]. For example in non-canonical TGFβ signaling, TAK1 activation leads to signaling via x-linked inhibitor of apoptosis protein (XIAP) or TRAF6, resulting in either JNK, p38, NF-kB or calcineurin activation [425-427]. Apart from all these distinct signals, TAK1 activation can also be a result of osmotic shock, hypoxia or DNA damage [428-430]. So although TAK1 is mainly associated with the innate immune response, it is also involved in many other processes [431]. In the experiments in chapter 2, OA synovium conditioned medium is used to simulate an inflammatory environment. It was found that inhibition of TAK1 results in partial counteraction of the inflammatory effect of OA synovium conditioned medium, indicating that there are factors in OA synovium conditioned medium that can activate TAK1. In control conditions in which the TAK1 inhibitor is present and no OA synovium conditioned medium is added, no inhibition of chondrogenesis of hBMSCs was observed. This indicates that the non-canonical TGFβ signaling via TAK1 does not play a major role in hBMSC chondrogenesis at the stage in the process where the TAK1 inhibitor and the OA synovium conditioned medium were added. It was also found that initiation of chondrogenesis was necessary before addition of the TAK1 inhibitor to prevent the negative effect of the conditioned medium. This can be an indication that TAK1 plays a different role in different stages of chondrogenesis. In order to study this, for example an experiment in which the TAK1 inhibitor is added to hBMSCs at different stages of chondrogenesis can be performed. Western blots

for different downstream targets of TAK1 on samples treated with the TAK1 inhibitor or untreated samples could reveal the specific TAK1 associated pathways that are involved at specific stages of chondrogenesis of hBMSCs.

As mentioned, it is unknown how the specific downstream signaling after initial TAK1 activation is selected. Since the rate at which chondrogenesis of adult hBMSCs takes place is known to vary between different donors, it could be challenging to determine the optimal window of administration for the TAK1 inhibitor to exert its protective effect for inflammatory signals. If this timing is not correct, it is possible that pathways that are crucial for successful chondrogenesis might be compromised. These issues need to be addressed for translation to clinical applications. The inhibitors need to be administered in such way that the formation of cartilaginous tissue is not prevented, for example by treatment of inflammation prior to placement of cells in a cartilage defect, or by tissue engineering cartilaginous constructs in vitro.

In the synovium, macrophages of different subtypes are present. Monocytes are stimulated towards an inflammatory M1 macrophage phenotype, or to a tissue repair M2 macrophage phenotype by for example IFNγ and IL4 respectively [255, 268]. M1 are known to produce various inflammatory factors. In synovial fluid of OA patients factors like IL6, RANTES and macrophage derived chemokine (MDC) that can be produced by M1 macrophages, were found to be present in higher levels than in synovial fluid of healthy controls [373]. **Chapter 4** reports proof of principle that synovial macrophages are responsible for the negative effect of OA synovium by showing that a macrophage-enriched population isolated from OA synovium had an effect on hBMSC chondrogenesis similar to OA synovium conditioned medium. A fibroblast-enriched population did not have this effect. Using peripheral blood derived monocytes that were stimulated towards an M1 or M2 phenotype, conditioned media were prepared that were used as a model system for subtypes of synovial macrophages. Together, these findings lead to the conclusion that M1 synovial macrophages play a key role in the negative effect of OA synovium on hBMSC chondrogenesis. This implicates that targeting synovial M1 macrophages might be an effective treatment for inflammation in OA and potentially also in joint inflammation that is not associated with OA. To study this, OA synovial explants could be treated with anti-inflammatory mediators prior to preparation of synovium conditioned medium. This conditioned medium could be added to chondrogenically differentiating hBMSCs to verify the effectiveness of the treatment.

It was reported in literature that significantly more IL1β, bFGF and IGF were present in synovial fluid of patients with focal cartilage defects than in the synovial fluid of healthy patients [374]. Synovial fluid of patients with focal cartilage defects as well as that of

OA patients was reported to contain significantly more IL6 than synovial fluid of healthy persons [375]. In **chapter 4** it is reported that high levels of IL6 were present in OA synovium conditioned medium as well as in M1 macrophage conditioned medium. It is now evident that relevant levels of inflammatory cytokines can be present in OA joints as well as in joints with focal cartilage defects. This implicates that the negative effects of inflammatory cytokines play important roles in both an OA environment as well as in case of focal cartilage defects. In focal cartilage defects, inflammation needs to be treated adequately in order to provide a favorable environment for cell-based cartilage regeneration.

**The effect of the osteochondral environment on chondrogenesis**

The osteochondral model that is presented in **chapter 5** was validated using bovine chondrocytes. This approach was chosen to provide optimal conditions for cartilage regeneration, allowing testing of different cell-based methods as a validation of the model. As expected, these passage 0 chondrocytes spontaneously formed cartilaginous tissue when they were directly seeded into osteochondral defects. In contrast to our hypothesis, hBMSCs also spontaneously formed cartilaginous tissue without supplementing TGFβ when placed in alginate in osteochondral defects as described in **chapter 6**. As we found that blocking of TGFβ signaling in different ways did not inhibit this effect, we concluded that factors other than TGFβ must be responsible for this effect. The subchondral bone was found to be responsible for the production and secretion of these factors. Due to the tightly balanced interplay of osteoblasts and osteoclasts, factors are released by remodeling bone. The role of the subchondral bone as an active joint tissue is gaining more and more attention [24, 25].

***Stable cartilage formation***

The tendency of hBMSCs to undergo terminal differentiation is advantageous for bone tissue engineering, however it is highly undesired for cartilage regeneration. The embryonic process of endochondral bone formation is mimicked by using chondrogenically differentiated hBMSCs as a template for later mineralization and bone formation [79, 319]. Addition of growth and differentiation factor 5 (GDF5), which is associated with the chondrogenic phase of embryonic endochondral bone formation, to hBMSCs undergoing chondrogenesis resulted in increased hypertrophy [376]. To induce chondrogenesis of hBMSCs in vitro, a specific chondrogenic medium containing TGFβ is required [72, 73]. BMP2 is known to act synergistically in this process [377]. However, both of these factors are also involved in intracellular pathways that are associated with terminal differentiation and bone formation. TGFβ can exert its effect on hBMSCs via two distinct intracellular pathways. The canonical binding of TGFβ to the ALK5 receptor results in Smad2/3 phosphorylation, which results in a signal transduction cascade associated with chondrogenesis [378]. Alternatively,

TGFβ can bind to the ALK1 receptor, which initiates the canonical BMP signaling pathway via Smad1/5/8 phosphorylation [379-381]. This results in the expression of hypertrophy related factors such as runt-related transcription factor 2 (Runx2), causing up-regulation of collagen type X, MMPs and VEGF, leading to terminal differentiation [326, 382]. The balance in TGFβ signaling via both of these pathways determines the composition of the cartilaginous tissue. Inhibition of the alternative pathway is known to decrease the expression and production of hypertrophic factors, where inhibition of the canonical pathway results in up regulation of these factors [378, 383]. However, timing in relation to the stage of chondrogenesis of hBMSCs was found to play a critical role in this process [314].

It is possible that an imbalance of TGFβ signaling via the canonical Smad2/3 route and the non-canonical Smad1/5/8 pathway is responsible for hypertrophy [314, 384-386]. Potentially, in vitro conditions stimulate this imbalance unintended [77]. Inhibition of the non-canonical TGFβ signaling pathway by prevention of Smad1/5/8 phosphorylation has been shown to decrease the hypertrophy of hBMSCs, although full prevention of hypertrophy was not achieved [314]. TGFβ is the key inducer of chondrogenesis of hBMSCs in vitro however it does not prevent hypertrophy [74, 75, 387]. An indication that differences in the balance of TGFβ signaling may play a role is that different subtypes of TGFβ were found to affect the extent of hypertrophy of chondrogenic hBMSCs, where TGFβ1 had the least effect on mineralization, although roughly 75% of the sequences are equal [310, 388]. It has been suggested that, although the subtypes use the same receptors to induce intracellular signaling, specific interactions with the TGFβ receptors may be responsible for differences in intracellular signaling cascades [389]. The affinity of TGFβ to bind to either the canonical or non-canonical receptor should be studied in order to gain understanding of the balance and interplay between both signaling pathways. Homozygous TGFβ1 or 2 knockout results in perinatal or fetal mortality and severe developmental defects [390, 391]. Experiments involving for example gene silencing in hBMSCs using specific short hairpin RNA (shRNA) or small interfering RNA (siRNA) for TGFβ or its receptors can provide clues on this. Conditional knockout mice, in which knockout of specific genes is regulated by pharmaceutical compounds, can also be used to study the balance of the pathways used for TGFβ signaling.

Parathyroid hormone related protein (PTHrP) is also known to suppress hypertrophy by suppression of Indian Hedgehog (Ihh): increased hypertrophy has been reported in knockout of PTHrP or its receptor [392-394]. In hBMSCs undergoing chondrogenesis administration of PTHrP results in down-regulation of hypertrophy. However mixed effects are reported on the effect of PTHrP on chondrogenesis of hBMSCs: down-regulation as well as up-regulation of collagen type 2 was described [80, 81, 83]. This might have been caused by

the use of different culture media, resulting in distinct intracellular signaling of PTHrP [395]. Wingless/Int (Wnt) signaling by prevention of β-catenin degradation results in stimulation of Runx2 expression. It also stimulates hypertrophy in hBMSCs undergoing chondrogenesis [396]. Inhibition of canonical Wnt signaling resulted in up-regulation of collagen type 2 and aggrecan expression in hBMSCs, where it did not decrease collagen type X expression [397]. Various other processes have been associated with hypertrophy, such as integrin binding and several epigenetic features as reviewed by Studer et al [395]. Altogether, chondrogenesis of hBMSCs and the accompanying hypertrophy is a tightly balanced process over which at this moment no full control is possible in vitro.

Culture of hBMSCs in the osteochondral model as described in **chapter 6** appears to decrease the expression of the hypertrophy related genes collagen type X and ALP. It is possible that this is due to the fact that lower levels of TGFβ are secreted by the osteochondral biopsies than are normally present in chondrogenic medium, affecting the balance of TGFβ signaling. Since the subchondral bone secretes a cocktail of factors, it is possible that together these factors provide an environment that favors stable cartilage formation. To study which factors are responsible for this effect, the factors that are secreted by the subchondral bone could be identified using for example multiplex ELISA. However, the use of this technique requires a selection of factors one wants to study. Since the composition of the cocktail of factors is unknown, a technique that screens broader might be more suitable, for example mass spectrometry.

**Biomaterials**

It has become more and more evident, the micro-environment in which hBMSCs reside plays an essential role in chondrogenesis. Until recently, it was thought that providing a 3D environment and specific mechanical properties were the most important features for biomaterials suitable for cartilage regeneration. The spontaneous chondrogenesis of hBMSCs in the osteochondral model which was not dependent of TGFβ (**chapter 6)** was found to be micro-environment dependent (**chapter 7)**. When fibrin was used to encapsulate the hBMSCs instead of alginate, chondrogenesis of hBMSCs in the osteochondral model was decreased in vitro. Upon subcutaneous implantation of the system in nude mice, this effect was even stronger since barely any safranin O positive repair tissue was formed by hBMSCs in fibrin in the osteochondral model. The occasional bone formation was almost exclusively observed when fibrin was used. It is likely that the interaction of different factors as well as cell-material interactions differ between alginate and fibrin, thereby causing differences in exposure of the hBMSCs to factors secreted by the subchondral bone in **chapter 7**.

Various biochemical and biophysical characteristics of biomaterials affect hBMSC chondrogenesis. For example a higher crosslinking density of HA based hydrogels was found to increase hypertrophy of hBMSCs ondergoing chondrogenesis [398]. Addition of chondroitin sulphate to polyethyleneglycol (PEG) gels was found to decrease collagen type X and Runx2 expression [85]. Modification of alginate in such way that it became sulphated was found to efficiently bind TGFβ1 and this functionalized material induced chondrogenesis of encapsulated hBMSCs [399]. It is possible that in hBMSCs in alginate in the osteochondral model in **chapter 7**, the off set of chondrogenesis and thereby the deposition of GAGs takes place earlier than in fibrin, thereby causing a stimulating environment that further enhances chondrogenesis and prevents hypertrophy. This may explain both the observed chondrogenesis and cartilaginous tissue formation by hBMSCs in alginate in the osteochondral model and the occasional bone formation observed in fibrin described in **chapter 7**.

Since stable cartilage formation using hBMSCs has not yet been achieved using the currently available culture models and biomaterials, the development of sophisticated biomaterials should continue. Using evolving chemical techniques, specialized biomaterials for cartilage regeneration are being developed. The HA-pNIPAM hydrogel that was used in **chapter 7** is an example of such a sophisticated biomaterial. The thermoresponsive characteristics of this material, being liquid at temperatures below 32$^{o}$C and gel at temperatures above 32$^{o}$C, make this material interesting for future clinical applications, as it allows minimal invasive application by injection followed by in situ gelation. Since this material was still under development when it was used for the experiments described in **chapter 7**, it requires further extensive optimization of protocols for use in experiments. The prerequisite temperature could not be guaranteed upon subcutaneous implantation in mice. Further development of this gel, for example making its gellation non-reversible, would make the use of this material for cartilage regeneration purposes more feasible. Sophisticated materials like the HA-pNIPAM hydrogel can be of vital importance for hBMSC-based cartilage regeneration. These materials could be tailored in such way that they induce chondrogenesis of hBMSCs, help in prevention of hypertrophy or counteract inflammation. This could be achieved by incorporation of growth factors or drugs, by using functionalized chemical groups or by specific chemical and physical properties, such as density, surface micro-architecture or the extent of crosslinking. Although many different biomaterials have been and are being developed, thorough testing for their suitability for cartilage regeneration is required. The optimal biophysical characteristics such as density, viscosity and features like functionalized groups and growth factor binding should be determined. The models described in this thesis provide platforms that can be used for such screenings in both focal cartilage lesions as well as in an inflammatory environment.

**Mechanical loading**

An issue that is not addressed in this thesis is mechanical loading. During the last decade, many bioreactors have been developed that aim to increase the quality of tissue engineered cartilaginous constructs by applying mechanical loading as reviewed by Grad et al [400]. Initially, bioreactors were designed to apply either compression or shear stress [89, 401-403]. Presently, more complex bioreactors exist that are capable of applying both compression and shear stresses, simulating the movements of a ball-socket joint like the hip [404, 405]. Upon loading of the joint, the hydrostatic pressure in cartilage increases due to the GAGs that retain water in the cartilage ECM. As reviewed by Elder et al, applying hydrostatic pressure on tissue engineered constructs has been shown to increase cartilaginous ECM production [406]. However, the amount of load applied, the exact patterns in which it is applied and the type of construct are critical factors that determine the outcome of the experiment [407]. For example the same pattern applied on chondrocytes in monolayer or on cartilage explants contradictively resulted in inhibition or stimulation of GAG production respectively [407]. Also donor age and the biomaterial used as a scaffold were found to affect the response of chondrocytes and hBMSCs to mechanical loading [408-410].

For successful cell-based cartilage regeneration, taking mechanical loading into account appears to be inevitable. However, this poses some requirements that might be hard to meet when working with explants or biopsies. The exact thickness, flatness of the surface and the state of the native cartilage and/or bone can strongly affect the exact distribution of mechanical forces and thereby the exact mechanical loading experienced on cell level can strongly vary within samples or among samples. This can lead to mixed and incomparable results. To be able to use the osteochondral biopsy model described in **chapter 5**, the methods to create the osteochondral biopsies should be optimized in order to obtain more uniformly shaped biopsies. However, this would be very challenging, since for example the flat area on the surface of the cartilage of the proximal sesamoid bones of the metacarpal phalangeal joint is often not sufficiently sized to be able to obtain a completely flat surfaced biopsy. This indicates that it is not only a technical challenge; it is also dependent on the biological material that is available.

Mechanical loading is not only of importance for in vitro generation of tissue engineered cartilage constructs. It is also important for cell-based patient treatments without prior in vitro generation of constructs. The specific rehabilitation programs that are used for patients that were treated with microfracture or ACI reflect this. Initially, weight bearing Is limited for several weeks, after which weight bearing is gradually increased [50, 55, 57, 58, 142, 157]. In the development of future cell-based treatments for cartilage regeneration, these rehabilitation protocols should be further analyzed.

**Translation towards cell-based clinical applications**

Although direct translation of the research described in this thesis towards clinical cell-based cartilage regeneration is not yet possible, important issues for future developments have been identified. For cartilage regeneration in an inflammatory environment, whether this is caused by OA or by a recent trauma, induction of chondrogenesis of hBMSCs appears to be necessary prior to exposure to the catabolic environment for successful treatment using inhibitors of inflammatory pathways. One approach could be to treat the joint with anti-inflammatory agents prior to placement of hBMSCs in the cartilage lesion to decrease the levels of inflammatory cytokines to as low levels as possible. This anti-inflammatory treatment should specifically target M1 synovial macrophages. Subsequently hBMSCs can be placed in the cartilage lesion, accompanied by treatment with the inhibitors. This would still be necessary, since every violation of joint integrity, no matter how small, causes at least some sort of inflammatory response. It has been shown that certain NSAIDs affect chondrogenically differentiating healthy hBMSCs and OA hBMSCs in different ways: hypertrophy in healthy hBMSCs is decreased, where there is no effect in OA hBMSCs [411]. The creation of tissue-engineered cartilage in vitro prior to implantation poses problems with integration into surrounding tissue. Treatment with enzymes prior to placement of constructs can help to favor integration. Since it is known that mature cartilage results in less efficient integration than young cartilage, one could aim to tissue engineer cartilaginous constructs in such way that chondrogenesis is not fully complete to favor integration.

In order to use undifferentiated hBMSCs, it is required to study whether pre-treatment of the joint to decrease the inflammation and use of the inhibitors is sufficient to allow chondrogenesis of hBMSCs. This could be simulated in vitro using the models described in this thesis. The osteochondral biopsies could be pre-exposed to OA synovium conditioned medium before the hBMSCs are placed in the simulated defects to simulate pre-existing inflammation. Different conditions could be applied: continuation or stop of exposure to OA synovium conditioned medium, treatment with anti-inflammatory compounds prior to placement of the hBMSCs and conditions in which hBMSCs in different stages of chondrogenesis are used. All conditions can be executed with and without using the JAK1/TAK inhibitors. One could also think of the use of human OA material to prepare osteochondral biopsies. However, this poses some practical issues. The quality of the material that is left over from total knee replacement surgeries highly varies in quality. In some cases, there is barely any cartilage left, where in other cases there is some cartilage left of relatively good quality. The use of human osteochondral material to obtain biopsies could cause problems in reproducibility. Pre-treatment of bovine osteochondral biopsies using OA synovium conditioned medium or inflammatory cytokines would provide more reproducible experimental conditions.

As mentioned, hBMSCs are, apart from their multilineage differentiation potential, also known for their immunomodulatory capacity [412]. Making use of this capacity could also pose interesting treatment options for tissue regeneration in an inflammatory environment. One could think of approaches in which hBMSCs are implanted into the joint at two time points: firstly to modulate the ongoing inflammation and secondly for cartilage regeneration. It is evident that studies are required that address critical issues for such an approach. Again, specific timing of both interventions is very likely to be of high importance. First steps in this direction are already described in literature. Adipose tissue derived MSCs and hBMSCs were found to decrease hypertrophy of OA chondrocytes in vitro [413]. Intra-articular injection of umbilical cord derived MSCs in a rat arthritis model was shown to successfully decrease joint inflammation [414]. However, mixed results were found in immunomodulatory capacity of hBMSCs in response to exposure to OA synovial fluid due to donor variations [415]. It is evident that before the immunomodulatory capacity of hBMSCs can be combined with cartilage tissue engineering strategies, more research is required to gain more understanding and control of this interesting phenomenon.

Patient specific treatments may provide the ultimate treatment for cartilage damage. Tailor made treatment protocols could be established by measuring the extent of inflammation in the affected joint for example by multiplex ELISA. Depending on the levels of inflammatory cytokines and ratios between different cytokines, appropriate anti-inflammatory treatment can be initiated. Screening of autologous hBMSCs for example by FACS or microarray analysis can be part of the protocol to identify patient specific characteristics. In the future it might even be possible to assess the chondrogenic differentiation capacity of the patients hBMSCs using these methods. Sophisticated biomaterials could be used for delivery of hBMSCs as well as to stimulate stable chondrogenesis of hBMSCs. Together, this could result in optimal articular conditions allowing hBMSC-based cartilage regeneration.

To use hBMSCs for cell-based treatments for cartilage regeneration, full understanding of the process of stable cartilage formation is essential. Once extensive studies on hBMSC chondrogenesis have provided control over stable chondrogenic differentiation and the roles of the osteochondral environment and inflammation are further elucidated, high quality randomized clinical trials are required. Apart from short-term outcome, long-term follow up should be included to be able to eventually determine not only the effect on cartilage regeneration, but also on the possible development of early OA.

Although clinical application is not realistic yet, the research described in this thesis has provided important clues on the effects of the osteochondral environment and inflammatory processes on hBMSC chondrogenesis. The complex environment of the joint should not be

underestimated and the models described in this thesis can assist to unravel all interactions. Use of these and other models may lead to a better understanding of the mechanism of chondrogenic differentiation of hBMSCs in an osteochondral and/or inflammatory environment. In the future, this may lead to the development of hBMSC-based treatments for cartilage regeneration that may eventually prevent the development of early OA.

## Summary

In contrast to many other tissues, cartilage lacks a self-regenerative potential. Untreated cartilage lesions are generally believed to progress towards osteoarthritis (OA). Several treatments for cartilage lesions are in clinical use for over 20 years, but the molecular mechanisms behind it are largely unknown and the clinical outcomes are variable. In order to improve current treatment procedures as well as to develop new treatment strategies, models are needed to elucidate the mechanisms involved in cell-based cartilage regeneration. This thesis aimed to provide such models. The effects of different tissues present in articular joints on cartilage regeneration using human bone marrow derived mesenchymal stem cells (hBMSCs) were studied.

**Chapter 2** provides a review of clinically used strategies and the future potential of regenerative medicine for cartilage regeneration. Randomized clinical trials (RCTs) on microfracture, osteochondral autograft transplantation and autologous chondrocyte implantation (ACI) apply specific inclusion criteria, resulting in a study population with focal cartilage lesions without other joint problems. The results of treatments can therefore be different in other patient populations. Chondrocytes have several drawbacks for use in cell-based cartilage regeneration including the creation of joint defects to obtain the cells and their tendency to dedifferentiate in vitro. Therefore hBMSCs are promising because of their chondrogenic differentiation capacity. Apart from their cartilage regeneration potential, hBMSCs have a trophic activity, which can potentially be used to counteract the catabolic environment present in OA joints.

OA is often accompanied by synovial inflammation, leading to a catabolic joint environment. The inflammatory factors secreted by OA synovium negatively affect chondrogenesis of hBMSCs. In **chapter 3 and 4**, synovial inflammation is modeled in vitro using conditioned medium prepared from OA synovium. In **chapter 3** it is demonstrated that there are factors in OA synovium conditioned medium that signal via the TAK1 and JAK signaling pathways which are involved in the negative effect of OA synovium on hBMSC chondrogenesis, since inhibitors of these pathways partly counteract the negative effect of OA synovium. In **chapter 4** a potential role in this process is identified for M1 macrophages that are present in OA synovium. This is demonstrated using both OA synovium conditioned medium as well as peripheral blood derived monocytes stimulated towards M1 and M2 macrophages as model systems. Like OA synovium conditioned medium, M1 macrophage conditioned medium was found to negatively affect hBMSC chondrogenesis, where M2 macrophage conditioned medium did not. Together, these chapters indicate that synovial inflammation should be treated to allow optimal hBMSC chondrogenesis for cartilage regeneration. New treatments

should aim to prevent inflammatory cytokine secretion by M1 macrophages or signaling of these factors for example using TAK1 and JAK inhibitors.

Traumatic cartilage lesions that do not violate the subchondral bone do not heal, where some fibrocartilaginous tissue is formed when the subchondral bone is involved. This implicates that apart from articular cartilage, the subchondral bone is an important joint tissue. Conventional in vitro models for cartilage regeneration often do not take the subchondral bone into account. In **chapter 5** we describe the development and validation of an osteochondral in vitro model that provides a representative environment to reproducibly study cell-based cartilage regeneration in vitro. This model is used to study hBMSC chondrogenesis in **chapter 6**. The subchondral bone was identified as key inducer of chondrogenesis of hBMSCs, making exogenous supplementation of TGFβ to this system no longer necessary. Interestingly, the use of an antibody against TGFβ as well as inhibiting canonical TGFβ signaling indicated that factors other than TGFβ are also secreted by the subchondral bone and responsible for the induction of chondrogenesis of hBMSCs in a simulated osteochondral environment in vitro. These findings underline the complexity of the osteochondral environment, making the use of representative models essential to evaluate strategies for cell-based cartilage regeneration.

The effect of the osteochondral environment on hBMSC-based cartilage regeneration was further studied in **chapter 7** by comparing three different hydrogels for their capacity to support chondrogenesis of hBMSCs. The well-known biomaterials alginate and fibrin as well as a sophisticated hyaluronic acid based material with thermoresponsive characteristics were tested. Where no differences were observed upon conventional in vitro culture, the use of the osteochondral model described in **chapter 5** revealed differences in chondrogenesis of hBMSCs encapsulated in the different hydrogels. These findings are confirmed in in vivo experiments. This stresses the fact that the micro-environment in which hBMSCs reside is critical for successful regeneration of cartilage.

The findings described in this thesis provide important clues for the future enhancement of cell-based cartilage regeneration. Placed in scientific context in **chapter 8**, it becomes clear that to develop new treatments or to improve current strategies, a multifactorial approach is essential. To achieve successful cartilage regeneration, the catabolic environment that can be present both in case of focal cartilage defects as well as in OA needs to be taken into account. Inflammation should preferably be treated prior to deployment of hBMSCs to provide optimal conditions to allow cartilage regeneration.

## Nederlandse samenvatting

Kraakbeen heeft, in tegenstelling tot veel andere weefsels, niet de capaciteit om zichzelf te herstellen. De algemene opvatting is dat onbehandelde kraakbeendefecten leiden tot de ontwikkeling van artrose. Verschillende behandelingsmethodes voor kraakbeenschade worden al ruim 20 jaar toegepast, hoewel de moleculaire mechanismen die er aan ten grondslag liggen nog niet volledig bekend zijn en de klinische resultaten zijn variabel. Om bestaande behandelingen te kunnen verbeteren en om nieuwe behandelingen te kunnen ontwikkelen, zijn modellen nodig om de werkingsmechanismen te onderzoeken. Dit proefschrift beschrijft modellen, waarmee de invloed wordt onderzocht van verschillende weefsels die aanwezig zijn in gewrichten op kraakbeenvorming door humane uit beenmerg geïsoleerde mesenchymale stamcellen (hBMSCs).

In **hoofdstuk 2** wordt een overzicht gegeven van klinisch toegepaste technieken en de potentie van regeneratieve geneeskunde voor kraakbeenherstel. Voor gerandomiseerde klinische studies over de microfractuurtechniek, osteochondrale autograft transplantatie en autologe chondrocyten implantatie (ACI) wordt gebruik gemaakt van zeer specifieke inclusiecriteria, wat resulteert in populaties patiënten met kraakbeendefecten zonder andere gewrichtsproblemen. Het is mogelijk dat de resultaten in andere patiëntenpopulaties niet vergelijkbaar zijn. Het gebruik van chondrocyten gaat gepaard met het creëren van gewrichtsdefecten om cellen te verkrijgen en chondrocyten dedifferentiëren bij expansie in vitro. Vanwege deze nadelen van het gebruik van chondrocyten en omdat ze kunnen differentiëren naar kraakbeen, zijn hBMSCs interessant om te gebruiken voor kraakbeenregeneratie. Daarnaast zouden hBMSCs ook gebruikt kunnen worden om te katabole omgeving die aanwezig is artrotische gewrichten tegen te gaan, vanwege hun immuun-modulatoire capaciteiten.

Artrose gaat vaak samen met synoviale ontsteking, wat leidt tot een katabole gewrichtsomgeving. Het is bekend dat ontstekingsfactoren die worden uitgescheiden door artrotisch synovium een negatief effect hebben op de kraakbeenvorming door hBMSCs. In de **hoofdstukken 3 en 4** wordt synoviale ontsteking gesimuleerd door geconditioneerd medium van artrotisch synovium te gebruiken. In **hoofdstuk 3** wordt duidelijk dat er factoren worden uitgescheiden door artrotisch synovium die gebruik maken van TAK1 en JAK signaleringsroutes. Deze factoren zijn betrokken bij het negatieve effect van artrotisch synovium op kraakbeenvorming door hBMSCs. Inhibitoren voor deze signaleringsroutes konden het effect van artrotisch synovium deels tegen gaan. Een mogelijke rol van synoviale M1 macrofagen wordt geïdentificeerd in **hoofdstuk 4**. Monocyten geïsoleerd uit perifeer bloed werden gestimuleerd tot M1 of M2 macrofagen en gebruikt om geconditioneerd

medium te maken. Net als voor synovium geconditioneerd medium, blijkt M1 geconditioneerd medium een negatief effect op de kraakbeenvorming door hBMSCs te hebben, waar M2 geconditioneerd medium dit effect niet heeft. Deze hoofdstukken geven aan dat het van belang is om synoviale ontsteking te behandelen om te zorgen dat hBMSCs onder optimale omstandigheden kraakbeen kunnen regenereren. Nieuwe behandelmethodes zouden tot doel kunnen hebben om de uitscheiding van ontstekingscytokines door synoviale M1 macrofagen te voorkomen of voorkomen dat deze cytokines effect hebben door gebruik te maken van TAK1 en JAK inhibitoren.

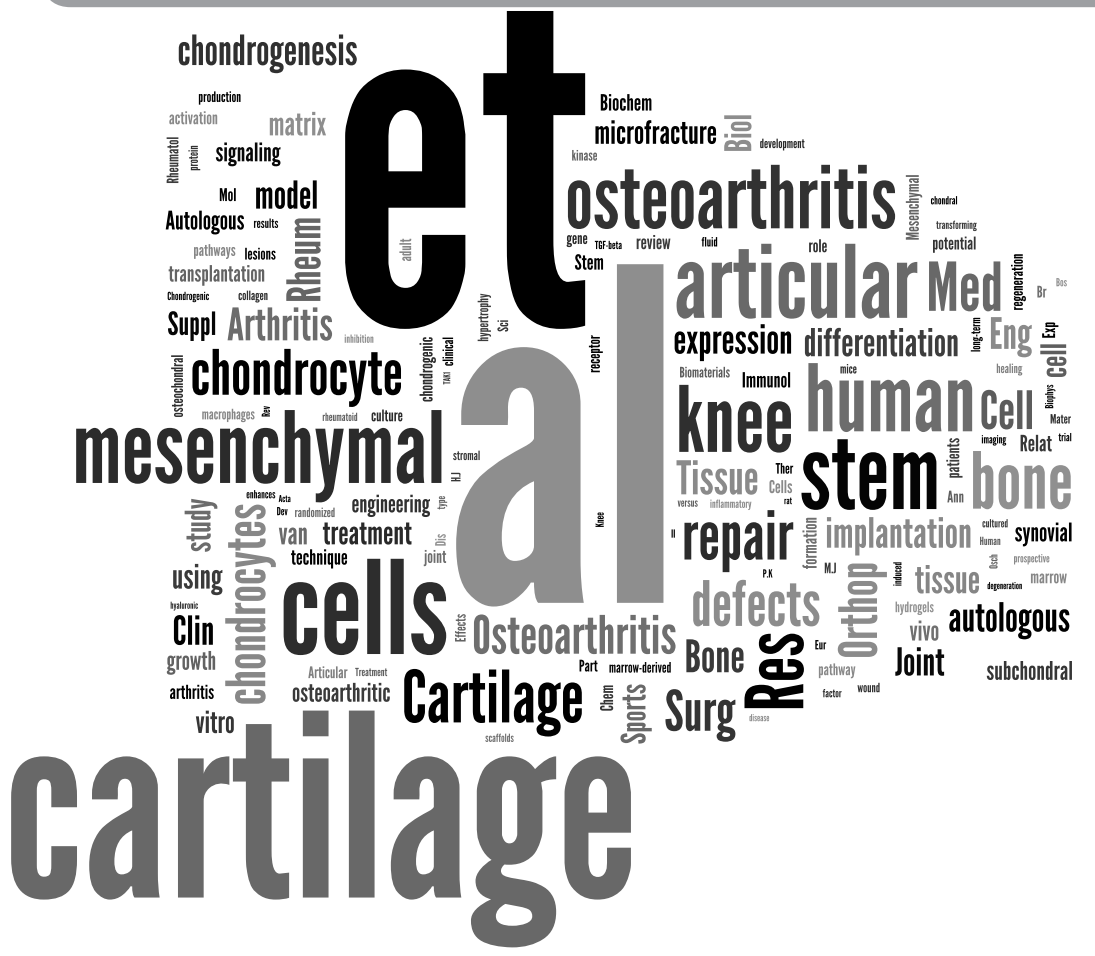
Traumatische kraakbeendefecten die niet tot op het subchondrale bot reiken, herstellen niet, waar in diepere defecten wat fibreus kraakbeenachtig weefsel gevormd wordt. Dit suggereert dat naast kraakbeen, het subchondrale bot een belangrijk gewrichtsweefsel is, wat in conventionele kweeksystemen vaak buiten beschouwing wordt gelaten. In **hoofdstuk 5** wordt de ontwikkeling en validatie van een osteochondraal kweekmodel beschreven, wat in een meer representatieve simulatie van de gewrichtsomgeving voorziet. Met dit model kan op reproduceerbare wijze kraakbeenregeneratie met behulp van cellen onderzocht worden in vitro. Dit model is gebruikt om kraakbeenregeneratie met hBMSCs te onderzoeken in **hoofdstuk 6**. Het subchondrale bot is geïdentificeerd als belangrijkste stimulator van kraakbeenvorming door hBMSCs, wat het toevoegen van TGFβ aan dit kweeksysteem overbodig maakt. Opvallend is dat het gebruik van een antilichaam tegen TGFβ en het voorkomen van intracellulaire signaaltransductie van TGFβ aangeven dat het waarschijnlijk is dat andere factoren dan TGFβ verantwoordelijk zijn voor het stimuleren van kraakbeenvorming door hBMSCs in een osteochondrale omgeving. Deze resultaten benadrukken de complexiteit van de osteochondrale omgeving en geven het essentiële belang aan van representatieve modellen om kraakbeenregeneratie met behulp van cellen te onderzoeken.

Het effect van de osteochondrale omgeving op kraakbeenregeneratie met hBMSCs is verder onderzocht in **hoofdstuk 7** door drie verschillende hydrogelen te vergelijken op basis van hun capaciteiten om kraakbeenvorming te ondersteunen. De bekende biomaterialen alginaat en fibrine, en een nieuw materiaal wat gebaseerd is op hyaluronzuur met thermoresponsieve eigenschappen zijn onderzocht. Bij conventionele kweek in vitro werden geen verschillen tussen de materialen gevonden. Als het osteochondrale model uit **hoofdstuk 5** wordt gebruikt, worden er verschillen gevonden tussen de materialen. Deze resultaten zijn bevestigd met in vivo experimenten. Dit benadrukt het feit dat de micro-omgeving waarin hBMSCs zich bevinden van cruciaal belang is voor succesvolle kraakbeenregeneratie.

De resultaten die in dit proefschrift worden beschreven geven belangrijke inzichten voor de verbetering van kraakbeenregeneratie met behulp van cellen. Als de bevindingen in wetenschappelijke context worden geplaatst in **hoofdstuk 8,** wordt duidelijk dat bij de ontwikkeling van nieuwe behandelingen of verbetering van bestaande behandelingen een multi-factoriele aanpak essentieel is. Om regeneratie van kraakbeen te verkrijgen, is het nodig dat de katabole omgeving die aanwezig kan zijn in zowel artrotische gewrichten als bij focale kraakbeendefecten, behandeld wordt. Om de gewrichtsomgeving gunstig te maken voor kraakbeenherstel met behulp van hBMSCs zou synoviale inflammatie eerst behandeld moeten worden voordat hBMSCs in een kraakbeendefect geplaatst worden.

# Chapter 9

## References



## References


1. Gabriel, S.E., C.S. Crowson, and W.M. O'Fallon, *Costs of osteoarthritis: estimates from a geographically defined population.* J Rheumatol Suppl, 1995. 43
2. Buckwalter, J.A. and H.J. Mankin, *Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation.* Instr Course Lect, 1998. 47
3. Hunziker, E.B., *Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects.* Osteoarthritis Cartilage, 2002. 10(6)
4. Messner, K. and J. Gillquist, *Cartilage repair. A critical review.* Acta Orthop Scand, 1996. 67(5)
5. Muir, H., *The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules.* Bioessays, 1995. 17(12)
6. Gentili, C. and R. Cancedda, *Cartilage and bone extracellular matrix.* Curr Pharm Des, 2009. 15(12)
7. Setton, L.A., D.M. Elliott, and V.C. Mow, *Altered mechanics of cartilage with osteoarthritis: human osteoarthritis and an experimental model of joint degeneration.* Osteoarthritis Cartilage, 1999. 7(1)
8. Mow, V.C., C.C. Wang, and C.T. Hung, *The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage.* Osteoarthritis Cartilage, 1999. 7(1)
9. Williamson, A.K., A.C. Chen, and R.L. Sah, *Compressive properties and function-composition relationships of developing bovine articular cartilage.* J Orthop Res, 2001. 19(6)
10. Hardingham, T., B.C. Heng, and P. Gribbon, *New approaches to the investigation of hyaluronan networks.* Biochem Soc Trans, 1999. 27(2)
11. Poole, A.R., et al., *Composition and structure of articular cartilage: a template for tissue repair.* Clin Orthop Relat Res, 2001(391 Suppl)
12. Stockwell, R.A., *Chondrocytes.* J Clin Pathol Suppl (R Coll Pathol), 1978. 12
13. Scanzello, C.R., A. Plaas, and M.K. Crow, *Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound?* Curr Opin Rheumatol, 2008. 20(5)
14. Bondeson, J., et al., *The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis.* Arthritis Res Ther, 2006. 8(6)
15. Cancedda, R., et al., *Developmental control of chondrogenesis and osteogenesis.* Int J Dev Biol, 2000. 44(6)
16. Goldring, M.B., K. Tsuchimochi, and K. Ijiri, *The control of chondrogenesis.* J Cell Biochem, 2006. 97(1)
17. Burr, D.B., *The importance of subchondral bone in the progression of osteoarthritis.* J Rheumatol Suppl, 2004. 70
18. Henrotin, Y., L. Pesesse, and C. Sanchez, *Subchondral bone in osteoarthritis physiopathology: state-of-the art and perspectives.* Biomed Mater Eng, 2009. 19(4-5)
19. Brandt, K.D., et al., *Yet more evidence that osteoarthritis is not a cartilage disease.* Ann Rheum Dis, 2006. 65(10)
20. Peterfy, C.G., et al., *Whole-Organ Magnetic Resonance Imaging Score (WORMS) of the knee in osteoarthritis.* Osteoarthritis Cartilage, 2004. 12(3)
21. Mankin, H.J., et al., *Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data.* J Bone Joint Surg Am, 1971. 53(3)
22. Day, J.S., et al., *Adaptation of subchondral bone in osteoarthritis.* Biorheology, 2004. 41(3-4)
23. Hill, C.L., et al., *Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis.* Ann Rheum Dis, 2007. 66(12)
24. Karsdal, M.A., et al., *Should subchondral bone turnover be targeted when treating osteoarthritis?* Osteoarthritis Cartilage, 2008. 16(6)
25. Alcaraz, M.J., et al., *New molecular targets for the treatment of osteoarthritis.* Biochem Pharmacol, 2010. 80(1)
26. Botter, S.M., et al., *ADAMTS5-/- mice have less subchondral bone changes after induction of osteoarthritis through surgical instability: implications for a link between cartilage and subchondral bone changes.* Osteoarthritis Cartilage, 2009. 17(5)

27. Sniekers, Y.H., et al., *A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models.* BMC Musculoskelet Disord, 2008. 9
28. Zhang, Y. and J.M. Jordan, *Epidemiology of osteoarthritis.* Rheum Dis Clin North Am, 2008. 34(3)
29. Wang, Y., et al., *Relationship between body adiposity measures and risk of primary knee and hip replacement for osteoarthritis: a prospective cohort study.* Arthritis Res Ther, 2009. 11(2)
30. Englund, M., P.T. Paradowski, and L.S. Lohmander, *Association of radiographic hand osteoarthritis with radiographic knee osteoarthritis after meniscectomy.* Arthritis Rheum, 2004. 50(2)
31. Cawston, T.E. and A.J. Wilson, *Understanding the role of tissue degrading enzymes and their inhibitors in development and disease.* Best Pract Res Clin Rheumatol, 2006. 20(5)
32. Plaas, A., et al., *Aggrecanolysis in human osteoarthritis: confocal localization and biochemical characterization of ADAMTS5-hyaluronan complexes in articular cartilages.* Osteoarthritis Cartilage, 2007. 15(7)
33. Moos, V., et al., *Immunohistological analysis of cytokine expression in human osteoarthritic and healthy cartilage.* J Rheumatol, 1999. 26(4)
34. Farahat, M.N., et al., *Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis.* Ann Rheum Dis, 1993. 52(12)
35. Benito, M.J., et al., *Synovial tissue inflammation in early and late osteoarthritis.* Ann Rheum Dis, 2005. 64(9)
36. Beekhuizen, M., et al., *Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: establishment and characterization of a long-term cartilage-synovium coculture.* Arthritis Rheum, 2011. 63(7)
37. Goldring, M.B. and F. Berenbaum, *The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide.* Clin Orthop Relat Res, 2004(427 Suppl)
38. Pountos, I., et al., *NSAIDS inhibit in vitro MSC chondrogenesis but not osteogenesis: implications for mechanism of bone formation inhibition in man.* J Cell Mol Med, 2011. 15(3)
39. Walker-Bone, K., et al., *Regular review: medical management of osteoarthritis.* BMJ, 2000. 321(7266)
40. Hunter, D.J. and D.T. Felson, *Osteoarthritis.* BMJ, 2006. 332(7542)
41. Lane, N.E., *Pain management in osteoarthritis: the role of COX-2 inhibitors.* J Rheumatol Suppl, 1997. 49
42. Ucar, D., et al., *Intra-articular hyaluronic Acid as treatment in elderly and middle-aged patients with knee osteoarthritis.* Open Rheumatol J, 2013. 7
43. Suarez, J., et al., *Why do revision knee arthroplasties fail?* J Arthroplasty, 2008. 23(6 Suppl 1)
44. De Bari, C. and F. Dell'accio, *Mesenchymal stem cells in rheumatology: a regenerative approach to joint repair.* Clin Sci (Lond), 2007. 113(8)
45. Hunziker, E., et al., *Translation from research to applications.* Tissue Eng, 2006. 12(12)
46. Kuo, C.K., et al., *Cartilage tissue engineering: its potential and uses.* Curr Opin Rheumatol, 2006. 18(1)
47. Shapiro, F., S. Koide, and M.J. Glimcher, *Cell origin and differentiation in the repair of full-thickness defects of articular cartilage.* J Bone Joint Surg Am, 1993. 75(4)
48. Mankin, H.J., *The response of articular cartilage to mechanical injury.* J Bone Joint Surg Am, 1982. 64(3)
49. Brittberg, M., et al., *Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation.* N Engl J Med, 1994. 331(14)
50. Shah, M.R., et al., *Articular cartilage restoration of the knee.* Bull NYU Hosp Jt Dis, 2007. 65(1)
51. Marlovits, S., et al., *Cartilage repair: generations of autologous chondrocyte transplantation.* Eur J Radiol, 2006. 57(1)
52. Saris, D.B., et al., *Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture.* Am J Sports Med, 2008. 36(2)
53. Brittberg, M., *Autologous chondrocyte implantation--technique and long-term follow-up.* Injury, 2008. 39 Suppl 1
54. Drobnic, M., et al., *Debridement of cartilage lesions before autologous chondrocyte implantation by open or transarthroscopic techniques: a comparative study using post-mortem materials.* J Bone Joint Surg Br, 2010. 92(4)
55. Steadman, J.R., et al., *[The microfracture technic in the management of complete cartilage defects in the knee joint].* Orthopade, 1999. 28(1)

56. Steinwachs, M.R., T. Guggi, and P.C. Kreuz, *Marrow stimulation techniques.* Injury, 2008. 39 Suppl 1
57. Mithoefer, K., et al., *Chondral resurfacing of articular cartilage defects in the knee with the microfracture technique. Surgical technique.* J Bone Joint Surg Am, 2006. 88 Suppl 1 Pt 2
58. Steadman, J.R., W.G. Rodkey, and J.J. Rodrigo, *Microfracture: surgical technique and rehabilitation to treat chondral defects.* Clin Orthop Relat Res, 2001(391 Suppl)
59. Knutsen, G., et al., *Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial.* J Bone Joint Surg Am, 2004. 86-A(3)
60. Knutsen, G., et al., *A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years.* J Bone Joint Surg Am, 2007. 89(10)
61. Bartlett, W., et al., *Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation for osteochondral defects of the knee: a prospective, randomised study.* J Bone Joint Surg Br, 2005. 87(5)
62. Horas, U., et al., *Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial.* J Bone Joint Surg Am, 2003. 85-A(2)
63. Bentley, G., et al., *A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee.* J Bone Joint Surg Br, 2003. 85(2)
64. Dozin, B., et al., *Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial.* Clin J Sport Med, 2005. 15(4)
65. Gudas, R., et al., *Osteochondral autologous transplantation versus microfracture for the treatment of articular cartilage defects in the knee joint in athletes.* Knee Surg Sports Traumatol Arthrosc, 2006. 14(9)
66. Gooding, C.R., et al., *A prospective, randomised study comparing two techniques of autologous chondrocyte implantation for osteochondral defects in the knee: Periosteum covered versus type I/III collagen covered.* Knee, 2006. 13(3)
67. Saris, D.B., et al., *Treatment of symptomatic cartilage defects of the knee: characterized chondrocyte implantation results in better clinical outcome at 36 months in a randomized trial compared to microfracture.* Am J Sports Med, 2009. 37 Suppl 1
68. Pelttari, K., et al., *Secretion of matrix metalloproteinase 3 by expanded articular chondrocytes as a predictor of ectopic cartilage formation capacity in vivo.* Arthritis Rheum, 2008. 58(2)
69. Friedenstein, A.J., et al., *Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method.* Exp Hematol, 1974. 2(2)
70. Friedenstein, A.J., et al., *Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues.* Transplantation, 1968. 6(2)
71. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells.* Science, 1999. 284(5411)
72. Johnstone, B., et al., *In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells.* Exp Cell Res, 1998. 238(1)
73. Mackay, A.M., et al., *Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow.* Tissue Eng, 1998. 4(4)
74. Pelttari, K., et al., *Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice.* Arthritis Rheum, 2006. 54(10)
75. Mueller, M.B. and R.S. Tuan, *Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells.* Arthritis Rheum, 2008. 58(5)
76. Ichinose, S., et al., *Detailed examination of cartilage formation and endochondral ossification using human mesenchymal stem cells.* Clin Exp Pharmacol Physiol, 2005. 32(7)
77. Hellingman, C.A., W. Koevoet, and G.J. van Osch, *Can one generate stable hyaline cartilage from adult mesenchymal stem cells? A developmental approach.* J Tissue Eng Regen Med, 2012. 6(10)
78. Solchaga, L.A., et al., *Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells.* Tissue Eng Part A, 2010. 16(3)
79. Farrell, E., et al., *Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair?* Tissue Eng Part C Methods, 2009. 15(2)
80. Kafienah, W., et al., *Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients.* Arthritis Rheum, 2007. 56(1)

81. Kim, Y.J., H.J. Kim, and G.I. Im, *PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs.* Biochem Biophys Res Commun, 2008. 373(1)
82. Barry, F., et al., *Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components.* Exp Cell Res, 2001. 268(2)
83. Weiss, S., et al., *Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells.* J Cell Physiol. 223(1)
84. Stoop, R., *Smart biomaterials for tissue engineering of cartilage.* Injury, 2008. 39 Suppl 1
85. Varghese, S., et al., *Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells.* Matrix Biol, 2008. 27(1)
86. Diduch, D.R., et al., *Marrow stromal cells embedded in alginate for repair of osteochondral defects.* Arthroscopy, 2000. 16(6)
87. Solchaga, L.A., et al., *Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage.* J Orthop Res, 1999. 17(2)
88. Wayne, J.S., et al., *In vivo response of polylactic acid-alginate scaffolds and bone marrow-derived cells for cartilage tissue engineering.* Tissue Eng, 2005. 11(5-6)
89. Angele, P., et al., *Cyclic, mechanical compression enhances chondrogenesis of mesenchymal progenitor cells in tissue engineering scaffolds.* Biorheology, 2004. 41(3-4)
90. Bosnakovski, D., et al., *Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis.* Biotechnol Bioeng, 2006. 93(6)
91. Caterson, E.J., et al., *Three-dimensional cartilage formation by bone marrow-derived cells seeded in polylactide/alginate amalgam.* J Biomed Mater Res, 2001. 57(3)
92. Hauselmann, H.J., et al., *Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads.* Matrix, 1992. 12(2)
93. Lee, C.R., et al., *Fibrin-polyurethane composites for articular cartilage tissue engineering: a preliminary analysis.* Tissue Eng, 2005. 11(9-10)
94. Macchiarini, P., et al., *Clinical transplantation of a tissue-engineered airway.* Lancet, 2008. 372(9655)
95. D'Este, M., M. Alini, and D. Eglin, *Single step synthesis and characterization of thermoresponsive hyaluronan hydrogels.* Carbohydr Polym, 2012. 90(3)
96. Wakitani, S., et al., *Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees.* Osteoarthritis Cartilage, 2002. 10(3)
97. Haasper, C., et al., *Tissue engineering of osteochondral constructs in vitro using bioreactors.* Injury, 2008. 39 Suppl 1
98. Hendriks, J., J. Riesle, and C.A. van Blitterswijk, *Co-culture in cartilage tissue engineering.* J Tissue Eng Regen Med, 2007. 1(3)
99. Frisbie, D.D., M.W. Cross, and C.W. McIlwraith, *A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee.* Vet Comp Orthop Traumatol, 2006. 19(3)
100. Hayami, T., et al., *Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis.* Bone, 2006. 38(2)
101. Kamekura, S., et al., *Osteoarthritis development in novel experimental mouse models induced by knee joint instability.* Osteoarthritis Cartilage, 2005. 13(7)
102. Glasson, S.S., T.J. Blanchet, and E.A. Morris, *The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse.* Osteoarthritis Cartilage, 2007. 15(9)
103. Siebelt, M., et al., *Hsp90 inhibition protects against biomechanically induced osteoarthritis in rats.* Arthritis Rheum, 2013. 65(8)
104. Siebelt, M., et al., *Quantifying osteoarthritic cartilage changes accurately using in vivo microCT arthrography in three etiologically distinct rat models.* J Orthop Res, 2011. 29(11)
105. Kyostio-Moore, S., et al., *STR/ort mice, a model for spontaneous osteoarthritis, exhibit elevated levels of both local and systemic inflammatory markers.* Comp Med, 2011. 61(4)
106. Yamamoto, K., et al., *Morphological studies on the ageing and osteoarthritis of the articular cartilage in C57 black mice.* J Orthop Surg (Hong Kong), 2005. 13(1)

107. Mankin, H.J., *Chondrocyte transplantation--one answer to an old question.* N Engl J Med, 1994. 331(14)
108. Brittberg, M. and L. Peterson, *Introduction to an articular cartilage classification.* ICRS Newsletter, 1998. 1
109. Hjelle, K., et al., *Articular cartilage defects in 1,000 knee arthroscopies.* Arthroscopy, 2002. 18(7)
110. Aroen, A., et al., *Articular cartilage lesions in 993 consecutive knee arthroscopies.* Am J Sports Med, 2004. 32(1)
111. Noyes, F.R., et al., *Arthroscopy in acute traumatic hemarthrosis of the knee. Incidence of anterior cruciate tears and other injuries.* J Bone Joint Surg Am, 1980. 62(5)
112. Hardaker, W.T., Jr., W.E. Garrett, Jr., and F.H. Bassett, 3rd, *Evaluation of acute traumatic hemarthrosis of the knee joint.* South Med J, 1990. 83(6)
113. Maffulli, N., et al., *Acute haemarthrosis of the knee in athletes. A prospective study of 106 cases.* J Bone Joint Surg Br, 1993. 75(6)
114. Curl, W.W., et al., *Cartilage injuries: a review of 31,516 knee arthroscopies.* Arthroscopy, 1997. 13(4)
115. Shelbourne, K.D., S. Jari, and T. Gray, *Outcome of untreated traumatic articular cartilage defects of the knee: a natural history study.* J Bone Joint Surg Am, 2003. 85-A Suppl 2
116. Outerbridge, R.E., *The etiology of chondromalacia patellae.* J Bone Joint Surg Br, 1961. 43-B
117. Linden, B., *Osteochondritis dissecans of the femoral condyles: a long-term follow-up study.* J Bone Joint Surg Am, 1977. 59(6)
118. Widuchowski, W., et al., *Long-term clinical and radiological assessment of untreated severe cartilage damage in the knee: a natural history study.* Scand J Med Sci Sports, 2010
119. Gelber, A.C., et al., *Joint injury in young adults and risk for subsequent knee and hip osteoarthritis.* Ann Intern Med, 2000. 133(5)
120. Yen, Y.M., et al., *Treatment of osteoarthritis of the knee with microfracture and rehabilitation.* Med Sci Sports Exerc, 2008. 40(2)
121. Gomoll, A.H., et al., *Triad of cartilage restoration for unicompartmental arthritis treatment in young patients: meniscus allograft transplantation, cartilage repair and osteotomy.* J Knee Surg, 2009. 22(2)
122. Lawrence, R.C., et al., *Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II.* Arthritis Rheum, 2008. 58(1)
123. Michaud, C.M., et al., *The burden of disease and injury in the United States 1996.* Popul Health Metr, 2006. 4
124. Hootman, J.M. and C.G. Helmick, *Projections of US prevalence of arthritis and associated activity limitations.* Arthritis Rheum, 2006. 54(1)
125. Hangody, L., et al., *Mosaicplasty for the treatment of articular defects of the knee and ankle.* Clin Orthop Relat Res, 2001(391 Suppl)
126. Bobic, V., *[Autologous osteo-chondral grafts in the management of articular cartilage lesions].* Orthopade, 1999. 28(1)
127. Hangody, L., et al., *Autologous osteochondral grafting--technique and long-term results.* Injury, 2008. 39 Suppl 1
128. Richter, W., *Mesenchymal stem cells and cartilage in situ regeneration.* J Intern Med, 2009. 266(4)
129. Buckwalter, J.A., *Articular cartilage injuries.* Clin Orthop Relat Res, 2002(402)
130. Brittberg, M. and L. Peterson, *Introduction to an articular cartilage classification.* ICRS newsletter, 1998. 1(8)
131. Pridie, K.H., *A method of resurfacing osteoarthritic knee joints.* J Bone Joint Surg Br, 1959. 41
132. Steadman, J.R., W.G. Rodkey, and K.K. Briggs, *Microfracture: Its history and experience of the developing surgeon.* Cartilage, 2010. 1(2)
133. Frisbie, D.D., et al., *Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses.* Vet Surg, 1999. 28(4)
134. Frisbie, D.D., et al., *Early events in cartilage repair after subchondral bone microfracture.* Clin Orthop Relat Res, 2003(407)
135. Frisbie, D.D., et al., *Effects of calcified cartilage on healing of chondral defects treated with microfracture in horses.* Am J Sports Med, 2006. 34(11)
136. Morisset, S., et al., *IL-1ra/IGF-1 gene therapy modulates repair of microfractured chondral defects.* Clin Orthop Relat Res, 2007. 462

137. Mithoefer, K., et al., *The microfracture technique for the treatment of articular cartilage lesions in the knee. A prospective cohort study.* J Bone Joint Surg Am, 2005. 87(9)
138. Brown, W.E., et al., *Magnetic resonance imaging appearance of cartilage repair in the knee.* Clin Orthop Relat Res, 2004(422)
139. Wilk, K.E., L.C. Macrina, and M.M. Reinold, *Rehabilitation following microfracture of the knee.* Cartilage, 2010. 1(2)
140. Steadman, J.R., et al., *Microfracture technique for full-thickness chondral defects: technique and clinical results.* Op Tech Orthop, 1997. 7
141. Hagerman, G.R., J.A. Atkins, and C. Dillman, *Rehabilitation of chondral injuries and chronic degenerative arthritis of the knee in the athlete.* Oper Tech Sports Med, 1995. 3
142. Gill, T.J., *The treatment of articular cartilage defects using microfracture and debridement.* Am J Knee Surg, 2000. 13(1)
143. Mithoefer, K., et al., *Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: an evidence-based systematic analysis.* Am J Sports Med, 2009. 37(10)
144. Gille, J., et al., *Mid-term results of Autologous Matrix-Induced Chondrogenesis for treatment of focal cartilage defects in the knee.* Knee Surg Sports Traumatol Arthrosc,
145. Hoemann, C.D., et al., *Chitosan-glycerol phosphate/blood implants improve hyaline cartilage repair in ovine microfracture defects.* J Bone Joint Surg Am, 2005. 87(12)
146. Chevrier, A., et al., *Chitosan-glycerol phosphate/blood implants increase cell recruitment, transient vascularization and subchondral bone remodeling in drilled cartilage defects.* Osteoarthritis Cartilage, 2007. 15(3)
147. Buschmann, M.D., et al., *Cartilage repair with chitosan-glycerol phosphate stabilized blood clots.* Cartilage repair strategies, 2007
148. Shive, M.S., et al., *BST-CarGel: In Situ ChondroInduction for Cartilage Repair.* Op Tech Orthop, 2006. 16
149. Dorotka, R., et al., *Repair of articular cartilage defects treated by microfracture and a three-dimensional collagen matrix.* Biomaterials, 2005. 26(17)
150. Strauss, E., et al., *The efficacy of intra-articular hyaluronan injection after the microfracture technique for the treatment of articular cartilage lesions.* Am J Sports Med, 2009. 37(4)
151. Strauss, E.J., et al., *Augmentation strategies following the microfracture technique for repair of focal chondral defects.* Cartilage, 2010. 1(2)
152. Kramer, J., et al., *In vivo matrix-guided human mesenchymal stem cells.* Cell Mol Life Sci, 2006. 63(5)
153. Sohn, D.H., et al., *Effect of gravity on localization of chondrocytes implanted in cartilage defects.* Clin Orthop Relat Res, 2002(394)
154. Drobnic, M., et al., *Debridement of cartilage lesions before autologous chondrocyte implantation by open or transarthroscopic techniques: a comparative study using post-mortem materials.* J Bone Joint Surg Br. 92(4)
155. Simon, T.M. and D.W. Jackson, *Articular cartilage: injury pathways and treatment options.* Sports Med Arthrosc, 2006. 14(3)
156. Krishnan, S.P., et al., *Who is the ideal candidate for autologous chondrocyte implantation?* J Bone Joint Surg Br, 2006. 88(1)
157. Peterson, L., et al., *Autologous chondrocyte transplantation. Biomechanics and long-term durability.* Am J Sports Med, 2002. 30(1)
158. Roberts, S., et al., *Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology.* Arthritis Res Ther, 2003. 5(1)
159. Moseley, J.B., Jr., et al., *Long-term durability of autologous chondrocyte implantation: a multicenter, observational study in US patients.* Am J Sports Med, 2010. 38(2)
160. Browne, J.E., et al., *Clinical outcome of autologous chondrocyte implantation at 5 years in US subjects.* Clin Orthop Relat Res, 2005(436)
161. Giannoni, P., et al., *Species variability in the differentiation potential of in vitro-expanded articular chondrocytes restricts predictive studies on cartilage repair using animal models.* Tissue Eng, 2005. 11(1-2)
162. Tuan, R.S., *Stemming cartilage degeneration: adult mesenchymal stem cells as a cell source for articular cartilage tissue engineering.* Arthritis Rheum, 2006. 54(10)

163. Safran, M.R. and K. Seiber, *The evidence for surgical repair of articular cartilage in the knee.* J Am Acad Orthop Surg, 2010. 18(5)
164. Bekkers, J.E., M. Inklaar, and D.B. Saris, *Treatment selection in articular cartilage lesions of the knee: a systematic review.* Am J Sports Med, 2009. 37 Suppl 1
165. Vasiliadis, H.S., J. Wasiak, and G. Salanti, *Autologous chondrocyte implantation for the treatment of cartilage lesions of the knee: a systematic review of randomized studies.* Knee Surg Sports Traumatol Arthrosc,
166. Vavken, P. and D. Samartzis, *Effectiveness of autologous chondrocyte implantation in cartilage repair of the knee: a systematic review of controlled trials.* Osteoarthritis Cartilage. 18(6)
167. Ruano-Ravina, A. and M. Jato Diaz, *Autologous chondrocyte implantation: a systematic review.* Osteoarthritis Cartilage, 2006. 14(1)
168. Smith, G.D., G. Knutsen, and J.B. Richardson, *A clinical review of cartilage repair techniques.* J Bone Joint Surg Br, 2005. 87(4)
169. Wasiak, J., C. Clar, and E. Villanueva, *Autologous cartilage implantation for full thickness articular cartilage defects of the knee.* Cochrane Database Syst Rev, 2006. 3
170. Jadad, A.R., et al., *Assessing the quality of reports of randomized clinical trials: is blinding necessary?* Control Clin Trials, 1996. 17(1)
171. Noth, U., et al., *Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells.* J Orthop Res, 2002. 20(5)
172. Zuk, P.A., *The Adipose-derived stem cell: looking back and looking ahead.* Mol Biol Cell, 2010. 21(11)
173. De Bari, C., F. Dell'Accio, and F.P. Luyten, *Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age.* Arthritis Rheum, 2001. 44(1)
174. Wickham, M.Q., et al., *Multipotent stromal cells derived from the infrapatellar fat pad of the knee.* Clin Orthop Relat Res, 2003(412)
175. Bos, P.K., et al., *Cellular origin of neocartilage formed at wound edges of articular cartilage in a tissue culture experiment.* Osteoarthritis Cartilage, 2008. 16(2)
176. Dowthwaite, G.P., et al., *The surface of articular cartilage contains a progenitor cell population.* J Cell Sci, 2004. 117(Pt 6)
177. Bouffi, C., et al., *Multipotent mesenchymal stromal cells and rheumatoid arthritis: risk or benefit?* Rheumatology (Oxford), 2009. 48(10)
178. Caplan, A.I., *Why are MSCs therapeutic? New data: new insight.* J Pathol, 2009. 217(2)
179. Dahlke, M.H., et al., *Toward MSC in solid organ transplantation: 2008 position paper of the MISOT study group.* Transplantation, 2009. 88(5)
180. Le Blanc, K., et al., *Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study.* Lancet, 2008. 371(9624)
181. Passier, R., L.W. van Laake, and C.L. Mummery, *Stem-cell-based therapy and lessons from the heart.* Nature, 2008. 453(7193)
182. Pelttari, K., E. Steck, and W. Richter, *The use of mesenchymal stem cells for chondrogenesis.* Injury, 2008. 39 Suppl 1
183. Centeno, C.J., et al., *Safety and complications reporting on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique.* Curr Stem Cell Res Ther. 5(1)
184. Nejadnik, H., et al., *Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study.* Am J Sports Med, 2010. 38(6)
185. Dell'Accio, F., et al., *Expanded phenotypically stable chondrocytes persist in the repair tissue and contribute to cartilage matrix formation and structural integration in a goat model of autologous chondrocyte implantation.* J Orthop Res, 2003. 21(1)
186. Min, B.H., et al., *The fate of implanted autologous chondrocytes in regenerated articular cartilage.* Proc Inst Mech Eng H, 2007. 221(5)
187. Hirschmann, F., et al., *Vital marking of articular chondrocytes by retroviral infection using green fluorescence protein.* Osteoarthritis Cartilage, 2002. 10(2)
188. van Buul, G.M., et al., *Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI.* Contrast Media Mol Imaging, 2009. 4(5)
189. Farrell, E., et al., *Effects of iron oxide incorporation for long term cell tracking on MSC differentiation in vitro and in vivo.* Biochem Biophys Res Commun, 2008. 369(4)

190. Bos, P.K., et al., *Specific enzymatic treatment of bovine and human articular cartilage: implications for integrative cartilage repair.* Arthritis Rheum, 2002. 46(4)
191. Tew, S.R., et al., *The reactions of articular cartilage to experimental wounding: role of apoptosis.* Arthritis Rheum, 2000. 43(1)
192. Silverman, R.P., et al., *Adhesion of tissue-engineered cartilate to native cartilage.* Plast Reconstr Surg, 2000. 105(4)
193. Obradovic, B., et al., *Integration of engineered cartilage.* J Orthop Res, 2001. 19(6)
194. van de Breevaart Bravenboer, J., et al., *Improved cartilage integration and interfacial strength after enzymatic treatment in a cartilage transplantation model.* Arthritis Res Ther, 2004. 6(5)
195. Janssen, L.M., et al., *Short-duration enzymatic treatment promotes integration of a cartilage graft in a defect.* Ann Otol Rhinol Laryngol, 2006. 115(6)
196. Mochizuki, Y., V. Goldberg, and A. Caplan, *Enzymatical digestion for the repair of superficial articular cartilage lesions.* Trans Orthop Res Soc, 1993. 39
197. Hunziker, E.B. and E. Kapfinger, *Removal of proteoglycans from the surface of defects in articular cartilage transiently enhances coverage by repair cells.* J Bone Joint Surg Br, 1998. 80(1)
198. Lee, M.C., et al., *Adhesive force of chondrocytes to cartilage. Effects of chondroitinase ABC.* Clin Orthop, 2000(370)
199. Schaefer, D.B., et al., *Lubricin reduces cartilage--cartilage integration.* Biorheology, 2004. 41(3-4)
200. Tiderius, C.J., et al., *dGEMRIC (delayed gadolinium-enhanced MRI of cartilage) indicates adaptive capacity of human knee cartilage.* Magn Reson Med, 2004. 51(2)
201. Bashir, A., M.L. Gray, and D. Burstein, *Gd-DTPA2- as a measure of cartilage degradation.* Magn Reson Med, 1996. 36(5)
202. Maroudas, A., ed. *Physicochemical properties of articular cartilage*. Adult articular cartilage, ed. M. Freeman. Vol. 2. 1979, Pitman Medical: London.
203. Gray, M.L., et al., *2007 Elizabeth Winston Lanier Award Winner. Magnetic resonance imaging of cartilage glycosaminoglycan: basic principles, imaging technique, and clinical applications.* J Orthop Res, 2008. 26(3)
204. Trattnig, S., et al., *Three-dimensional delayed gadolinium-enhanced MRI of cartilage (dGEMRIC) for in vivo evaluation of reparative cartilage after matrix-associated autologous chondrocyte transplantation at 3.0T: Preliminary results.* J Magn Reson Imaging, 2007. 26(4)
205. Domayer, S.E., et al., *T2 mapping and dGEMRIC after autologous chondrocyte implantation with a fibrin-based scaffold in the knee: preliminary results.* Eur J Radiol, 2010. 73(3)
206. Vasiliadis, H.S., et al., *Autologous chondrocyte implantation in cartilage lesions of the knee: long-term evaluation with magnetic resonance imaging and delayed gadolinium-enhanced magnetic resonance imaging technique.* Am J Sports Med, 2010. 38(5)
207. Watanabe, A., et al., *Ability of dGEMRIC and T2 mapping to evaluate cartilage repair after microfracture: a goat study.* Osteoarthritis Cartilage, 2009. 17(10)
208. Yang, K.G., et al., *The effect of synovial fluid from injured knee joints on in vitro chondrogenesis.* Tissue Eng, 2006. 12(10)
209. Rodrigo, J.J., et al., *Effects of human knee synovial fluid on chondrogenesis in vitro.* Am J Knee Surg, 1995. 8(4)
210. Kruger, J.P., et al., *Chondrogenic differentiation of human subchondral progenitor cells is affected by synovial fluid from donors with osteoarthritis or rheumatoid arthritis.* J Orthop Surg Res, 2012. 7
211. Heldens, G.T., et al., *Catabolic factors and osteoarthritis-conditioned medium inhibit chondrogenesis of human mesenchymal stem cells.* Tissue Eng Part A, 2012. 18(1-2)
212. Sakurai, H., *Targeting of TAK1 in inflammatory disorders and cancer.* Trends Pharmacol Sci, 2012. 33(10)
213. Delaney, J.R. and M. Mlodzik, *TGF-beta activated kinase-1: new insights into the diverse roles of TAK1 in development and immunity.* Cell Cycle, 2006. 5(24)
214. Ninomiya-Tsuji, J., et al., *The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway.* Nature, 1999. 398(6724)
215. Sakurai, H., et al., *Functional interactions of transforming growth factor beta-activated kinase 1 with IkappaB kinases to stimulate NF-kappaB activation.* J Biol Chem, 1999. 274(15)
216. Darnell, J.E., Jr., I.M. Kerr, and G.R. Stark, *Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.* Science, 1994. 264(5164)

217. Schindler, C. and J.E. Darnell, Jr., *Transcriptional responses to polypeptide ligands: the JAK-STAT pathway.* Annu Rev Biochem, 1995. 64
218. Kisseleva, T., et al., *Signaling through the JAK/STAT pathway, recent advances and future challenges.* Gene, 2002. 285(1-2)
219. Murray, P.J., *The JAK-STAT signaling pathway: input and output integration.* J Immunol, 2007. 178(5)
220. O'Sullivan, L.A., et al., *Cytokine receptor signaling through the Jak-Stat-Socs pathway in disease.* Mol Immunol, 2007. 44(10)
221. Yoshimura, A., T. Naka, and M. Kubo, *SOCS proteins, cytokine signalling and immune regulation.* Nat Rev Immunol, 2007. 7(6)
222. Aaronson, D.S. and C.M. Horvath, *A road map for those who don't know JAK-STAT.* Science, 2002. 296(5573)
223. Ghoreschi, K., et al., *Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550).* J Immunol, 2011. 186(7)
224. Meyer, D.M., et al., *Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis.* J Inflamm (Lond), 2010. 7
225. van der Heijde, D., et al., *Tofacitinib (CP-690,550) in patients with rheumatoid arthritis receiving methotrexate: twelve-month data from a twenty-four-month phase III randomized radiographic study.* Arthritis Rheum, 2013. 65(3)
226. Burmester, G.R., et al., *Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: a randomised phase 3 trial.* Lancet, 2013. 381(9865)
227. Kurth, T., et al., *Chondrogenic potential of human synovial mesenchymal stem cells in alginate.* Osteoarthritis Cartilage, 2007. 15(10)
228. Farndale, R.W., D.J. Buttle, and A.J. Barrett, *Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue.* Biochim Biophys Acta, 1986. 883(2)
229. Beier, F. and R.F. Loeser, *Biology and pathology of Rho GTPase, PI-3 kinase-Akt, and MAP kinase signaling pathways in chondrocytes.* J Cell Biochem, 2010. 110(3)
230. Scharstuhl, A., et al., *Adenoviral overexpression of Smad-7 and Smad-6 differentially regulates TGF-beta-mediated chondrocyte proliferation and proteoglycan synthesis.* Osteoarthritis Cartilage, 2003. 11(11)
231. Hoffmann, A., et al., *Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors.* J Biol Chem, 2005. 280(29)
232. Yumoto, K., et al., *TGF-beta-activated kinase 1 (Tak1) mediates agonist-induced Smad activation and linker region phosphorylation in embryonic craniofacial neural crest-derived cells.* J Biol Chem, 2013. 288(19)
233. Hanafusa, H., et al., *Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression.* J Biol Chem, 1999. 274(38)
234. Nakamura, K., et al., *p38 mitogen-activated protein kinase functionally contributes to chondrogenesis induced by growth/differentiation factor-5 in ATDC5 cells.* Exp Cell Res, 1999. 250(2)
235. Jin, E.J., et al., *BMP-2-enhanced chondrogenesis involves p38 MAPK-mediated down-regulation of Wnt-7a pathway.* Mol Cells, 2006. 22(3)
236. Derynck, R. and Y.E. Zhang, *Smad-dependent and Smad-independent pathways in TGF-beta family signalling.* Nature, 2003. 425(6958)
237. Tuli, R., et al., *Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk.* J Biol Chem, 2003. 278(42)
238. Zuzarte-Luis, V., et al., *A new role for BMP5 during limb development acting through the synergic activation of Smad and MAPK pathways.* Dev Biol, 2004. 272(1)
239. Gunnell, L.M., et al., *TAK1 regulates cartilage and joint development via the MAPK and BMP signaling pathways.* J Bone Miner Res, 2010. 25(8)
240. Li, W.Q., F. Dehnade, and M. Zafarullah, *Oncostatin M-induced matrix metalloproteinase and tissue inhibitor of metalloproteinase-3 genes expression in chondrocytes requires Janus kinase/STAT signaling pathway.* J Immunol, 2001. 166(5)
241. Klatt, A.R., et al., *TAK1 downregulation reduces IL-1beta induced expression of MMP13, MMP1 and TNF-alpha.* Biomed Pharmacother, 2006. 60(2)

242. Bau, B., et al., *Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro.* Arthritis Rheum, 2002. 46(10)
243. Rogerson, F.M., et al., *Cytokine-induced increases in ADAMTS-4 messenger RNA expression do not lead to increased aggrecanase activity in ADAMTS-5-deficient mice.* Arthritis Rheum, 2010. 62(11)
244. El Mabrouk, M., J. Sylvester, and M. Zafarullah, *Signaling pathways implicated in oncostatin M-induced aggrecanase-1 and matrix metalloproteinase-13 expression in human articular chondrocytes.* Biochim Biophys Acta, 2007. 1773(3)
245. Temenoff, J.S. and A.G. Mikos, *Review: tissue engineering for regeneration of articular cartilage.* Biomaterials, 2000. 21(5)
246. Redman, S.N., S.F. Oldfield, and C.W. Archer, *Current strategies for articular cartilage repair.* Eur Cell Mater, 2005. 9
247. Murphy, J.M., et al., *Stem cell therapy in a caprine model of osteoarthritis.* Arthritis Rheum, 2003. 48(12)
248. Yoo, J.U., et al., *The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells.* J Bone Joint Surg Am, 1998. 80(12)
249. Pessler, F., et al., *The synovitis of "non-inflammatory" orthopaedic arthropathies: a quantitative histological and immunohistochemical analysis.* Ann Rheum Dis, 2008. 67(8)
250. Stevens, A.L., et al., *Nitric oxide enhances aggrecan degradation by aggrecanase in response to TNF-alpha but not IL-1beta treatment at a post-transcriptional level in bovine cartilage explants.* Osteoarthritis Cartilage, 2008. 16(4)
251. Legendre, F., et al., *JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of Type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of SOX9 expression.* J Biol Chem, 2003. 278(5)
252. Beekhuizen, M., et al., *Osteoarthritic synovial tissue inhibits proteoglycan production in human osteoarthritic cartilage; Establishment and characterisation of a long-term coculture.* Arthritis Rheum, 2011
253. Beekhuizen, M., et al., *Inhibition of oncostatin M in osteoarthritic synovial fluid enhances GAG production in osteoarthritic cartilage repair.* Eur Cell Mater, 2013. 26
254. Bondeson, J., et al., *The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis.* Arthritis Rheum, 2010. 62(3)
255. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. 8(12)
256. Hofkens, W., et al., *Liposomal targeting of prednisolone phosphate to synovial lining macrophages during experimental arthritis inhibits M1 activation but does not favor M2 differentiation.* PLoS One, 2013. 8(2)
257. Petro, T.M., S.S. Chen, and R.B. Panther, *Effect of CD80 and CD86 on T cell cytokine production.* Immunol Invest, 1995. 24(6)
258. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization.* Trends Immunol, 2004. 25(12)
259. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.* J Immunol, 2006. 177(10)
260. Pfander, D., et al., *Tenascin and aggrecan expression by articular chondrocytes is influenced by interleukin 1beta: a possible explanation for the changes in matrix synthesis during osteoarthritis.* Ann Rheum Dis, 2004. 63(3)
261. Malfait, A.M., et al., *Intra-articular injection of tumor necrosis factor-alpha in the rat: an acute and reversible in vivo model of cartilage proteoglycan degradation.* Osteoarthritis Cartilage, 2009. 17(5)
262. Aida, Y., et al., *IL-6 and soluble IL-6 receptor stimulate the production of MMPs and their inhibitors via JAK-STAT and ERK-MAPK signalling in human chondrocytes.* Cell Biol Int, 2012. 36(4)
263. Shakibaei, M., et al., *Suppression of NF-kappaB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: Implications for the treatment of osteoarthritis.* Biochem Pharmacol, 2007. 73(9)
264. Poree, B., et al., *Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1.Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter.* J Biol Chem, 2008. 283(8)
265. Koch, A.E., P.J. Polverini, and S.J. Leibovich, *Stimulation of neovascularization by human rheumatoid synovial tissue macrophages.* Arthritis Rheum, 1986. 29(4)

266. Kumagai, K., et al., *Pretreatment of plastic Petri dishes with fetal calf serum. A simple method for macrophage isolation.* J Immunol Methods, 1979. 29(1)
267. van Osch, G.J., et al., *Monoclonal antibody 11-fibrau: a useful marker to characterize chondrocyte differentiation stage.* Biochem Biophys Res Commun, 2001. 280(3)
268. Grotenhuis, N., et al., *A culture model to analyze the acute biomaterial-dependent reaction of human primary macrophages.* Biochem Biophys Res Commun, 2013. 433(1)
269. Farndale, R.W., C.A. Sayers, and A.J. Barrett, *A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures.* Connect Tissue Res, 1982. 9(4)
270. Enobakhare, B.O., D.L. Bader, and D.A. Lee, *Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue.* Anal Biochem, 1996. 243(1)
271. Cheung, D.L., et al., *Contrasting effects of interferon-gamma and interleukin-4 on the interleukin-6 activity of stimulated human monocytes.* Immunology, 1990. 71(1)
272. Hart, P.H., et al., *Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2.* Proc Natl Acad Sci U S A, 1989. 86(10)
273. Martinez-Pomares, L., et al., *Analysis of mannose receptor regulation by IL-4, IL-10, and proteolytic processing using novel monoclonal antibodies.* J Leukoc Biol, 2003. 73(5)
274. Clockaerts, S., et al., *Peroxisome proliferator activated receptor alpha activation creases inflammatory and destructive responses in osteoarthritic cartilage.* Osteoarthritis Cartilage, 2011. 19(7)
275. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. 3(6)
276. Drutman, S.B., J.C. Kendall, and E.S. Trombetta, *Inflammatory spleen monocytes can upregulate CD11c expression without converting into dendritic cells.* J Immunol, 2012. 188(8)
277. Tsuchida, A.I., et al., *Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model.* Arthritis Res Ther, 2012. 14(6)
278. Gierman, L.M., et al., *Profiling the secretion of soluble mediators by end stage osteoarthritis synovial tissue explants reveals a reduced responsiveness to an inflammatory trigger.* PLoS One, 2013. 8(5)
279. Tsuneyoshi, Y., et al., *Functional folate receptor beta-expressing macrophages in osteoarthritis synovium and their M1/M2 expression profiles.* Scand J Rheumatol, 2012. 41(2)
280. Freytes, D.O., et al., *Macrophages modulate the viability and growth of human mesenchymal stem cells.* J Cell Biochem, 2013. 114(1)
281. van Buul, G.M., et al., *Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture.* Osteoarthritis and Cartilage, 2012. 20(10)
282. Kim, J. and P. Hematti, *Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages.* Exp Hematol, 2009. 37(12)
283. Zhang, Q.Z., et al., *Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing.* Stem Cells, 2010. 28(10)
284. Holladay, C., et al., *Functionalized Scaffold-mediated Interleukin 10 Gene Delivery Significantly Improves Survival Rates of Stem Cells In Vivo.* Mol Ther, 2011
285. Le Blanc, K., et al., *HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells.* Exp Hematol, 2003. 31(10)
286. Bos, P.K., van Melle, M.L., van Osch, G.J.V.M., *Articular cartilage repair and the evolving role of regenerative medicin.* Open access surgery, 2010. 3
287. van Osch, G.J., et al., *Cartilage repair: past and future--lessons for regenerative medicine.* J Cell Mol Med, 2009. 13(5)
288. Lowry, O.H., et al., *The quantitative histochemistry of brain. II. Enzyme measurements.* J Biol Chem, 1954. 207(1)
289. Erlebacher, A. and R. Derynck, *Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype.* J Cell Biol, 1996. 132(1-2)
290. Scheven, B.A., et al., *Differentiation kinetics of osteoclasts in the periosteum of embryonic bones in vivo and in vitro.* Anat Rec, 1986. 214(4)
291. Sniekers, Y.H., et al., *Estrogen modulates iodoacetate-induced gene expression in bovine cartilage explants.* J Orthop Res, 2010. 28(5)

292. Jenniskens, Y.M., et al., *Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFbeta2 and FGF2.* Osteoarthritis Cartilage, 2006. 14(11)
293. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. 25(4)
294. Mueller-Rath, R., et al., *In vivo cultivation of human articular chondrocytes in a nude mouse-based contained defect organ culture model.* Biomed Mater Eng, 2007. 17(6)
295. Schuller, G.C., et al., *An in vivo mouse model for human cartilage regeneration.* J Tissue Eng Regen Med, 2008. 2(4)
296. Iwai, R., et al., *Ex vivo cartilage defect model for the evaluation of cartilage regeneration using mesenchymal stem cells.* J Biosci Bioeng, 2011. 111(3)
297. Secretan, C., K.M. Bagnall, and N.M. Jomha, *Effects of introducing cultured human chondrocytes into a human articular cartilage explant model.* Cell Tissue Res, 2010. 339(2)
298. Botter, S.M., et al., *Cartilage damage pattern in relation to subchondral plate thickness in a collagenase-induced model of osteoarthritis.* Osteoarthritis Cartilage, 2008. 16(4)
299. Sniekers, Y.H., et al., *Oestrogen is important for maintenance of cartilage and subchondral bone in a murine model of knee osteoarthritis.* Arthritis Res Ther, 2010. 12(5)
300. Sniekers, Y.H., et al., *Development of osteoarthritic features in estrogen receptor knockout mice.* Osteoarthritis Cartilage, 2009. 17(10)
301. Intema, F., et al., *Subchondral bone remodeling is related to clinical improvement after joint distraction in the treatment of ankle osteoarthritis.* Osteoarthritis Cartilage, 2011
302. Zhang, L., et al., *Enhancement of subchondral bone quality by alendronate administration for the reduction of cartilage degeneration in the early phase of experimental osteoarthritis.* Clin Exp Med, 2011
303. Pfander, D. and K. Gelse, *Hypoxia and osteoarthritis: how chondrocytes survive hypoxic environments.* Curr Opin Rheumatol, 2007. 19(5)
304. Bos, P.K., et al., *Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model.* Osteoarthritis Cartilage, 2001. 9(4)
305. Bos, P.K., J.A. Verhaar, and G.J. van Osch, *Age-related differences in articular cartilage wound healing: a potential role for transforming growth factor beta1 in adult cartilage repair.* Adv Exp Med Biol, 2006. 585
306. Wolff, J., *The law of bone remodeling* translation of the german 1892 version. 1986, Berlin: Springer.
307. Niemeyer, P., et al., *Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint.* Am J Sports Med, 2008. 36(11)
308. Caplan, A.I., *Mesenchymal stem cells.* J Orthop Res, 1991. 9(5)
309. Bos, P.K., M.L. van Melle, and G.J. van Osch, *Articular cartilage repair and the evolving role of regenerative medicine.* Open Acces Surgery, 2010. 3
310. Cals, F.L., et al., *Effects of transforming growth factor-beta subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells.* J Tissue Eng Regen Med, 2012. 6(1)
311. Potier, E., J. Noailly, and K. Ito, *Directing bone marrow-derived stromal cell function with mechanics.* J Biomech, 2010. 43(5)
312. Khan, W.S., et al., *Bone marrow-derived mesenchymal stem cells express the pericyte marker 3G5 in culture and show enhanced chondrogenesis in hypoxic conditions.* J Orthop Res, 2010. 28(6)
313. Fischer, J., et al., *Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis.* Arthritis Rheum, 2010. 62(9)
314. Hellingman, C.A., et al., *Smad signaling determines chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification.* Tissue Eng Part A, 2011. 17(7-8)
315. Tay, L.X., et al., *Treatment outcomes of alginate-embedded allogenic mesenchymal stem cells versus autologous chondrocytes for the repair of focal articular cartilage defects in a rabbit model.* Am J Sports Med, 2012. 40(1)
316. Qi, Y., et al., *The restoration of full-thickness cartilage defects with mesenchymal stem cells (MSCs) loaded and cross-linked bilayer collagen scaffolds on rabbit model.* Mol Biol Rep, 2012. 39(2)

317. Li, Q., et al., *Comparing the chondrogenic potential in vivo of autogeneic mesenchymal stem cells derived from different tissues.* Artif Cells Blood Substit Immobil Biotechnol, 2011. 39(1)
318. Steck, E., et al., *Mesenchymal stem cell differentiation in an experimental cartilage defect: restriction of hypertrophy to bone-close neocartilage.* Stem Cells Dev, 2009. 18(7)
319. Farrell, E., et al., *In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells.* BMC Musculoskelet Disord, 2011. 12
320. de Vries-van Melle, M.L., et al., *An osteochondral culture model to study mechanisms involved in articular cartilage repair.* Tissue Eng Part C Methods, 2012. 18(1)
321. Emadedin, M., et al., *Intra-articular injection of autologous mesenchymal stem cells in six patients with knee osteoarthritis.* Arch Iran Med, 2012. 15(7)
322. Mokbel, A.N., et al., *Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model.* BMC Musculoskelet Disord, 2011. 12
323. Chen, J., et al., *Simultaneous regeneration of articular cartilage and subchondral bone in vivo using MSCs induced by a spatially controlled gene delivery system in bilayered integrated scaffolds.* Biomaterials, 2011. 32(21)
324. Yoshioka, T., et al., *Fate of bone marrow mesenchymal stem cells following the allogeneic transplantation of cartilaginous aggregates into osteochondral defects of rabbits.* J Tissue Eng Regen Med, 2011. 5(6)
325. Caplan, A.I. and D. Correa, *The MSC: an injury drugstore.* Cell Stem Cell, 2011. 9(1)
326. Cao, X. and D. Chen, *The BMP signaling and in vivo bone formation.* Gene, 2005. 357(1)
327. Urist, M.R., *Bone: formation by autoinduction.* Science, 1965. 150(3698)
328. van Blitterswijk, C.A., *Tissue Engineering*. 2008, London: Academic Press Elsevier.
329. Bolander, M.E., *Regulation of fracture repair by growth factors.* Proc Soc Exp Biol Med, 1992. 200(2)
330. Joyce, M.E., et al., *Role of growth factors in fracture healing.* Prog Clin Biol Res, 1991. 365
331. Krause, C., et al., *Distinct modes of inhibition by sclerostin on bone morphogenetic protein and Wnt signaling pathways.* J Biol Chem, 2010. 285(53)
332. Hernandez, R.M., et al., *Microcapsules and microcarriers for in situ cell delivery.* Adv Drug Deliv Rev, 2010. 62(7-8)
333. Johnstone, B., et al., *Tissue engineering for articular cartilage repair--the state of the art.* Eur Cell Mater, 2013. 25
334. Loeser, R.F., C.S. Carlson, and M.P. McGee, *Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage.* Exp Cell Res, 1995. 217(2)
335. Ghidoni, I., et al., *Alginate cell encapsulation: new advances in reproduction and cartilage regenerative medicine.* Cytotechnology, 2008. 58(1)
336. Thomas, S., *Alginate dressings in surgery and wound management: Part 3.* J Wound Care, 2000. 9(4)
337. Orive, G., et al., *Biocompatibility of microcapsules for cell immobilization elaborated with different type of alginates.* Biomaterials, 2002. 23(18)
338. Almqvist, K.F., et al., *Treatment of cartilage defects in the knee using alginate beads containing human mature allogenic chondrocytes.* Am J Sports Med, 2009. 37(10)
339. Basta, G., et al., *Long-term metabolic and immunological follow-up of nonimmunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: four cases.* Diabetes Care, 2011. 34(11)
340. Rokstad, A.M., et al., *Alginate microbeads are complement compatible, in contrast to polycation containing microcapsules, as revealed in a human whole blood model.* Acta Biomater, 2011. 7(6)
341. Ito, K., et al., *Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet-rich plasma.* Clin Oral Implants Res, 2006. 17(5)
342. Haleem, A.M., et al., *The Clinical Use of Human Culture-Expanded Autologous Bone Marrow Mesenchymal Stem Cells Transplanted on Platelet-Rich Fibrin Glue in the Treatment of Articular Cartilage Defects: A Pilot Study and Preliminary Results.* Cartilage, 2010. 1(4)
343. Liao, H.T., et al., *Combination of guided osteogenesis with autologous platelet-rich fibrin glue and mesenchymal stem cell for mandibular reconstruction.* J Trauma, 2011. 70(1)

344. Wu, X., J. Ren, and J. Li, *Fibrin glue as the cell-delivery vehicle for mesenchymal stromal cells in regenerative medicine.* Cytotherapy, 2012. 14(5)
345. Ryu, J.H., et al., *Implantation of bone marrow mononuclear cells using injectable fibrin matrix enhances neovascularization in infarcted myocardium.* Biomaterials, 2005. 26(3)
346. Falanga, V., et al., *Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds.* Tissue Eng, 2007. 13(6)
347. Bellamy, N., et al., *Viscosupplementation for the treatment of osteoarthritis of the knee.* Cochrane Database Syst Rev, 2006(2)
348. Divine, J.G., B.T. Zazulak, and T.E. Hewett, *Viscosupplementation for knee osteoarthritis: a systematic review.* Clin Orthop Relat Res, 2007. 455
349. Toole, B.P., *Hyaluronan in morphogenesis.* J Intern Med, 1997. 242(1)
350. Mortisen, D., et al., *Tailoring thermoreversible hyaluronan hydrogels by "click" chemistry and RAFT polymerization for cell and drug therapy.* Biomacromolecules, 2010. 11(5)
351. Peroglio, M., et al., *Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell encapsulation.* Eur Spine J, 2012. 21 Suppl 6
352. Malonzo, C., et al., *A papain-induced disc degeneration model for the assessment of thermo-reversible hydrogel-cells therapeutic approach.* J Tissue Eng Regen Med, 2013
353. de Vries - van Melle, M.L., Narcisi, R., Kops, N., Koevoet, J.L.M., Bos, P.K., Murphy, J.M., Verhaar, J.A.N., van der Kraan, P.M., van Osch, G.J.V.M., *Chondrogenesis of mesenchymal stem cells in an osteochondral environment is mediated by the subchondral bone* Tissue Eng 2013. accepted july 2013
354. Scotti, C., et al., *Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering.* Proc Natl Acad Sci U S A, 2010. 107(16)
355. Itay, S., A. Abramovici, and Z. Nevo, *Use of cultured embryonal chick epiphyseal chondrocytes as grafts for defects in chick articular cartilage.* Clin Orthop Relat Res, 1987(220)
356. Goa, K.L. and P. Benfield, *Hyaluronic acid. A review of its pharmacology and use as a surgical aid in ophthalmology, and its therapeutic potential in joint disease and wound healing.* Drugs, 1994. 47(3)
357. Ting, V., et al., *In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes.* Ann Plast Surg, 1998. 40(4)
358. Park, S.H., et al., *Chondrogenesis of rabbit mesenchymal stem cells in fibrin/hyaluronan composite scaffold in vitro.* Tissue Eng Part A, 2011. 17(9-10)
359. Ma, K., et al., *Variations in chondrogenesis of human bone marrow-derived mesenchymal stem cells in fibrin/alginate blended hydrogels.* Acta Biomater, 2012. 8(10)
360. Weisel, J.W., *Fibrinogen and fibrin.* Adv Protein Chem, 2005. 70
361. Farrell, M.J., E.S. Comeau, and R.L. Mauck, *Mesenchymal stem cells produce functional cartilage matrix in three-dimensional culture in regions of optimal nutrient supply.* Eur Cell Mater, 2012. 23
362. Erickson, I.E., et al., *Macromer density influences mesenchymal stem cell chondrogenesis and maturation in photocrosslinked hyaluronic acid hydrogels.* Osteoarthritis Cartilage, 2009. 17(12)
363. Grigolo, B., et al., *Transplantation of chondrocytes seeded on a hyaluronan derivative (hyaff-11) into cartilage defects in rabbits.* Biomaterials, 2001. 22(17) 364. Kavalkovich, K.W., et al., *Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system.* In Vitro Cell Dev Biol Anim, 2002. 38(8)
365. Facchini, A., et al., *Human chondrocytes and mesenchymal stem cells grown onto engineered scaffold.* Biorheology, 2006. 43(3-4)
366. Liao, E., et al., *Tissue-engineered cartilage constructs using composite hyaluronic acid/collagen I hydrogels and designed poly(propylene fumarate) scaffolds.* Tissue Eng, 2007. 13(3)
367. Rampichova, M., et al., *Fibrin/hyaluronic acid composite hydrogels as appropriate scaffolds for in vivo artificial cartilage implantation.* ASAIO J, 2010. 56(6)
368. Kim, I.L., R.L. Mauck, and J.A. Burdick, *Hydrogel design for cartilage tissue engineering: a case study with hyaluronic acid.* Biomaterials, 2011. 32(34)
369. Erickson, I.E., et al., *High mesenchymal stem cell seeding densities in hyaluronic acid hydrogels produce engineered cartilage with native tissue properties.* Acta Biomater, 2012. 8(8)

370. Woolf, A.D. and B. Pfleger, *Burden of major musculoskeletal conditions.* Bull World Health Organ, 2003. 81(9)
371. Felson, D.T., *Clinical practice. Osteoarthritis of the knee.* N Engl J Med, 2006. 354(8)
372. Sahni, M., et al., *FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway.* Genes Dev, 1999. 13(11)
373. Beekhuizen, M., et al., *An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid.* Osteoarthritis Cartilage, 2013. 21(7)
374. Schmal, H., et al., *In vivo quantification of intraarticular cytokines in knees during natural and surgically induced cartilage repair.* Cytotherapy, 2009. 11(8)
375. Tsuchida, A.I., et al., *Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model.* Arthritis Res Ther. 14(6)
376. Coleman, C.M., et al., *Growth differentiation factor-5 enhances in vitro mesenchymal stromal cell chondrogenesis and hypertrophy.* Stem Cells Dev. 22(13)
377. Toh, W.S., et al., *Combined effects of TGFbeta1 and BMP2 in serum-free chondrogenic differentiation of mesenchymal stem cells induced hyaline-like cartilage formation.* Growth Factors, 2005. 23(4)
378. Ferguson, C.M., et al., *Smad2 and 3 mediate transforming growth factor-beta1-induced inhibition of chondrocyte maturation.* Endocrinology, 2000. 141(12)
379. Goumans, M.J., et al., *Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors.* EMBO J, 2002. 21(7)
380. Goumans, M.J., et al., *Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling.* Mol Cell, 2003. 12(4)
381. Goumans, M.J., F. Lebrin, and G. Valdimarsdottir, *Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways.* Trends Cardiovasc Med, 2003. 13(7)
382. Retting, K.N., et al., *BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation.* Development, 2009. 136(7)
383. Yang, X., et al., *TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage.* J Cell Biol, 2001. 153(1)
384. Blaney Davidson, E.N., et al., *Expression of transforming growth factor-beta (TGFbeta) and the TGFbeta signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation.* Ann Rheum Dis, 2006. 65(11)
385. van der Kraan, P.M., et al., *TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads.* Osteoarthritis Cartilage, 2009. 17(12)
386. Blaney Davidson, E.N., et al., *Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice.* J Immunol, 2009. 182(12)
387. Sekiya, I., et al., *In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis.* Proc Natl Acad Sci U S A, 2002. 99(7)
388. Moses, H.L. and R. Serra, *Regulation of differentiation by TGF-beta.* Curr Opin Genet Dev, 1996. 6(5)
389. Radaev, S., et al., *Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily.* J Biol Chem. 285(19)
390. Memon, M.A., et al., *Transforming growth factor beta (TGFbeta1, TGFbeta2 and TGFbeta3) null-mutant phenotypes in embryonic gonadal development.* Mol Cell Endocrinol, 2008. 294(1-2)
391. Sanford, L.P., et al., *TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes.* Development, 1997. 124(13)
392. Vortkamp, A., et al., *Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein.* Science, 1996. 273(5275)
393. Mak, K.K., et al., *Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy.* Development, 2008. 135(11)
394. Lanske, B., et al., *PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth.* Science, 1996. 273(5275)
395. Studer, D., et al., *Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells.* Eur Cell Mater. 24

396. Dong, Y.F., et al., *Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor.* J Cell Physiol, 2006. 208(1)

397. Im, G.I., J.M. Lee, and H.J. Kim, *Wnt inhibitors enhance chondrogenesis of human mesenchymal stem cells in a long-term pellet culture.* Biotechnol Lett. 33(5)

398. Bian, L., et al., *The influence of hyaluronic acid hydrogel crosslinking density and macromolecular diffusivity on human MSC chondrogenesis and hypertrophy.* Biomaterials. 34(2)

399. Re'em, T., et al., *Chondrogenesis of hMSC in affinity-bound TGF-beta scaffolds.* Biomaterials. 33(3)

400. Grad, S., et al., *Physical stimulation of chondrogenic cells in vitro: a review.* Clin Orthop Relat Res. 469(10)

401. Campbell, J.J., D.A. Lee, and D.L. Bader, *Dynamic compressive strain influences chondrogenic gene expression in human mesenchymal stem cells.* Biorheology, 2006. 43(3-4)

402. Huang, C.Y., et al., *Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells.* Stem Cells, 2004. 22(3)

403. Mouw, J.K., et al., *Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells.* Stem Cells, 2007. 25(3)

404. Wimmer, M.A., et al., *Tribology approach to the engineering and study of articular cartilage.* Tissue Eng, 2004. 10(9-10)

405. Stoddart, M.J., L. Ettinger, and H.J. Hauselmann, *Enhanced matrix synthesis in de novo, scaffold free cartilage-like tissue subjected to compression and shear.* Biotechnol Bioeng, 2006. 95(6)

406. Elder, B.D. and K.A. Athanasiou, *Hydrostatic pressure in articular cartilage tissue engineering: from chondrocytes to tissue regeneration.* Tissue Eng Part B Rev, 2009. 15(1)

407. Parkkinen, J.J., et al., *Effects of cyclic hydrostatic pressure on proteoglycan synthesis in cultured chondrocytes and articular cartilage explants.* Arch Biochem Biophys, 1993. 300(1)

408. Carver, S.E. and C.A. Heath, *Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure.* Biotechnol Bioeng, 1999. 62(2)

409. Wenger, R., et al., *Hydrostatic pressure increases apoptosis in cartilage-constructs produced from human osteoarthritic chondrocytes.* Front Biosci, 2006. 11

410. Gavenis, K., et al., *Effects of cyclic hydrostatic pressure on the metabolism of human osteoarthritic chondrocytes cultivated in a collagen gel.* Artif Organs, 2007. 31(2)

411. Almaawi, A., et al., *Effect of acetaminophen and nonsteroidal anti-inflammatory drugs on gene expression of mesenchymal stem cells.* Tissue Eng Part A. 19(7-8)

412. Caplan, A.I. and J.E. Dennis, *Mesenchymal stem cells as trophic mediators.* J Cell Biochem, 2006. 98(5)

413. Maumus, M., et al., *Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis.* Stem Cell Res. 11(2)

414. Greish, S., et al., *Human umbilical cord mesenchymal stem cells as treatment of adjuvant rheumatoid arthritis in a rat model.* World J Stem Cells. 4(10)

415. Leijs, M.J., et al., *Effect of Arthritic Synovial Fluids on the Expression of Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro Study.* Front Immunol. 3

416. Shim, J.H. et al., *TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo,* Genes Dev 2005. 19

417. Akira, S., *Toll-like receptor signaling,* J Biol Chem 2003. 278

418. Ono, K. et al., *An evolutionarily conserved motif in the TAB1 C-terminal region is necessary for interaction with and activation of TAK1 MAPKKK,* J Biol Chem 2001. 276

419. Cheung, P.C., Nebreda, A.R. and Cohen, P., *TAB3, a new binding partner of the protein kinase TAK!,* Biochem J 2003. 378

420. Ishitani, T. et al., *Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling,* EMBO J 2003. 22

421. Kulathu, Y. et al., *Two-sided ubiquitin binding explains specificity of the TAB2 NZF domain,* Nat Struct Mol Biol 2009. 16

422. Xia, Z.P. et al., *Direct activation of protein kinases by unanchored polyubiquitin chains,* Nature 1999. 461

423. Kim, S.I. et al., *Transforming growth factor-β (TGF-β1) activates TAK1 via TAB1-mediated autophosphorylation, independent of TGF-β receptor kinase activity in mesangial cells,* J Biol Chem 2009. 284

424. Ishitani, T. et al., *The TAK1-NLK-MAPK-related pathway antagonizes signaling between β-catenin and transcription factor TCF,* Nature 1999. 399

425. Mao, R. et al., *TAK1 lysine 158 is required for TGF-β-induced TRAF6-mediated Smad-independent IKK/NF-kB and JNK/AP-1 activation,* Cell Signal 2011. 23

426. Liu, Q., Busby, J.C. and Molkentin, J.D., *Interaction between TAK1-TAB1-TAB2 and RCAN1-calcineurin defines a signaling nodal control point,* Nat Cell Biol 2009. 11

427. Yamaguchi, K. et al., *XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signalin pathway,* EMBO J 1999. 18

428. Cheung, P.C. et al., *Feedback control of the protein kinase TAK1 by SAPK2a/p38a,* EMBO J 2003. 22

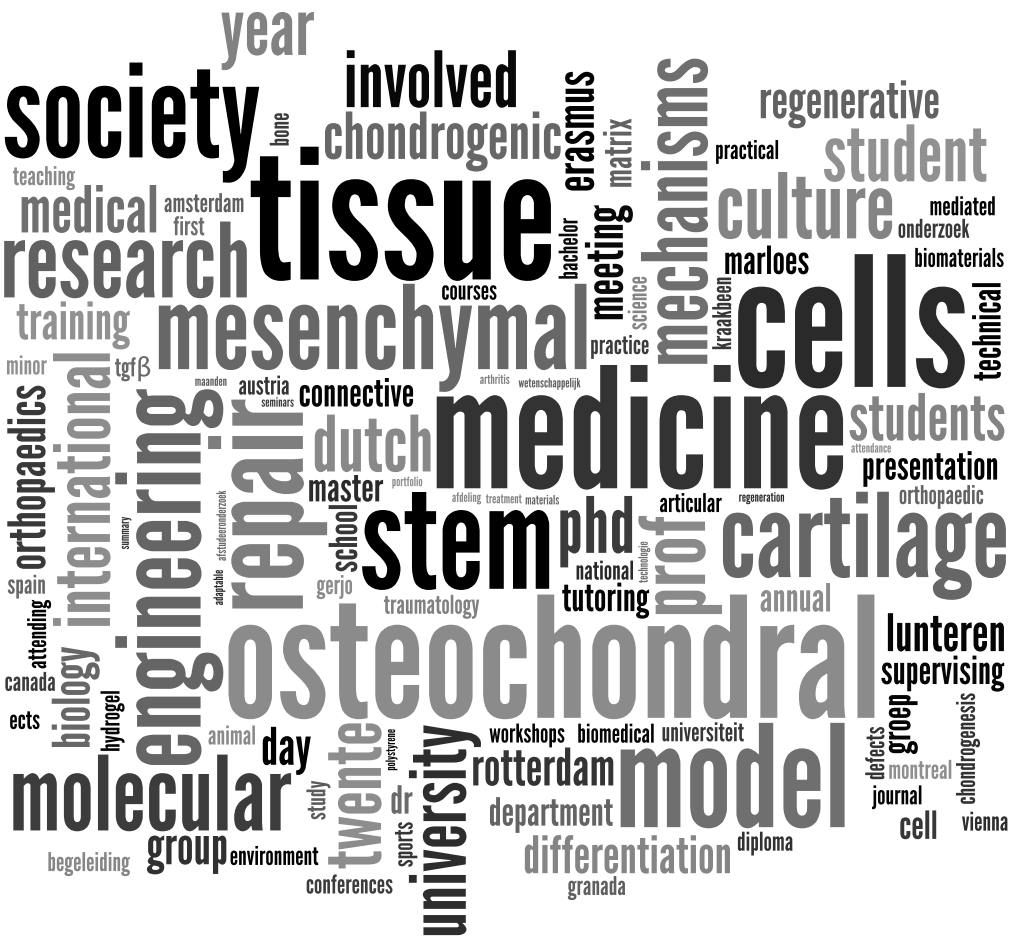
429. Culver, C. et al., *Mechanism of hypoxia-induced NF-kB,* Mol Cell Biol 2010. 30

430. Yang, Y. et al., *A cytosolic ATM/NEMO/RIP1 complex recruits TAK1 to mediate NF-kB and p38 mitogen-activated protein kinase (MAPK)/MAPK-activated protein 2 responses to DNA damage,* Mol Cell Biol 2011. 31

431. Dai, L. et al., *TAK1, more than just innate immunity,* IUBMB Life 2012. 64(10)

# Chapter 10

## Appendices



## PhD Portfolio

### Summary of PhD training and teaching

| | |
|---|---|
| Name PhD student: Marloes de Vries – van Melle<br>Erasmus MC Department: Orthopaedics<br>Research School: Molecular Medicine | PhD period: May 2009 – july 2013<br>Promotors: Prof. dr. Gerjo van Osch,<br>Prof. dr. Jan Verhaar<br>Co-promotor: Dr. Peter van der Kraan |

| 1. PhD training | Year | Workload (ECTS) |
|---|---|---|
| **General courses** | | |
| - Laboratory animal science | 2010 | 4 |
| - Biomedical English writing and presentation | 2011 | 4 |
| - Biobusiness Summer School, Amsterdam | 2012 | 2 |
| - Good Manufacturing Practice, Leiden | 2013 | 1 |
| **Specific courses (e.g. Research school, Medical Training)** | | |
| - Animal anatomy, University of Utrecht | 2009 | 4 |
| - Biomedical research techniques, Molecular Medicine | 2009 | 2 |
| - Biosafety, Fontys University of Applied Sciences, Eindhoven | 2011 | 1 |
| - Hydrogel handling practical training, AO Research Institute, Davos | 2011 | 1 |
| **Seminars and workshops** | | |
| - Journal club Connective Tissue, Cells and Repair group (36x) | 2009 - 2013 | 1 |
| - Workshops PhD Day, Erasmus MC, Rotterdam | 2011 | |
| **Presentations** | | >5 |
| - Research group meeting Orthopaedics Research Lab (9x) | 2009 – 2013 | |
| - Research group meeting Connective Tissue, Cells and Repair group (17x) | 2009 – 2013 | |
| - Department of Orthopaedics Annual Science day | | |
| - Department of Orthopaedics Research Committee | 2010 | |
| - GAMBA consortium meeting (3x) | 2011<br>2010 – 2013 | |
| **(Inter)national conferences – attendance / poster presentation** | | |
| - Stem Cells in Development and Disease, Amsterdam | 2009 | 1 |
| - Mesenchymal Stem Cells in Solid Organ Transplantation, Rotterdam | 2009 | 1 |
| - Annual Molecular Medicine Day, Rotterdam (3x)<br>Mechanisms involved in chondrogenic differentiation of mesenchymal stem cells in an osteochondral in vitro model, 2012 | 2010 – 2012 | 3 |
| - Dutch Society for Matrix Biology, Lunteren (3x) | 2010 – 2012 | 3 |
| - Tissue Engineering and Regenerative Medicine International Society, Granada, Spain | 2011 | 1 |
| - Dutch Society for Biomaterials and Tissue Engineering | 2011 | 1 |
| - International Cartilage Repair Society, Montreal, Canada<br>In vitro chondrogenesis of mesenchymal stem cells in an osteochondral environment is not solely mediated by TGFβ | 2012 | 1 |
| - Nambio Cost meeting, Vienna, Austria<br>Mechanisms involved in chondrogenic differentiation of mesenchymal stem cells in an osteochondral in vitro model | 2012 | 1 |
| - Tissue Engineering and Regenerative Medicine International Society, Vienna, Austria Mechanisms involved in chondrogenic differentiation of mesenchymal stem cells in an osteochondral in vitro model | 2012 | 1 |

| **1. PhD training** Continued | | |
|---|---|---|
| | **Year** | **Workload (ECTS)** |
| **(Inter)national conferences – podium presentation** | | >5 |
| - An osteochondral culture model to study treatment of cartilage defects of different depths, Dutch Society for Matrix Biology, Lunteren | 2010 | |
| - Studying mechanisms involved in articular cartilage repair using an osteochondral culture model, Annual Molecular Medicine Day, Rotterdam | 2011 | |
| - Studying mechanisms involved in articular cartilage repair using an osteochondral culture model, Tissue Engineering and Regenerative Medicine International Society, Granada, Spain | 2011 | |
| - An osteochondral culture model to study cartilage repair mechanisms in vitro, Dutch Society for Biomaterials and Tissue Engineering, Lunteren | 2011 | |
| - Mechanisms involved in chondrogenic differentiation of mesenchymal stem cells in an osteochondral in vitro model, International Cartilage Repair Society, Montreal, Canada | 2012 | |
| - In vitro chondrogenesis of mesenchymal stem cells in an osteochondral environment is not solely mediated by TGFβ, Dutch Society for Matrix Biology, Lunteren | 2012 | |
| **Other** | | |
| - Travel grant Dutch Arthritis Association | 2012 | |
| - Reviewer for international journals:<br>European Cells and Materials (2x)<br>Journal of Tissue Engineering and Regenerative Medicine<br>Cartilage | 2011 - 2013 | 2 |
| **2. Teaching** | | |
| **Lecturing** | | |
| - Cartilage lectures for third year medical students attending the minor "Orthopaedic Sports Traumatology" | 2012 | 1 |
| **Supervising practicals and excursions, Tutoring** | | |
| - Tutoring first year medical students | 2009-2010 | 2 |
| - Tutoring first year medical students for "introduction in working practice" | 2010 | 1 |
| - Supervising practical assignment for third year medical students attending the minor "Orthopaedic Sports Traumatology" | 2012 | 1 |
| **Supervising Master students** | | |
| - Osteochondral culture model, Stefan Sandker, student Technical Medicine, University of Twente | 2010 | 3 |
| - A highly adaptable osteochondral culture model towards simulating microfracturing in vitro, René van der Bel, student Technical Medicine, University of Twente | 2011 | 3 |
| - Optimizing of the preparation, cell encapsulation and analysis of hydrogels, Lizette Utomo, student Technical Medicine, University of Twente | 2011 | 3 |
| - Modulating synovial macrophage phenotypes to improve stem cell based cartilage repair, Johannes Lehmann, student Molecular Medicine, Erasmus MC | 2012 | 5 |
| - Comparing the ability of three hydrogel carriers to support repair of osteochondral defects by mesenchymal stem cells in an in vitro model, Maria Tihaya, student Molecular Medicine, Erasmus MC | 2012 | 5 |

## Curriculum Vitae

Marloes van Melle werd geboren op 18 juli 1984 in Deventer. Zij groeide op in Twello en ging in Apeldoorn naar het Stedelijk Gymnasium, waar zij in 2002 haar diploma behaalde. Daarna startte zij de studie biomedische technologie aan de Universiteit Twente. Voor haar bachelor opdracht deed zij haar eerste ervaring op met wetenschappelijk onderzoek tijdens een stage bij het laboratorium klinische chemie van het Medisch Spectrum Twente in samenwerking met de afdeling polymeerchemie en biomaterialen van de Universiteit Twente. Dit resulteerde in de bachelor scriptie “The proliferation behavior of mesenchymal stem cells on tissue culture polystyrene and polymeric scaffolds”. Ondertussen begon zij met de master molecular, cellular & tissue engineering. In 2008 kreeg zij de kans om drie maanden onderzoek te doen naar het effect van lage zuurstofspanning en serum op de differentiatie naar kraakbeen van uit embryonale stamcellen afgeleide mesenchymale progenitor cellen. Dit onderzoek werd uitgevoerd bij de molecular cell biology groep van prof. Anders Lindahl van het Sahlgrenska University Hospital in Göteborg, Zweden. Haar interesse in kraakbeen en bot tissue engineering leidde tot het afstudeeronderzoek bij de tissue regeneration groep onder begeleiding van prof. Clemens van Blitterswijk en associate prof. Jan de Boer. Met dit onderzoek, getiteld “Endochondral bone formation: a chondrogenic approach for bone tissue engineering” behaalde ze in maart 2009 haar master diploma. In mei 2009 begon het promotieonderzoek bij de connective tissue, cells & repair groep onder begeleiding van prof. Gerjo van Osch, prof. Jan Verhaar en dr. Peter van der Kraan. In 2010 trouwde Marloes met Rogier de Vries en in december 2012 werd hun dochter Frederique geboren. Naar verwachting wordt in mei 2014 hun tweede kindje geboren.

## Dankwoord

Professor van Osch, Gerjo, zonder jouw supervisie zou dit boekje niet bestaan! Even bij je binnen lopen voor een vraag kan altijd en als ik even vast zat met schrijven waren jouw snelle blik en wat (soms onleesbare ;-)) aantekeningen genoeg voor nieuwe inspiratie! Bedankt voor alle kansen die je me hebt gegeven om mezelf te ontwikkelen, zowel op wetenschappelijk als persoonlijk vlak.

Professor Verhaar, bedankt voor de kans om mijn promotieonderzoek bij uw afdeling orthopaedie uit te voeren. Uw klinische kijk op de zaken heb ik heel erg gewaardeerd, deze was altijd goed voor nieuwe inzichten en heeft bijgedragen aan de volledigheid van dit proefschrift. Bedankt voor uw begeleiding en input voor mijn onderzoek.

Mijn co-promotor Peter van der Kraan, soms reageerde je nog sneller op onderzoeksplannen of manuscripten dan Gerjo, ik wist niet dat dat mogelijk was! Bedankt voor de gastvrijheid op jouw laboratorium voor het uitvoeren van de experimenten samen met Henk. Werkbesprekingen waren altijd erg interessant en nuttig of ze nou in Nijmegen, Rotterdam of op Utrecht Centraal plaats vonden. Bedankt voor al je input en de prettige samenwerking.

All the partners of the GAMBA consortium: thank you for the nice cooperation and interesting project meetings. In particular I would like to thank Mauro Alini, David Eglin, Matteo D'Este, Mary Murphy, Niamh Fahy and Eric Farrel. Mauro, thank you for having me over in your lab in Davos for the training on the thermoresponsive hydrogel. David, thanks for all your support and sharing your expertise in the hydrogel both in your lab and in Rotterdam. Matteo, thank you for your suggestions on the hydrogel and for the logistics in sending material to Rotterdam. Mary, Niamh and Eric, thank you for the nice cooperation on the experiments and manuscript on the effect of synovial macrophages on MSC chondrogenesis.

Alle co-auteurs, bedankt voor de prettige samenwerking. In het bijzonder wil ik graag Koen Bos, Henk van Beuningen en Roberto Narcisi bedanken. Koen, bedankt voor de fijne samenwerking bij het schrijven van het review artikel en voor jouw bijdrage als klinisch begeleider van de TG studenten. Jouw interesse in translationeel onderzoek is van grote waarde. Henk, bedankt voor je inzet bij het uitvoeren van de experimenten met onze cellen in jullie lab, ook al hebben ze helaas niet tot een artikel geleid. Gelukkig is dat wel gelukt met de remmer-experimenten! Bedankt voor alle moeite die je in de experimenten en het manuscript hebt gestopt. Roberto, being the ultimate western blot king, you managed to analyse my tricky biopsy-cultured alginate samples! Thanks a lot for all the effort you've put in this, for your assistance in histological scoring and for your input on the manuscript.

En natuurlijk de studenten die ik met veel plezier heb begeleid: TG studenten Stefan Sandker, René van der Bel en Lizette Utomo en de molecular medicine studenten Johannes Lehmann en Maria Tihaya. Stefan en René, de pilot experimenten die jullie gedaan hebben zijn de voorzet voor hoofdstuk 6 in mijn proefschrift geweest, bedankt voor de fijne samenwerking en alle praktische suggesties die het leven op het lab soms net een beetje makkelijker hebben gemaakt. Stefan, succes met de combinatie van je promotie en je opleiding tot klinisch perfusionist en René, succes met je promotie! Lizette, jouw stage was het begin van hoofdstuk 7. Het was niet makkelijk om het werken met de hydrogel goed op poten te krijgen, maar door jouw gedrevenheid en creativiteit zijn we tot het protocol gekomen! Je kwam terug voor je afstudeerstage bij Mieke en nu begin je met je eigen promotie. Heel veel succes! Maria, jij ging verder waar Lizette gebleven was. Je hebt wat praktische trucs bedacht om met de hydrogel te werken. En ondanks dat het soms wat stressvol was vanwege de combinatie met geneeskunde en soms wat fysieke tegenslagen, heb je je stage goed afgerond en heeft jouw werk een groot deel van hoofdstuk 7 gevormd. Heel veel succes in de kliniek! Johannes, although your project was challenging, this did not scare or demotivate you at all! You managed to set up a protocol to isolate synovial macrophages, which was a major contribution to chapter 4. Your creativity and graphical skills are impressive, which will be of great use for your own PhD project.

Wendy, Nicole en Sandra, het lab kan niet zonder jullie! Wendy en Nicole, naast celkweken, histologie en kletspraat hebben we ook nog een hoop hormonen gedeeld ☺ Wendy, heel erg bedankt voor al je hulp met de MSC kweken, het oogsten en analyseren en nog veel meer; ik mis het kletsen in de kweek al! Nicole, bedankt voor alle hulp bij de histologie en de muizen! Zonder jouw hulp, jouw connecties bij diverse afdelingen en je gulle lach waren deze experimenten niet gelukt ☺ Sandra, bedankt voor je hulp bij diverse administratieve zaken, de gezelligheid aan de koffietafel en natuurlijk bij het regelen van alles omtrent mijn promotie!

Mieke, met Nienke erbij noemde Gerjo ons de 3 musketiers, vandaar ook onze gezamenlijke afscheidsborrel, ook al ging ik eind juli weg en jij eind november ☺ Bedankt voor je gezelligheid, je directheid en het kletsen over van alles, van MSC tot muis tot cocktail. Heel veel succes met je opleiding bij de plastische! Kamergenoten Jasper en Rintje, bedankt voor de gezelligheid en het sparren over allerlei onderwerpen! Heel veel succes met jullie klinische carrière! (oud)CTCR collega's Yvonne, Mairead, Wu, Maarten, Gerben en Stefan bedankt voor de gezelligheid op het lab, tijdens de CTCR dagen, bij het maken van het filmpje voor Gerjo en bedank voor al jullie input bij werkbesprekingen. Andere ortho-collega's Michiel, Anna, Ruud, Marjan, Marianne, Erwin, Johan, Harrie, Holger, Esther en verdere oud collega's bedankt voor de gezelligheid op het lab en bij de cake van de week!

Nienke en Martine, mijn paranimfen. Nienke, op het lab hebben we elkaar leren kennen en vanaf de eerste dag was het alsof we elkaar al jaren kenden. Door de jaren heen hebben we letterlijk lief en leed gedeeld, je bent een heel erg dierbare vriendin geworden! Ik ben erg blij dat jij bij mijn promotie naast mij staat. Bedankt voor de geweldige vriendschap, die spontane etentjes en oppasavondjes gaan we hopelijk nog vele jaren doen ☺ Martine, allebei in Enschede gestudeerd net als onze mannen en toch elkaar pas in Rotterdam leren kennen, hoe maf is dat? Geweldig om de herkenning te zien als onze mannen weer met verhalen over de olieboeren thuis komen. En daar zitten we dan, mevrouw Janssen en mevrouw de Vries in de vinex-wijk; zullen we er toch maar voor gaan zorgen dat de rest van het stereo-type niet klopt? Ik hoop dat onze kleintjes het net zo goed met elkaar kunnen vinden als wij!

Papa, mama, Linda, Arjan, Luuk en Sem; Wim, Renée, Floor, Patrick, Tim en Sanne, bedankt voor jullie interesse en betrokkenheid bij mijn onderzoek en alle perikelen er om heen. Papa en mama, waar ik het ook over had, of waar ik ook mee bezig was, jullie hebben me altijd gesteund en aangemoedigd. Bedankt dat jullie me geleerd hebben om nooit op te geven en niet zomaar de makkelijkste weg te kiezen!

Lieve Frederique, wat zijn wij blij dat jij er bent. Ons vrolijke lichtpuntje, jij laat ons zien wat nou echt belangrijk is in het leven. We hopen dat jouw broertje of zusje straks net zo lief, blij, vrolijk en gezellig wordt als jij!

Lieve Rogier, mijn steun en toeverlaat, mijn rots, mijn sparringpartner, mijn allerliefste! Bedankt dat je altijd in mij geloofd hebt, mij altijd gesteund hebt en mij altijd hebt geholpen waar dat nodig was. Samen hebben we deze reis gemaakt, het was niet altijd makkelijk, maar samen kunnen we de hele wereld aan! Ik heb alle vertrouwen in alle reizen die nog komen gaan, samen met Frederique, haar broertje of zusje en onze twee harige monsters ☺