Microenvironment of osteoarthritic cartilage and subchondral bone influences chondrogenic differentiation, extracellular matrix production and composition of bone marrow-derived stem cells and articular chondrocytes

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Abbreviations

3D	Three-dimensional
ACAN	Aggrecan gene
ALP	Alkaline phosphatase
APS	Ammonium peroxodisulfate solution
ASC	Adipose derived stem cells
BCA	Bicinchoninic acid
bFGF	basic fibroblast growth factor
BMSC	Bone marrow derived stem cells
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ch	Chondrocytes
Col	Collagen
COMP	Cartilage oligomeric matrix protein
Ct	Threshold cycle
Da	Dalton
DAPI	4´,6´-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco´s modified eagle´s medium
DMMB	Dimethylmethylene blue
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	mixture of deoxyribonucleotides dATP, dCTP, dGTP, dTTP
E. coli	Escherichia coli
ECM	Extracellular matrix
EDTA	Ethylene-diamine-tetra acetic acid

ELISA	enzyme linked immunosorbent assay
et al.	et alii (from Latin: and others)
EtOH	Ethanol
E∞	equilibrium modulus
F	monocultures (without subchondral bone explants)
FACS	Fluorescence activated cell sorting
FB	co- and tricultures with subchondral bone explants
FC	co- and tricultures with articular cartilage explants
FCS	Fetal calf serum
g	gravitation
GAG	Glycosaminoglycan
H_A	aggregate modulus
IBMX	3-isobutyl-1-methylxanthine
IGF	Insulin-like growth factor
Ihh	Indian hedgehog homolog
IL	Interleukin
k	hydraulic permeability
kb	kilo bases
LC-MS	Liquid chromatography–mass spectrometry
LDH	Lactate dehydrogenase
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization- Time Of Flight
Mixed	mixed cultures of BMSC and chondrocytes
MMP	Matrix metalloprotease
mRNA	messenger RNA
MW	molecular weight
n	number of independent samples
N	Newton

NB	normal bone
OA	osteoarthritis
oBMSC	ovine bone marrow derived stem cells
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PTHrP	Parathyroid hormone related peptide
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sox	Sex related homeobox containing transcription factors
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-α
Tris	Tris(hydroxymethyl)-aminomethan
U	units

Abstract

Objective

Osteoarthritis (OA) is characterized by an imbalance in cartilage and subchondral bone homeostasis, which could be potentially treated or improved by cell-based therapies. In the present study, a reproducible *in vitro* coculture model was established to evaluate the influence of normal and OA-cartilage or subchondral bone explants on chondrogenic differentiation of human bone marrow derived stem cells (BMSC), human adipose derived stem cells (ASC) and phenotype of OA-chondrocytes. Signals from the articular cartilage or the underlying subchondral bone were hypothesized to induce phenotypic shifts and an altered re- and differentiation potential in cocultured BMSC, ASC and differentiated chondrocytes from OA patients. To provide a chondrogenic environment, cells were embedded in fibrin gel and kept in chondrogenic medium for up to 28 days.

Methods

A reproducible coculture model of ASC, BMSC, mixed cultures (BMSC and chondrocytes in equal ratio) and chondrocytes embedded in fibrin gel seeded on articular cartilage or subchondral bone explants (= co- and tricultures) compared with monocultured cells in fibrin gel without explants was established. Human OA-tissues, OA-chondrocytes and BMSC were derived from patients subjected to arthroplasty. Normal (healthy) human tissues were received from knees of rare trauma affected donors (treated for sports accidents). ASC were isolated from subcutaneous fat tissue obtained from patients undergoing elective body contouring procedures. Ovine cartilage, chondrocytes and BMSC were obtained from normal (healthy) pasture sheep. Gene expression analysis, biochemical assays (ELISA, Dot-blot, DMMB, Hydroxyproline), immunofluorescence staining and biomechanical tests were used to characterize the properties of newly generated extracellular matrix (ECM) from chondrocytes and chondrogenically differentiated ASC and BMSC.

Results

In general, all cell regimens cocultured with OA-explants exhibited reduced gene expression patterns of collagens I, II, III and X in comparison with monocultures. Significant lower levels of collagen I and II protein (BMSC) and significant lower collagen I and III protein (mixed cultures) were detected in cartilage co- or tricultures, while co- and triculture with subchondral bone inhibited collagens in general. In contrast, no changes in glycosaminoglycan (GAG) synthesis were observed for cartilage co- and tricultures, while reduced GAG production was observed in subchondral bone co- and triculture lysates. Repetition of key experiments with normal ASC confirmed inhibitory effects for OA-subchondral bone. Co- and triculture with normal cartilage or subchondral bone explants showed no

or only reduced inhibitory effects on chondrogenic differentiation or collagen gene expression. In addition, biomechanical properties of the OA-cartilage or subchondral bone co- and tricultured cell-fibrin gels were affected. All co- and triculture regimens tended to exhibit lower Young´s modulus and aggregate modulus compared with monocultures. In contrast, hydraulic permeability seemed to be higher in co- and tricultures. Supernatants of cartilage and subchondral bone co- and tricultures contained significant higher IL-1β, IL-6 and IL-8 levels, as well as significant more soluble GAGs compared with controls. In general, stimulation of monocultures with IL-1β induced matrix metalloproteinase (MMP)2, and MMP3 and reduced collagens I, II and X gene expression and led to a downregulation of aggrecan gene expression. Stimulation with IL-6 reduced aggrecan, MMP3 and MMP13 gene expression and mainly reduced collagen I, II and III gene expression of BMSC and/or chondrocytes. In contrast, IL-8 stimulation of mixed and chondrocytes monocultures had only little effects, while IL-8 stimulated BMSC showed reduced collagen I, II and III gene expression.

Conclusions

Taken together our results suggest an inhibitory effect of factors from the microenvironment of OA-cartilage and subchondral bone on production of collagens. This indicates a distinct modulatory influence, which affects the composition of the *de novo* produced ECM from cocultured cells and leads to impaired mechanical strength and biochemical properties of the newly formed matrix. Experiments with ASC, normal cartilage and subchondral bone explants either might hint to disease status induced effects (OA vs. trauma) or to an effect caused by different mean age of cell and tissue donors. Soluble signal factors, i.e. pro-inflammatory cytokines (including IL-1β and IL-6), released from OA-cartilage, OA-subchondral bone, chondrocytes and osteoblasts, might partly mediate these effects on newly formed extracellular matrix properties. Thus, the microenvironment of neighbored OA-cartilage seems to provide both: promoting and inhibiting signals for BMSC differentiation and suggests that the balance of these factors determines the destiny of BMSC. This knowledge can be used to develop new strategies for cell based cartilage regeneration.

Zusammenfassung auf Deutsch

Die Mikroumgebung von arthrotischem Knorpel und subchondralem Knochengewebe beeinflusst die chondrogene Differenzierung sowie die ECM Produktion und Zusammensetzung von BMSC und artikulären Chondrozyten

Charakteristisch für Osteoarthrose (OA) ist ein Ungleichgewicht der Knorpel- und subchondralen Knochen Homöostase. Die Behandlung und der Verlauf dieser Erkrankung könnte durch zellbasierte Therapien deutlich verbessert werden. In dieser Studie wurde ein reproduzierbares *in vitro* Kokulturmodell entwickelt, um den Einfluss von OA-Knorpel und -Knochen Explantaten auf die chondrogene Differenzierung von adipogenen Stammzellen (ASC), von humanen mesenchymalen Stammzellen aus dem Knochenmark (BMSC) und den Phänotyp von OA-Chondrozyten zu untersuchen. Vermutlich verursachen Signalfaktoren aus dem artikulären Knorpel und der darunterliegenden subchondralen Knochenschicht einen Shift im Phänotyp oder eine Veränderung im (Re-) Differenzierungspotential von ASC, BMSC und differenzierten Chondrozyten. Um eine chondrogene Mikroumgebung zu schaffen, wurden die Zellen in Fibringel eingebettet und bis zu 28 Tage in chondrogenem Medium kultiviert.

In einem Kokulturmodell wurden in Fibringel eingebettete ASC, BMSC, Chondrozyten oder ein Gemisch aus BMSC und Chondrozyten (zu gleichen Teilen) entweder zusammen mit osteoarthrotischem Knorpel oder subchondralem Knochen kultiviert (= Ko- und Trikultur) und mit den entsprechenden Zellen in Monokultur ohne Explantat verglichen. Alle humanen OA-Gewebestücke wurden aus Patienten entnommen, die einer Arthroplastik unterzogen wurden. Gesundes humanes Gewebe wurde aus Knien von Unfallpatienten gewonnen, die wegen einer Sportverletzung behandelt wurden. ASC wurden aus Unterhautfettgewebe isoliert, das von Patienten einer kosmetischen Fettabsaugung stammt. Genexpressionsanalysen, biochemische Assays (ELISA, Dot-blot, DMMB, Hydroxyprolin), Immunfluoreszenzfärbungen und biomechanische Tests wurden verwendet, um die Eigenschaften der neu gebildeten Matrix von Chondrozyten und chondrogen differenzierten BMSC zu bestimmen.

Im Allgemeinen zeigten alle kokultivierten Zellbedingungen verminderte Genexpression von Kollagen I, II, III und X verglichen mit Monokulturen. Auf Proteinebene konnte signifikant weniger Kollagen I und II (BMSC) und signifikant weniger Kollagen I und III (gemischte Kulturen) in den Knorpel-Ko- und Trikulturen nachgewiesen werden. Die Ko- und Trikultur mit subchondralem Knochen zeigte eine generelle Verminderung der Expression aller Kollagene. Im Gegensatz dazu konnte keine Änderung in

der Glykosaminoglykan (GAG) Synthese der Knorpel Ko- und Trikultur nachgewiesen werden, während in der Ko- und Trikultur mit subchondralem Knochen temporär eine Reduktion der GAG Synthese beobachtet werden konnte. Die Wiederholung von Schlüsselexperimenten mit gesunden ASC konnte die inhibitorischen Effekte induziert durch Ko- und Trikultur mit OA-subchondralem Knochen bestätigen. Ko- und Trikultur mit normalem Knorpel oder subchondralem Knochen zeigte keine oder nur verminderte inhibierende Effekte auf die chondrogene Differenzierung oder Gen Expression von Kollagen. Die biomechanischen Eigenschaften der kokultivierten Fibringele waren ebenfalls beeinträchtigt. Alle Ko- und Trikulturbedingungen zeigten tendenziell ein geringeres Elastizitätsmodul und Aggregationsmodul im Vergleich zur Monokultur. Im Gegensatz dazu schien die hydraulische Permeabilität in den Ko-und Trikulturen höher zu sein. Die Überstände von Knorpel- und Knochen Ko- und Trikulturen enthielten im Vergleich mit den Kontrollen signifikant mehr IL-1β, IL-6 und IL-8, und auch mehr lösliche GAGs, die größtenteils von den Explantaten stammten. Die Stimulation der Monokulturen mit IL-1β induzierte im Allgemeinen *MMP2*, *MMP3* und *MMP13* und reduzierte die Genexpression der Kollagene I, II und X sowie von Aggrekan. Die Stimulation mit IL-6 verminderte die Aggrekan, *MMP3* und *MMP13* Genexpression und größtenteils auch der Kollagene I, II und III in BMSC und/oder Chondrozyten. Im Gegensatz dazu hatte die Stimulation mit IL-8 nur geringe Effekte auf gemischte und Chondrozyten Monokulturen, während in den IL-8 stimulierten BMSC eine Reduktion der Kollagene I, II und III beobachtet werden konnte.

Zusammenfassend deuten die Ergebnisse dieser Studie auf einen inhibitorischen Effekt von Faktoren aus der Mikroumgebung von OA-Knorpel oder -subchondralem Knochen auf die Kollagenproduktion hin. Diese Ergebnisse lassen auf einen modulierenden Einfluss schließen, der zu verminderten mechanischen und biochemischen Eigenschaften der neu gebildeten extrazellulären Matrix führt. Die Experimente mit ASC sowie mit normalen Knorpel und subchondralen Knochen Explantaten könnten entweder auf krankheitsbedingte Effekte (OA vs. Trauma) hinweisen oder auf einen Effekt aufgrund des unterschiedlichen Durchschnittsalters der Zell- und Gewebe Spender. Vermutlich regulieren lösliche Signalfaktoren wie z.B. proinflammatorische Zytokine, abgegeben von OA-Knorpel, OA-subchondralem Knochen, Chondrozyten oder Osteoblasten, zumindest teilweise diese negativen Effekte auf die chondrogene BMSC Differenzierung und die Qualität der neu gebildeten ECM. Dennoch birgt die direkte Mikroumgebung von OA-Knorpel beides: fördernde und inhibierende Signale der BMSC Differenzierung. Dies lässt darauf schließen, dass die Balance dieser Faktoren das Schicksal der BMSC bestimmt. Die in dieser Studie neu gewonnenen Erkenntnisse können in Zukunft dazu beitragen, neue Strategien für die zellbasierte Knorpelregeneration zu entwickeln und vor allem die Qualität des neu gebildeten Knorpels entscheidend zu verbessern.

1 State of the Art / General introduction

1.1 Epidemiology of Osteoarthritis (OA) in human joints

The human knee joint is one of the most important sources of pain in the western population, in people over 55 years old and nearly everybody over 65 years is affected by osteoarthritis (OA) (Hunter and Felson 2006). Possible causes for OA development are increasing age, mechanical stress, a high body mass index and a genetic component (Breedveld 2004). OA is a chronic joint disease and develops usually undetected many years before it is diagnosed. Notably, a correlation with pain is not mandatory and OA becomes painful in about 50% of patients (Pereira, Peleteiro et al. 2011). Still it is the leading cause of disability, which besides limitations in motility and quality of life is the high economic burden in form of direct and indirect health-related costs (Felson 2004; Hiligsmann, Cooper et al. 2013). Thus, ineffective treatments focusing only on symptoms - mainly pain reduction – should better include a change of lifestyle like weight reduction, adequate footwear and continuing exercises (Roddy and Doherty 2006). Anyhow, after diagnosis and radiographic confirmation for patients with severe end stage OA, a total replacement of the joint and implantation of a prosthesis is inevitable. Drawback in this regard is that the prosthesis has a limited lifespan and patients risk a revision surgery (Wiegant, van Roermund et al. 2013). Therefore, alternatives for successful OA-treatment are urgently required to regenerate cartilage tissue and avoid implantation of an artificial prosthesis.

1.2 Biology of articular cartilage

1.2.1 Organization of articular cartilage

Normal articular cartilage is accurately organized and consists of different zones **(Fig. 1)**; the outermost region is called lamina splendens, named after the light breaking reflections of parallel fibers (Hollander, Dickinson et al. 2010). The upper zone of cartilage is rich in thin collagen fibrils parallel to the articular surface, which protects the cartilage from shear forces. Characteristic is a high collagen content, with an increased collagen I content and lowest amount of aggrecan compared with deeper zones. Cells in the upper cartilage region are relatively flat, small and separated. Additionally, regulatory proteins like TGF-β1 and -3 as well as BMP1-6 (Anderson, Hodges et al. 2000; Yamane, Cheng et al. 2007) and MMPs (-1, -3, -8 and -13) are expressed but are only involved in matrix turnover, not in matrix degradation (Tetlow, Adlam et al. 2001; Tchetina 2011)

In the middle zone, resting round shaped chondrocytes are residing in chondrons, surrounded by thick, low organized fibers and an ECM consisting of high collagen II and aggrecan amounts (Pfander, Swoboda et al. 2001).

The deep zone of articular cartilage has the highest aggrecan and lowest water content and the collagen fibrils with the largest diameter, which are oriented perpendicular to the joint surface (Maroudas, Bayliss et al. 1980). Chondrocytes in the deep zone are enlarged and arranged in columns right angled to the cartilage surface (Pearle, Warren et al. 2005).

The deep zone merges into the hypertrophic zone, which is separated by the so-called tidemark region from calcified cartilage that links the cartilage layer with the subchondral bone. The calcified cartilage zone is rich in hydroxylapatit crystals and hypertrophy markers like collagen X, ALP and MMP13 are expressed (Aigner, Hemmel et al. 2001; Pearle, Warren et al. 2005).

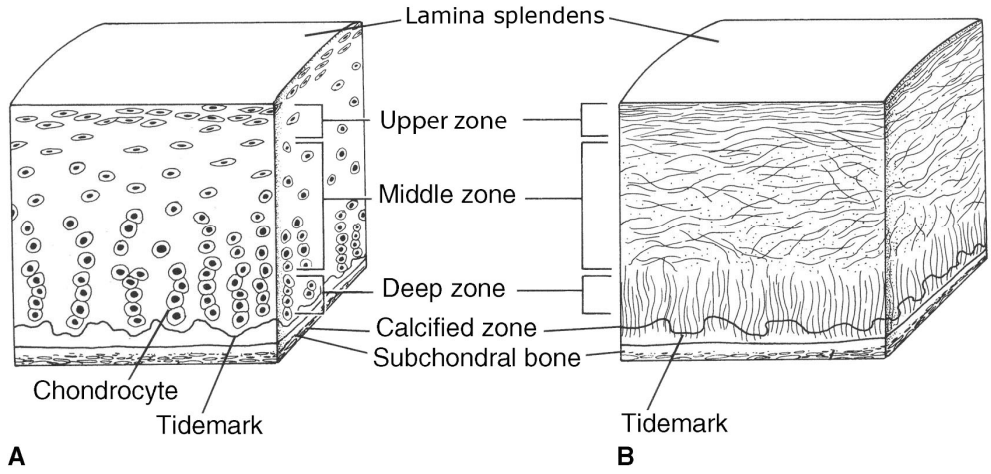


Figure 1: Schematic overview of articular cartilage
Articular cartilage is characterized by a specific arrangement of chondrocytes and ECM components. It is divided into several different zones beginning with the lamina splendens at the articular joint surface, followed by the upper, middle and deep zone, which are separated by the tidemark from the calcified zone and the subchondral bone (Drawing is adopted from (Jazrawi, Alaia et al. 2011)).

1.2.2 Differences between normal and OA cartilage

Normal articular cartilage is characterized by a special ECM composition mainly based on proteoglycan (aggrecan) and collagens. Collagen II is the most dominant collagen, which forms a fibrillar structure together with other collagens (XI and IX) as well as with non-collagenous proteins (Hagg, Bruckner et al. 1998).

Mature normal articular cartilage has a low cell density as with the end of growth, articular chondrocytes are hardly proliferating and exhibit only a low metabolism. Nevertheless, articular cartilage shows a carefully balanced activity of matrix turnover with respect to cartilage ECM degradation and synthesis (Poole 1997; Buckwalter and Mankin 1998; Tchetina 2011). In general, intact hyaline cartilage shows only low gene expression of collagens I, II, VI, IX, X and XI but a high turnover of aggrecan (Hardingham, Fosang et al. 1994; Aigner and McKenna 2002; Poole 2003).

Importantly, hyaline cartilage has no innervations and no blood flow and thus is characterized by a low supply with nutrients and oxygen. Additionally neither pain nor overloading can be perceived (Benedek 2006). For exactly that reason, a high impact load is unfavorable, because it is able to provoke cartilage damage and bears the risk for OA development (Newberry, Mackenzie et al. 1998). The impact level and its tenure are important, because the time period of maximal compression is more fatal if it occurs abruptly than gradually despite of the same amplitude. During this kind of strain, rupture of the collagen meshwork could happen if a critical impact is reached (Newberry, Garcia et al. 1998; Torzilli, Grigiene et al. 1999). The rupture of cartilage ECM - typically for traumatic sports injuries - represents a severe structural damage of the joint and induces the beginning of structural cartilage degradation because of constant loading alterations. Additionally it causes post-traumatic cell death, mediates acute joint inflammation and initiates repair mechanisms like an increase of pro-inflammatory cytokines, basic fibroblast growth factor (bFGF), MMPs, aggrecanases, and release of matrix fragments (Buckwalter, Anderson et al. 2013).

Figure 2 shows an overview of initiation and progression of OA including responsible factors and changes in gene expression and phenotype of chondrocytes and cartilage. More detailed, in early OA, cells residing in arthritic cartilage regions become active and restart metabolism. Especially TGF-β and collagen II production is enhanced most probably as an attempt to repair destroyed cartilage (Kouri and Lavalle 2006). Additionally, collagenases, aggrecanases, cytokines, collagen X, Indian hedgehog homolog (Ihh) and caspase 3 are upregulated in early stage cartilage lesions. During progression of OA, the balance of cartilage turnover is disturbed and MMPs – especially MMP13 – aggrecanases, collagen X and alkaline phosphatase (ALP) are further upregulated. In combination with downregulation of Sox9, TGF-β, TNF-α and aggrecan expression, a dramatic loss of aggrecan occurs in the upper zone. Additionally, the natural zonal organization of cartilage breaks down and results in large areas of destroyed inflamed cartilage tissue (Tchetina, Squires et al. 2005; Appleton, McErlain et al. 2007; Tchetina 2011). These regions represent a unique OA-microenvironment with release of high amounts of pro-inflammatory cytokines like IL-1β, IL-6, IL-8, IL-10 or TNF (Moldovan, Pelletier et al. 2000; Lu, Evans et al. 2011), and growth factors like FGF, PTHrP or TGF-β as well as

ECM fragments like collagens, fibronectin and soluble GAGs (Tchetina, Kobayashi et al. 2007; Huang and Wu 2008).

Finally, destruction of cartilage is dominant in late OA and ECM production disappears because chondrocytes shift their phenotype from reparative to degradative resulting in total breakdown of cartilage (Kouri and Lavalle 2006). Besides a metabolic imbalance, activation of the whole endochondral ossification program starting with cell proliferation through articular chondrocyte hypertrophy and apoptosis has been identified as an important determinant of OA progression (Alsalameh, Amin et al. 2004; van den Berg 2011). Although the view of a generalized OA-chondrocyte hypertrophy is controversial, signaling molecules relevant for endochondral ossification may be involved in OA pathogenesis. Reactivation of embryonic differentiation pathways underscore the clinical relevance of chondrocyte differentiation associated processes for OA pathogenesis (Dreier 2010; Pitsillides and Beier 2011).

As a result, this alteration in ECM composition and turnover, mainly the loss of proteoglycan, leads to changes in biomechanical properties, like higher permeability and an increased water content causing a decrease in hydraulic pressure. Hence, the diminished stability in weight bearing regions contributes to an enhanced cartilage loss and promotes further stress, inflammation and cell death leading to more pressure on residing chondrocytes (Akizuki, Mow et al. 1987; Lai, Hou et al. 1991; Setton, Mow et al. 1994; Kouri and Lavalle 2006). Consequently, the microenvironment of OA-cartilage is not comparable with the microenvironment provided by normal articular cartilage.

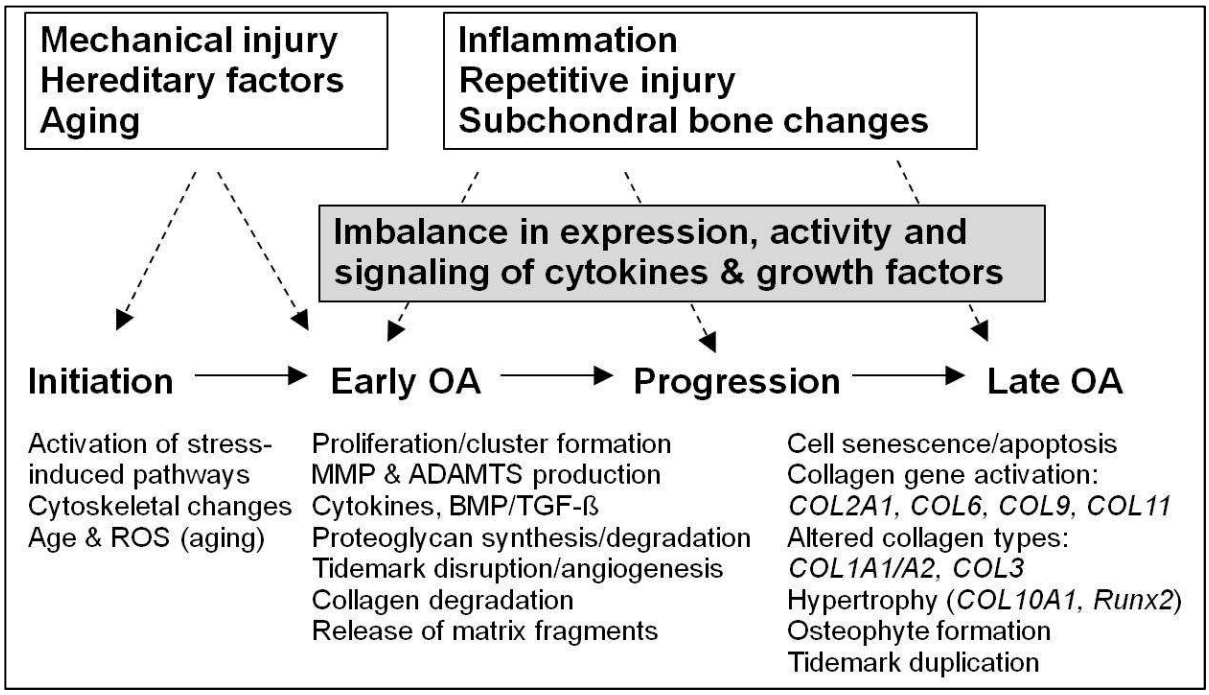


Figure 2: Scheme of initiation and progression of OA
Schematic overview of events initiating early OA and leading to progression of late OA. Possible contributing factors are in the boxes above and cellular and morphological changes are specified below (scheme is adopted from (Goldring and Goldring 2007)).

1.2.3 Biomechanical properties of cartilage

With every step we take our knee joint bears a multiple of our body weight and the articular cartilage has the important duty to distribute the load equally and frictionless over the whole joint. In return, normal articular cartilage has a unique composition of natural biphasic hyaline ECM which is mainly based on water (70-85%), proteoglycans (5-7%), foremost aggrecan, and collagens (10-20%) mainly collagen II (Mow, Holmes et al. 1984; Pearle, Warren et al. 2005). In the porous and permeable solid matrix, aggrecan is responsible for properties like elasticity and compressibility (Chambers, Cox et al. 2001; Lu and Mow 2008), while collagen II is in charge of shear and tensile properties of cartilage which enables a high viscoelastic and mechanical stability (Pearle, Warren et al. 2005). At last, water contributes 90% to load transmission on account of low permeability of cartilage. Pressurization of water provides an equal weight distribution and protects the solid phase of the matrix from load burden (Maroudas 1976; Mankin 1982; Soltz and Ateshian 1998).

In case of cartilage, diseases like OA, a rupture of tendons, damage of meniscus or lesions in the cartilage due to age, the normal/optimal distribution of weight is disturbed and the mechanical stimulation of additional local pressure on cartilage and chondrocytes can lead to alterations in cell-gene expression or to further cartilage wear out (Kouri, Aguilera et al. 2000; Abramson, Attur et al. 2006). Moreover, Salter et al. even suggest that OA-cartilage has a different sensing of the mechanical environment compared with normal cartilage leading to an inappropriate response to mechanical stimulation resulting in progression of disease by changes in the ECM and MMP gene expression (Salter, Hughes et al. 1992; Millward-Sadler, Wright et al. 2000; Salter, Millward-Sadler et al. 2002).

Key roles in biomechanical properties have, among others, the correct deposition of proteoglycans and collagens. Collagen fibrils which form the fibrillar matrix and proteoglycans which form the extra-fibrillar matrix determine the level of hydration and stability of the ECM and due to that the mechanical properties of articular cartilage. Therefore, GAG and collagen contents of ECM-tissue are useful for prediction of mechanical tissue properties (Steinert, Ghivizzani et al. 2007; Yan, Zhou et al. 2009).

In general, analysis of biomechanical properties covers attributes of stiffness, elasticity, compressibility and permeability (Busby, Grant et al. 2013). These attributes are dependent on load,

time and type of compression (Gannon, Nagel et al. 2012). Unconfined compression is a simple laboratory test method where no radial space limitations occur and specimens were compressed uniaxial (Hatami-Marbini and Etebu 2013). Confined compression is performed in a confining chamber, which simulates the *in situ* situation. That means the top and bottom of a cylindrical specimen is limited in space and only e.g. buffer solution is able to disappear via an effluent. Therefore, the fibrin gel is loaded hydrostatically to the desired confining pressure on all sides. The resulting measured parameters are used for calculation of common specimen properties (Mow and Guo 2002; Busby, Grant et al. 2013).

Biomechanical properties of a tissue are defined by the following parameters: Young´s modulus, aggregate modulus and hydraulic permeability. More detailed, Young´s modulus provides information about the stiffness and is performed under unconfined compression. Aggregate modulus provides information about the compressibility and hydraulic permeability about the flow rate of water (both applied under confined compression) (Mow and Guo 2002; Busby, Grant et al. 2013).

1.3 Biology of subchondral bone

1.3.1 Organization of subchondral bone

The so-called subchondral bone of the human knee joint is located beneath the layer of articular cartilage (Sanchez, Deberg et al. 2005). In contrast to articular cartilage, which has only one resident cell type (chondrocytes), subchondral bone consists of different cell types: osteoblasts, osteocytes, osteoclasts, and due to the bone marrow also of HSC and BMSC. The main cell-type, osteoblasts, are bone forming cells with an active metabolism secreting ECM components like collagen I which accounts for about 90% of the non mineralized bone matrix. The remaining 10% of non-mineralized bone matrix consists of glycoproteins and proteoglycans. Osteoblasts are also responsible for mineralization of the matrix, by secretion of proteins that are essential for mineralization like alkaline phosphatase (ALP), osteocalcin and osteopontin. When osteoblasts are trapped in the ECM they have produced by themselves, they become osteocytes (Grabowski 2009). Recent discoveries show that osteocytes are multifunctional cells, with important regulatory functions in bone and mineral homeostasis (Dallas, Prideaux et al. 2013; Li, Song et al. 2014). They are perceptive to mechanotransduction and turn mechanical stress into endocrine signals, which regulate bone remodeling of osteoclasts and osteoblasts (Bonewald 2002; Dallas, Prideaux et al. 2013). Another important cell type in bone are osteoclasts, which arise through fusion of hematopoietic cells of the monocyte/macrophage lineage and thus are characterized by numerous cell nuclei. Osteoclasts are in charge of bone resorption and remodeling and can be found next to remodeling bone surfaces

were they secrete acid and proteolytic enzymes forming a resorption lacuna. They are known to be attracted by chondrocytes, which release MMPs and generate fragments of ECM molecules that allure osteoclasts and promote vascularization (Grabowski 2009).

Since the adjective ‘‘subchondral’’ is vague, in the present study the subchondral bone is defined as a layer of bony lamella beneath the calcified cartilage, which are separated by the cement line **(Fig. 3).** Though the structure of the subchondral bone is very inconsistent, it always consists of a thin woven layer of the subchondral bone plate and the subarticular spongiosa (trabecular bone), which expands into the marrow cavity, a supporting three-dimensional structure with impact absorbing properties (van der Harst, Brama et al. 2004). Notably, the structure and assortment of the mineral content and the organic bone matrix assigns properties of subchondral bone like absorption capacity or distribution and transfer of load (Meunier and Boivin 1997; Day, Ding et al. 2001; Donnelly, Chen et al. 2010).

Subchondral bone primarily is based on collagen I fibrils which form parallel sheets continuing to the lamellae of the bone trabeculae (Inoue 1981), which are arranged in right angles to the joint surface and are perpendicular crossed by smaller trabeculae (Meachim and Allibone 1984). In a section tangential to the articular surface, little spaces built of joined plates look like a honeycomb. Further from the surface, these spaces enlarge and progressively extend right angular to the articular surface to form the subarticular spongiosa (Singh 1978). A direct connection of the uncalcified cartilage with the spongiosa cavities is provided by little spaces that form an osteochondral connection via vascular channels (Green, Martin et al. 1970; Inoue 1981; Milz and Putz 1994).

In contrast to the tidemark, which is crossed by collagen fibrils, no collagen fibrils cross the cement line. Importantly, sympathetic (Grassel 2014) and sensory (Ashraf and Walsh 2008) nerve fibers can grow along with subchondral blood vessels through canals in the subchondral bone plate into the calcified cartilage, causing nutrients and factors to reach chondrocytes in the calcified zone. Calcified cartilage nutrition depends on diffusion, however, a high density of channel formation in mechanically stressed zones suggests a particularly good blood supply as a response to long-term stress (Berry, Thaeler-Oberdoerster et al. 1986; Madry, van Dijk et al. 2010).

Variations in natural organization concern the shape of the cement line, the thickness, density and composition of the subchondral bone plate as well as number and type of perforations going into the calcified cartilage (Madry, van Dijk et al. 2010). Additionally, variations in the trabecular structure and in mechanical properties are reported in weight bearing vs. non-weight bearing areas (Bullough, Yawitz et al. 1985; Armstrong, Read et al. 1995).

In summary, the subchondral bone has two main functions: it contributes to shock absorption during mechanical stress and it maintains the articular cartilage with nutrients.

1.3.2 Differences of normal and OA subchondral bone

Research has focused on chondrocytes and cartilage as mediators of OA but there is strong evidence that also other cells and tissues of the joint like synovium or subchondral bone are influenced in OA pathogenesis (Sanchez, Deberg et al. 2005). Because of a close connection to articular cartilage via channels in the cement line, subchondral bone is not just an essential impact absorber, it additionally influences articular cartilage metabolism.

In early OA, the connecting interface between cartilage and subchondral bone is characterized by high rates of bone remodeling and turnover, mainly in the damaged regions. Proliferation of bone cells leads to thickening of the subchondral plate and diminishes the mineral density of subchondral bone, which influences its load bearing properties (Dore, Quinn et al. 2009). In this case, bone deformation under load is raised and a decrease in the elastic modulus can be observed. Under impact, these reduced biomechanical properties (Day, Ding et al. 2001) can lead to cracks or local defects in the subchondral bone plate, where a continuous pressure of water from the cartilage into the subarticular spongiosa causes bone resorption (Aspenberg and Van der Vis 1998).

Altered load distribution is followed by changes in anatomy demonstrating that the subchondral bone plate responds sensitively to modifications in the local environment. Since the subchondral bone plate provides mechanical and metabolic functions and is a dynamic location of remodeling, mechanical distress in the interphase of cartilage and bone results in enhanced OA progression (Kouri, Aguilera et al. 2000; Goldring and Goldring 2007), namely an increased remodeling and turnover of subchondral bone, thinning trabecular structures, subchondral bone cysts at the borders of the joint, bone marrow lesions and sclerosis of subchondral plate (Burr 2004; Burr 2004; Felson and Neogi 2004; Karsdal, Leeming et al. 2008). Additionally, calcification of the tidemark region leads to a decrease in cartilage thickness and increase of subchondral plate thickness (**Fig. 3**) (Lane, Villacin et al. 1977; Hill, Gale et al. 2001; Burr 2004).

Importantly, an OA rat model of the osteochondral junction and tissue samples from OA patients show presence of sensory nerve fibers and expression of nerve growth factor (NGF) in the region of vascular channels. Therefore, osteochondral angiogenesis and sensory fibers probably can cause symptomatic pain (Suri, Gill et al. 2007; Walsh, Bonnet et al. 2007; Ashraf and Walsh 2008).

Taken together, each of both anatomically connected tissues - articular cartilage and subchondral bone - is affected by changes in the mechanical properties of the other one (Aspenberg and Van der Vis 1998; Van der Vis, Aspenberg et al. 1998). Therefore, OA has to be seen completely as a disease of the whole joint and not separately of single tissues as it involves both: the articular cartilage and the subchondral bone as shown in **figure 3**.

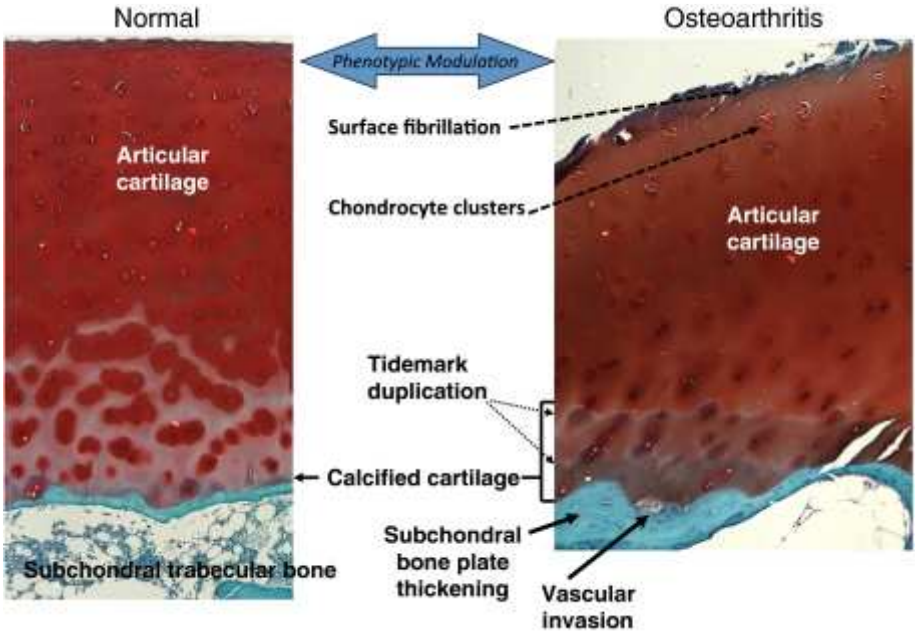


Figure 3: Anatomy of normal and osteoarthritic articular cartilage and subchondral bone
Normal human articular cartilage (left side) is separated by the tidemark from a thin zone of calcified cartilage. Followed by subchondral bone, wich is connected via numerous canals with the calcified cartilage. In contrast, osteoarthritic human articular cartilage (right side) shows clear signs of surface fibrillation and cartilage ECM degradation, in detail, increase of cartilage calcification, duplication of the tidemark and enhanced vascular invasion from subchondral bone (Drawing is adopted from (Goldring 2012)).

1.4 Biology of bone marrow derived mesenchymal stem cells (BMSC)

1.4.1 BMSC and their regenerative potential

There are in general two types of stem cells: embryonic stem cells and adult stem cells. The latter are a rich source for a variety of repair cells, which are able to help out in case of tissue injuries and replace damaged cells (Sylvester and Longaker 2004). They can be found in almost every tissue and can differentiate into a broad range of specialized cells. Stem cells isolated from respective tissues are predestined to differentiate into cells hosted along these lineages. In the bone marrow, even two different types of stem cells coexist, in particular hematopoietic stem cells (HSC) and mesenchymal

stem cells (MSC) (Sylvester and Longaker 2004). These adult bone marrow derived MSC (BMSC) are capable for differentiation *in vitro* after an appropriate stimulation into several different lineages and become amongst others adipocytes, osteoblasts or chondrocytes (Pittenger, Mackay et al. 1999; Kolf, Cho et al. 2007) **(Fig. 4)**.

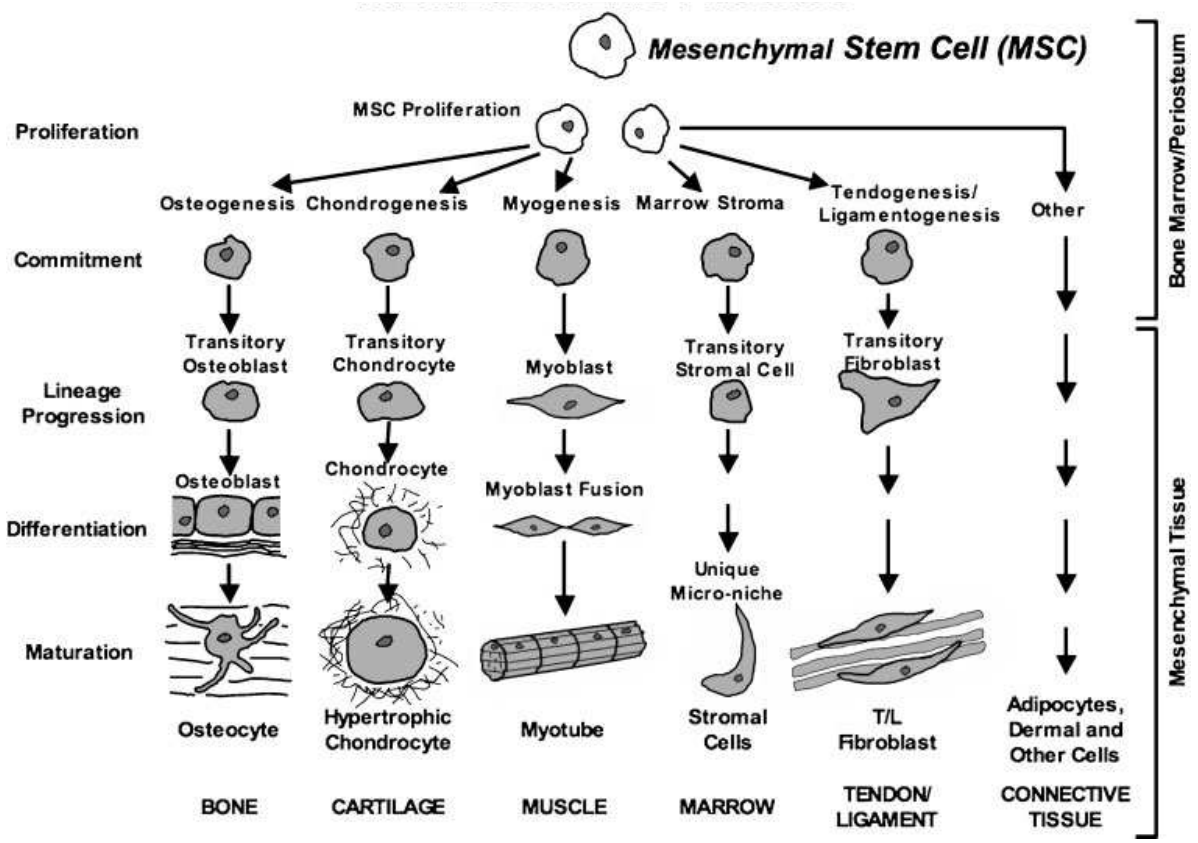


Figure 4: Regenerative potential of MSC
MSC have the potential to differentiate into a broad range of specialized cells and form new tissues like bone (osteocytes), cartilage (chondrocytes), muscle (myocytes), marrow stroma (marrow stromal cells), tendon/ligament (tenocytes/ligament cells), fat (adipocytes) and connective tissues (connective tissue cells). Growth factors and cytokines control differentiation into the different lineages. Therefore, therapeutically approaches in regenerative medicine, which use adult BMSC are promising as they facilitate tissue repair and regeneration of damaged tissue. (Drawing is adopted from (Caplan and Dennis 2006)).

The differentiation potential of BMSC, implanted into focal cartilage defects and their ability to produce a proper ECM, which integrates closely with the adjacent normal tissue is highly dependent on response of the BMSC to the tissue microenvironment. The microenvironment is provided by cell-cell interactions with surface proteins of neighbor cells via integrins or CD44 and by cell-matrix interactions with insoluble matrix molecules like collagens, glycoproteins and proteoglycans (Lutolf and Hubbell 2005; Steinert, Ghivizzani et al. 2007). Additionally soluble molecules released from the

neighboring tissue like growth factors (IGF I, TGF-β, FGF) (Denker, Nicoll et al. 1995), cytokines (IL-1, TNFα) (van den Berg, Joosten et al. 1999) and chemokines (IL-8) are important for proper differentiation (Garner, Stoker et al. 2011). Therefore, an important focus for successful differentiation lies on the interaction of BMSC and their microenvironment. A better understanding of these cues will facilitate regenerative therapies using adult BMSC, which have a promising future in musculoskeletal tissue repair.

1.4.2 Chondrogenic differentiation of BMSC

Regulation of chondrogenic differentiation of BMSC and production of a typical hyaline cartilage involves a variety of molecular mechanisms: stem cell intrinsic factors, paracrine factors derived from neighboring cells like undifferentiated BMSC, osteoblasts or chondrocytes and microenvironmental components independent from cells like availability of nutrients and oxygen, adhesion molecules, ECM epitopes or a 3D surrounding (Coimbra, Jimenez et al. 2004; Goldring, Tsuchimochi et al. 2006; Birmingham, Niebur et al. 2012). Additionally, chondrogenic differentiation of BMSC and deposition of new ECM can be induced *in vitro* by stimulation with growth factors like BMPs or TGF-β superfamily members in a controlled culture condition with accurately defined culture medium. Stimulated BMSC start upregulation of chondrocyte specific genes like fibronectin, N-cadherin, COMP, Collagen IX and during progressed differentiation also of typical articular proteins like collagen II, aggrecan, hyaluronan and chondroadherin (Majumdar, Wang et al. 2001; Sekiya, Larson et al. 2005; Park, Yang et al. 2009).

Molecular mechanisms during the tightly and complex regulated chondrogenic differentiation are not yet fully understood. BMSC pass through several maturation stages in which characteristic factors are activated. After initiation of chondrogenesis, BMSC start cell proliferation and differentiate to chondrocytes under the influence of growth and differentiation factors, like bFGF or bone morphogenetic protein (BMP). In early chondrogenesis, stem cells differentiate to resting chondrocytes with the largest increase in expression of genes coding for cartilage ECM macromolecules like proteoglycans, cartilage oligomeric matrix protein (COMP) and collagen II, IX and XI. In case of hyaline cartilage, this type of chondrocyte is stably maintained in the joint (Mastrogiacomo, Cancedda et al. 2001; Goldring, Tsuchimochi et al. 2006).

Further, interactions of transcription factors, such as Sox9 and Runx2 determine whether chondrocytes remain articular or undergo terminal differentiation before ossification (Mastrogiacomo, Cancedda et al. 2001; Goldring, Tsuchimochi et al. 2006). In embryonic development, chondrocyte differentiation processes during endochondral ossification resulting in

bone formation. Maturing chondrocytes proliferate, increase their metabolic activity and enlarge in size while they deposit collagen X and produce alkaline phosphatase (ALP). The late stage of terminal differentiation is marked by mineralization, vascularization and invasion of bone cells and bone marrow cells (Goldring, Tsuchimochi et al. 2006; Fischer, Dickhut et al. 2010). A common, yet unresolved problem during chondrogenesis of BMSC *in vitro* is that after 3-4 weeks most of the BMSC exhibit a hypertrophic phenotype.

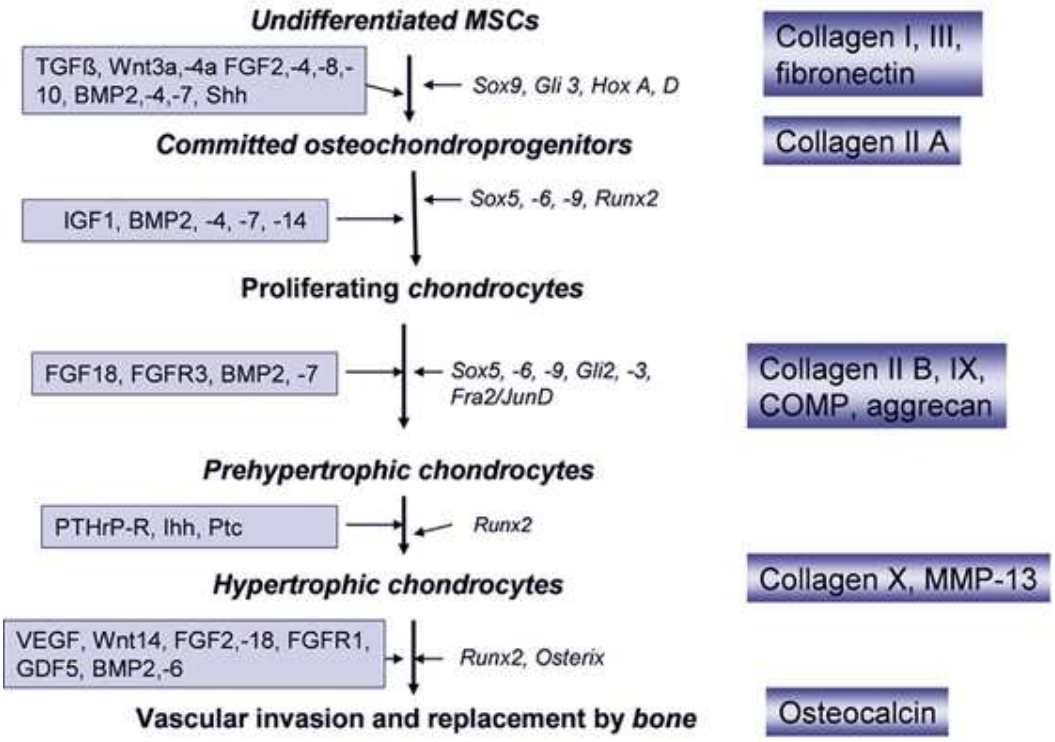


Figure 5: Chondrogenic differentiation during endochondral bone formation.
Schematic representation of different stages of chondrogenic differentiation of mesenchymal stem cells (MSC). Growth and differentiation factors are listed on the left side of the arrows and transcription factors on the right side. Stage specific ECM marker proteins are listed in blue boxes at the right side of the figure. (Drawing is adopted from (Grassel and Ahmed 2007))

In the early phase of chondrogenesis the most prominent transcription factors belong to the SOX family genes, mainly *SOX9*, *SOX5* and *SOX6* **(Fig. 5)** (Lefebvre and de Crombrugghe 1998; Sekiya, Tsuji et al. 2000). However, regulation of chondrogenesis is more complex and the progression of chondrogenesis is additionally governed by other transcription factors, DNA-binding proteins, nuclear receptors, matrix proteins, matrix modifiers as MMPs and adhesion molecules, many extracellular ligands and their receptors (Mundlos and Olsen 1997; de Crombrugghe, Lefebvre et al. 2000; Lefebvre, Behringer et al. 2001). Later, during terminal differentiation mainly PTHrP and Ihh

stimulate upregulation of collagen X, ALP and MMP13 in chondrocytes, which synthesize mineralized matrix followed by vascular invasion and cell death at the end **(Fig. 5)** (Barry, Boynton et al. 2001; Goldring, Tsuchimochi et al. 2006).

A better understanding of the mechanisms of chondrogenic differentiation and hypertrophy is fundamental for further stem cell based therapies, because for a successful cartilage repair a stable articular phenotype and prevention of terminal differentiation is essential. To obtain a high quality functional regenerative tissue with superior mechanical load capacity, proper differentiated BMSC which produce a highly organized functional ECM are required, as ECM components are strongly correlated to mechanical strength of engineered cartilage-like tissue (Mauck, Yuan et al. 2006).

1.4.3 BMSC as trophic mediators

Moreover, BMSC are trophic mediators, which secrete a variety of cytokines and growth factors that reflect their functional status in their specific microenvironment (Prockop 1997; Caplan and Dennis 2006; Kolf, Cho et al. 2007). These factors are able to feed back to the cell itself and govern the functional status and physiology by inducing intracellular signaling or by instructing another cell in the neighborhood to secrete functionally active factors (Caplan and Dennis 2006). A profound clinical use of BMSC may be due to their trophic effects, when BMSC at the site of an injury produce paracrine and autocrine acting factors that are known to suppress the local immune system, stimulate angiogenesis, reduce scar formation and apoptosis and enhance mitosis and differentiation of residing stem cells into regenerative tissue (Caplan and Dennis 2006). Additionally, the therapeutical potential of BMSC is provided by paracrine secretion of cytokines and chemokines like VEGF, FGF-2 or IL-6 via stimulation of survival pathways, induction of differentiation and stemness or regulation of anti-inflammatory effects (Burdon, Paul et al. 2011).

A recent study by Pricola et al. affirmed that IL-6 is probably a “stemness” factor, which contributes to the MSC undifferentiated status and that IL-6 is necessary for proliferation of MSC, protects from apoptosis and inhibits adipogenic and chondrogenic differentiation in an ERK1/2 dependent pathway (Pricola, Kuhn et al. 2009). BMSC used for therapy of chronically inflammatory diseases as OA have two main functions: Firstly, they can either differentiate into chondrocytes or they can mediate mitosis and differentiation of residing neighbor cells and secondly, BMSC can suppress apoptosis and inflammatory processes by release of trophic factors.

1.5 Cartilage repair and tissue engineering

1.5.1 Cartilage repair studies

Most tissues containing stem cells have an elaborated self-repair ambition, with exception of adult articular cartilage. Damaged articular cartilage reveals only minimal or low self-repair capacity, especially in defects of critical size and therefore it urgently requires potent tissue engineering-based therapeutic methods (Caplan, Elyaderani et al. 1997; Cheng, Hardingham et al. 2014).

Lars Peterson and Mats Brittberg were clinical pioneers, who performed the first cell based approach for treatment of full-thickness cartilage or osteochondral lesions via autologous chondrocyte implantation (ACI) in 1994 (Brittberg, Lindahl et al. 1994). Until now, there appeared to be many approaches with promising clinical results for cell based cartilage defect treatment of symptomatic osteochondral defects. Examples are autologous mosaicplasty (Reverte-Vinaixa, Joshi et al. 2013), matrix-assisted autologous chondrocyte transplantation (MACT) or microfracturing with or without biomaterial support. For autologous mosaicplasty, ACI and MACT, cartilage is harvested from a healthy non-weight bearing region of the joint and grafts or *ex vivo* expanded cells were re-implanted into the defect. Drawbacks of these methods are limited cell and graft availability as well as risk of donor morbidity (damage at the donor site) (Brittberg, Peterson et al. 2003; Bedi, Feeley et al. 2010). During microfracturing, little cavities were created in the subchondral bone leading to bleeding into the defect. Natural occurring stem cells and the developing fibrin-clot built the fundament of defect repair. Anyhow, recent studies showed a risk for promotion of fibrocartilage development by using this method (Harris, Siston et al. 2010; Holtzman, Theologis et al. 2010).

With increasing cell demand for regeneration of large damaged cartilage areas, chondrocytes need to be expanded *in vitro*, but chondrocytes de-differentiate and their chondrogenic capacities to produce stable cartilage ECM disappear (Mandelbaum, Browne et al. 2007). Hence, re-differentiation capacity of chondrocytes is desirable and studies showed that a 3D environment like it is provided by scaffolds or hydro gels is able to reactivate a chondrogenic phenotype after expansion (Endres, Neumann et al. 2012). An alternative autologous cell source to replace chondrocytes are chondrogenic differentiated BMSC, which have a high chondrogenic differentiation potential triggered by growth factors and cytokines (Caplan and Dennis 2006). Notably, regulatory obstacles in clinical use of BMSC-based treatments are impeding progress of tissue engineering strategies for cartilage repair (Grassel and Lorenz 2014).

Nevertheless, BMSC might be superior to expanded chondrocytes or cartilage grafts, as they are well expandable *in vitro*, retain their differentiation capacity over several passages and are capable of cartilage like matrix production in a proper environment. As a result, therapeutically approaches in

regenerative medicine which use adult BMSC, would be promising as they might facilitate tissue repair and regeneration of damaged cartilage.

1.5.2 Biomaterials and fibrin in tissue engineering

Interaction between BMSC and ECM components provides an instructive microenvironment and contributes to differentiation of BMSC into chondrocytes. This suggests a beneficial effect by using BMSC in conjunction with synthetic or natural scaffolds, which provide a 3D environment (Lutolf and Hubbell 2005; Lee, Yu et al. 2008). Additionally, a conductive effect of a 3D culture is given for chondrocytes, which maintain their articular character instead of de-differentiating in monolayer (Schagemann, Mrosek et al. 2006; Francioli, Candrian et al. 2010).

Several studies proved the suitability of different 3D culture models for cartilage repair, like collagen or gelatin scaffolds (George, Kuboki et al. 2006; Brochhausen, Sanchez et al. 2013) and hydrogels including photopolymerized hyaluronic acid, agarose (Williams, Kim et al. 2003), Matrigel, PuraMatrix (Dickhut, Gottwald et al. 2008) or alginate (Ho, Cool et al. 2010).

A commonly used hydrogel scaffold is based on fibrin (fibrin gel or fibrin glue) that encapsulates BMSC or chondrocytes and mimics the structure and function of a natural ECM by maintaining a round morphology of cells and thus facilitating deposition of new ECM components (Dickhut, Gottwald et al. 2008; Ho, Cool et al. 2010). Fibrin is a natural non-cytotoxic, biocompatible and biodegradable polymer that can be smoothly polymerized and casted from its basic constituents and has been in clinical use for several years. A big advantage of fibrin gel is a close contact of the implanted BMSC/chondrocytes to the defect tissue borders, as it can directly integrate. Additionally, various *in vivo* and *in vitro* studies demonstrate the potential of fibrin gels in the field of tissue engineering together with human or animal cells. The benefit of combining cells with fibrin is well established for cartilage regeneration, since numerous studies managed to successfully repair defects in animal models. For example, Fussenegger et al. accomplished to reconstruct cartilage in a rabbit-model using stabilized autologous fibrin-chondrocyte constructs (Fussenegger, Meinhart et al. 2003), Sims et al. produced a well formed cartilaginous matrix using bovine chondrocytes suspended in fibrin glue *in vivo* (Sims, Butler et al. 1998) and Mesa et al. demonstrated that ovine articular chondrocytes from 8 year old sheep can be rejuvenated *in vivo* when encapsulated in fibrin gel (Mesa, Zaporojan et al. 2006). Moreover, fibrin constructs containing rabbit BMSC which were provided with constant levels of growth factors during culture time enable the development of articular cartilage *in vitro* and *in vivo* (Park, Yang et al. 2009). Even a human pilot study using fibrin-ACI reports good clinical improvement 2 years after surgery with a favorable clinical outcome and

regenerated tissue shows evidence for similar characteristics to normal cartilage (Kim, Choi et al. 2010).

Depending on the experimental setup, some studies show that fibrin is degraded too fast accompanied with extensive shrinkage or replacement by fibrous tissue (Homminga, Buma et al. 1993; van Susante, Buma et al. 1999), while other groups were successful with cartilage reconstruction (Ting, Sims et al. 1998). Degradation of fibrin can be slowed by addition of high concentrations of antifibrinolytic substances like aprotinin and tranexamic acid or by elevating the fibrinogen/thrombin ratio causing alterations in the crosslinking during polymerization (Fussenegger, Meinhart et al. 2003).

Taken together, a 3D environment, as is provided by biodegradable porous scaffolds or hydro gels like fibrin gel, positively contributes to chondrogenic differentiation capacity and ECM production of BMSC and is beneficial for cartilage neogenesis (Williams, Kim et al. 2003; Erickson, Huang et al. 2009).

1.5.3 Coculture models in tissue engineering

1.5.3.1 Microenvironment of cartilage and chondrocytes

For long-term repair and regeneration of focal cartilage defects, chondrocytes or BMSC are implanted at the site of injury, however, not much attention has been paid to microenvironmental effects of neighboring cartilage or subchondral bone. This is specifically evident in diseases affecting diarthrodial joints as osteoarthritis (OA). The differentiation potential of BMSC, implanted into focal cartilage defects and their ability to produce a proper and stable ECM, which integrates closely with the adjacent normal tissue is highly dependent on response of the BMSC to the local tissue microenvironment provided by cell-cell and cell-matrix interactions and factors released from the neighboring tissue. Soluble and insoluble factors are working together to regulate cell commitment and tissue morphogenesis in native tissue (Lutolf and Hubbell 2005; Steinert, Ghivizzani et al. 2007; Leyh, Seitz et al. 2014 a).

Moreover, it has been demonstrated that BMSC are able to differentiate into a specific cell type, depending on the environment they are actually residing (Djouad, Delorme et al. 2007). Crosstalk between BMSC and cartilage ECM components could be a strongly determining factor for the differentiation of BMSC into chondrocytes. These interactions provide an instructive microenvironment suggesting a beneficial effect on differentiation of BMSC into chondrocytes (Lutolf and Hubbell 2005; Lee, Yu et al. 2008). In addition, normal articular rat cartilage microenvironment

enhances chondrogenic differentiation capacity of rat BMSC and leads to a higher collagen and glycosaminoglycan content in the ECM while at the same time it prevents hypertrophic differentiation (Ahmed, Dreier et al. 2007).

However, the effect of diseased cartilage and OA-chondrocytes on chondrogenic differentiation of BMSC is poorly understood. OA-chondrocytes secrete factors, such as pro-inflammatory cytokines and chemokines that are believed to have a negative effect on locally residing stem cells and inhibit cartilage repair *in vivo.* OA related cartilage lesions and fissures release high amounts of ECM degradation products like fragments of collagen, fibronectin or GAGs. So far, they have not been target for BMSC-based therapies, as this would imply to implant cells into the neighborhood of diseased tissue, where they are confronted with an altered microenvironment of the neighboring cartilage and subchondral bone tissue (Aung, Gupta et al. 2011).

Articular cartilage is unique because residing chondrocytes express a stable chondrogenic phenotype whereas in the growth plate or during endochondral ossification chondrocytes underwent terminal differentiation and hypertrophic cells are replaced by bone due to ossification of cartilage (Goldring, Tsuchimochi et al. 2006).

Recent studies demonstrate the ability of paracrine factors released by articular cartilage tissue or articular chondrocytes to induce chondrogenesis of BMSC and to suppress terminal differentiation and matrix calcification of growth plate chondrocytes *in vitro* and might stabilize articular differentiation of BMSC. Candidate factors are for example FGF-2, TGF-β or PTHrP (Bohme, Winterhalter et al. 1995; Jikko, Kato et al. 1999). Studies analyzing the effect of chondrocyte coculture on spontaneous chondrogenic differentiation of BMSC also show positive effects on collagen II gene and protein expression even without exogenous TGF-β supplementation, suggesting secreted factors that induce chondrogenic differentiation in BMSC (Vats, Bielby et al. 2006; Ahmed, Dreier et al. 2007; Lettry, Hosoya et al. 2010). However, the identity of these factors needs to be determined yet in order to develop protocols, which allow stabilization of the chondrogenic phenotype of BMSC and to reduce their tendency to undergo hypertrophy. Until now, numerous scientists have set up many different coculture studies using chondrocytes and BMSC to enlighten the accurate mechanism for chondrogenic induction and to find the best protocol for generation of a stable articular chondrocyte phenotype.

Interestingly, the ratio of cocultured BMSC and articular chondrocytes regulate whether differentiation proceeds towards a cartilaginous or osseous phenotype. For example, culturing articular chondrocytes with BMSC in a 2:1 ratio induces both phenotypes simultaneous in a 3D-alginate hydrogel construct indicating that chondrocytes provide the necessary factor(s) or cell

contact in both processes (Mo, Guo et al. 2009). Moreover, Bian et. al found that mixed cell populations of MSC and chondrocytes (4:1) reveal higher total collagen content than constructs of only one population, while collagen X deposition was significantly lower in the mixed populations than in MSC alone. Notably, culture of separate cell populations together in the same well did not improve biomechanical or biochemical properties, implying that close proximity is necessary (Bian, Zhai et al. 2011). This is in line with observations of Kang et al. where coculture of BMSC and chondrocytes in same ratios leads to newly engineered cartilage with the densest elastic fibers and highest Young's modulus as well as to cartilage matrix related gene expression profiles (Kang, Liu et al. 2012). Finally, Fischer et al. found that even conditioned medium from chondrocytes leads to significantly reduced expression ratios and deposition of collagen X relative to collagen II. This means direct coculture and soluble factors from chondrocytes can improve chondrogenesis and are able to prevent hypertrophy of BMSC (Fischer, Dickhut et al. 2010).

Taken together, there are many aspects, which manipulate the influence of chondrocytes on BMSC differentiation. The mixing ratio of both cell types, the coculture time and even the kind of coculture – direct, indirect or via conditioned medium – act together creating an unique condition for BMSC fate.

1.5.3.2 Microenvironment of subchondral bone and bone cells

Bone is a central organ of the body: it supports structural stability, carries mechanical load, provides hostage for hematopoiesis, is a depot for minerals and regulates tissue development, remodeling and repair. Several cell types with different functions, namely osteoblasts, osteocytes, osteoclasts, and due to the bone marrow HSC and BMSC, are residing in the subchondral bone. Several studies including these bone cells suggest that they are not only responsible for bone formation and turnover but also have regulatory paracrine functions. Therefore, an influence on metabolism, differentiation and ECM production of neighboring cells like chondrocytes or BMSC is likely. The most prominent bone cell type used for cartilage or chondrocyte coculture studies are osteoblasts.

For example, Westacott et al. shows that subchondral osteoblasts are able to modulate the metabolism of chondrocytes and can induce phenotypic shifts in OA-chondrocytes (Westacott, Webb et al. 1997). *In vitro* cocultures of OA-chondrocytes with OA-osteoblasts reveal increased IL-6 and IL-8 levels, induced inhibition of aggrecan production and a concomitant significant increase in *MMP13* synthesis in chondrocytes. Cocultured osteoblasts also decrease *COL2A1*, *SOX9* and *PTHrP/PTH-receptor* gene expression in chondrocytes, which was considered as a phenotypic shift towards the hypertrophic stage (Jiang, Nicoll et al. 2005; Sanchez, Deberg et al. 2005; Sanchez, Deberg et al. 2005;

Sanchez, Deberg et al. 2008). Further, Lin et al. show that articular chondrocytes cocultured with osteoblasts reveal a phenotypic shift to hypertrophic chondrocytes and that this effect is enhanced by using mechanically stressed ostoblasts. These results indicate that osteoblasts can cause alterations in cartilage metabolism and provide a possible explanation for the onset and progression of OA (Lin, Tanaka et al. 2010). Bianco et al. even suggest that hypertrophic chondrocytes are able to become osteoblast-like cells, but solely if they reside close to the osteogenic tissue border (Bianco, Cancedda et al. 1998). In contrast, a study from Jiang et al. revealed negative effects of chondrocyte derived factors on osteoblast mineralization in coculture, leading to reduced biomechanical properties of newly produced ECM (Jiang, Nicoll et al. 2005). These studies suggest that there are regulatory influneces of osteoblasts concerning chondrocyte metabolism and phenotype. Maybe also a cooperation of signals from both cell types are necessary in a variety of normal processes and both are inevitable for regeneration of cartilage and mineralized tissue (Nakaoka, Hsiong et al. 2006). Indeed, soluble factors are exchanged from subchondral bone and articular cartialge via diffusion through channels or vascular invasion (Burr and Radin 2003), what could explain the influence of subchondral bone factors on distant articular cartilage (Amin, Huntley et al. 2009; Pan, Zhou et al. 2009; Findlay 2010), and suggests that residing cells of both tissues are able to influence each other (Guevremont, Martel-Pelletier et al. 2003; Sanchez, Deberg et al. 2005). Presence of such factors is postulated by several groups, which report an altered phenotype of OA-osteoblasts compared with normal osteoblasts, including enhanced secretion of ALP, IL-6 and TGF-β1 (Mansell, Tarlton et al. 1997; Massicotte, Lajeunesse et al. 2002; Neilson, White et al. 2004).

Anyhow, though there are several coculture studies with osteoblasts concerning their influence on chondrocytes and their matrix generation, studies with osteoblasts and BMSC under chondrogenic differentiation are marginal. Moreover, the role of osteocytes or osteoclasts in chondrogenesis of BMSC or their influence on chondrocytes is mostly still unknown. However, osteoclasts are reported to secrete TNF and other para- and outocrine cytokines, such as IL-6 and IL-1 contributing to inflammatory processes of the bone (Boyce, Yao et al. 2009). Therefore, also osteoclasts might contribute to communication of subchondral bone with chondrocytes or BMSC via secreted factors.

Further, not only bone cells but also subchondral bone matrix itself is likely to provide regulatory features. Especially in case of OA, the increased turnover of subchondral bone leads to an elevated release of matrix bound factors into the microenvironment (Canalis, McCarthy et al. 1988). For example, demineralized bone matrix is known to contain factors like BMP-1 to BMP-7, TGF-β1 and TGF-β2, acidic fibroblast growth factor (FGF), basic FGF and insulin-like growth factor- I (IGF-I), which possibly mediate induction of chondrogenesis in MSC *in vitro* (Syftestad and Caplan 1984; Denker, Nicoll et al. 1995). There is also evidence of diffusion permitting the exchange of soluble substances,

Thus, the microenvironment of OA-subchondral bone has likely an influence on BMSC differentiation and matrix generation capacities, as implanted stem cells may respond to specific ECM components and present regulatory factors. To analyze the differentiation of BMSC in this special surrounding, a coculture system that promotes diffusion of soluble and paracrine factors is needed (Grassel and Ahmed 2007; Hwang, Varghese et al. 2008; Bian, Zhai et al. 2011).

Taken together, bone cells should not only be seen as structural mediators but also as trophic mediators creating a special microenvironment for chondrocytes. *In vitro* coculture of osteoblasts and chondrocytes even suggests reciprocal instructive interactions of both cell types. Thus indicating the essential role of subchondral bone for maintaining articular cartilage and joint homeostasis, the role of normal and OA-subchondral bone for molecular regulatory mechanisms and signaling pathways leading to BMSC differentiation are still unidentified.

1.6 Animals in cartilage trauma repair models

1.6.1 Animal models in cartilage repair

Experiments with normal cartilage are very important for a more detailed knowledge of the influence of unaffected articular cartilage tissue on BMSC differentiation and the differences that occur in OA-cartilage. Unfortunately, normal human cartilage is hard to obtain for ethical reasons. In this case, mammalian animal models provide good requisites to investigate cartilage trauma because many aspects like the genomic, cellular and immunologic structure are close to the human system (Schulze-Tanzil, Muller et al. 2009). Therefore, animal tissues and cells are an alternative, not only for *in vivo*, but also for *in vitro* studies. However, some aspects have to be considered before adopting results obtained from animal studies to the human system. Humans and animals have a different evolutionary background, sometimes resulting in differences in genetic and proteomic regulation (Greek and Menache 2013). For example, studies concerning responsiveness on BMPs to induce chondrogenesis are contradictory in different species partly because of variations in distinctive receptors (Osyczka, Diefenderfer et al. 2004; Hennig, Lorenz et al. 2007). However, in most cases mammalian animal models correspond to physiological processes and mechanisms in humans.

Especially in the field of cartilage tissue engineering differences in anatomy of the joint like size, load and weight distribution as well as features like cartilage thickness are essential. Additionally, distribution of load is important because commonly used animals are quadruped and not biped like humans (McLure, Fisher et al. 2012). Further, Stockwell et al. show that human articular cartilage is thicker compared with most animals, were it increases proportional to body mass of mouse, rat, cat,

rabbit, dog, sheep, man, and cow (Stockwell 1971). Therefore, in vivo studies with large animal models like sheep or swine are probably more suitable for comparison with human than studies with small animal models like rat or rabbit. Interestingly the cellular density decreases with increasing body mass, but biochemical composition of cartilage is surprisingly constant. Therefore, small animals with a high cell density have a benefit in natural cartilage repair *in vivo* (Malda, de Grauw et al. 2013).

Additionally, Taylor et al. show species-specific differences in re-differentiation potential of passaged chondrocytes for bovine chondrocytes compared with human chondrocytes (Taylor, Ahmed et al. 2010). Subsequently, choice of cell species is as important as age of the donor and culture conditions for further interpretation of animal coculture results (Otsuki, Grogan et al. 2010). Some studies even successfully use xenogeneic human BMSC for treatment of an animal recipient. Lack of immune reaction to the xenogeneic regenerated cartilage is possibly due to immunosuppressive activities of BMSC (Li, Chiang et al. 2009).

Taken together, *in vivo* cartilage defect repair is successful in a variety of animal studies like goat, sheep or swine using embedded BMSC, suggesting a future use for minimally invasive implantation and engineering of novel cartilage like tissue for humans (Williams, Kim et al. 2003; Li, Chiang et al. 2009; Zscharnack, Hepp et al. 2010).

1.6.2 Ovine cartilage repair models

Ovine cartilage models are a very potent and often used tool in biomedical research for studying normal articular cartilage tissues in large animals. Ovine BMSC (oBMSC) share similar properties with human BMSC like capability for growth factor induced proliferation or differentiation into multiple stromal lineages and they express stem cell associated surface markers (McCarty, Gronthos et al. 2009).

Usually, studies use ovine cartilage or animal models to study the treatment of osteochondral defects *in vivo* or *in vitro* with re-implantation of autologous BMSC into the defect (McLure, Fisher et al. 2012). For example, Mrugala et al. established an ovine patella defect model and embedded BMSC into a chitosan matrix resulting in a cartilage like tissue successfully filling the defect after 9 weeks (Mrugala, Bony et al. 2008). In another study, *ex vivo* chondrogenic pre-differentiated BMSC/collagen I gel constructs are implanted into an ovine osteochondral defect model. MACT using pre-differentiated MSC shows potential for repair of chronic osteochondral defects including collagen II deposition and chondrogenic characteristic of cells (Zscharnack, Hepp et al. 2010).

Several *in vitro* and *in vivo* studies prove usage of ovine cells embedded in hydrogel - especially fibrin for successful cartilage repair. For example re-differentiation potential of de-differentiated expanded ovine chondrocytes in PGA-fibrin scaffolds show formation of cartilaginous matrix accompanied by induction of collagen II and aggrecan (Endres, Neumann et al. 2012). Munirah et al. reveal that implantation of autologous chondrocyte-fibrin constructs results in hyaline-like cartilage regeneration *in vivo* (Munirah, Samsudin et al. 2007). Finally, Schagemann et al. conclude that a 3D hydrogel culture keeps chondrocytes in a differentiated status and offers a chondrogenic environment for *in vitro* cartilage regeneration (Schagemann, Mrosek et al. 2006).

Taken together, normal ovine tissue and chondrocytes or BMSC embedded in a 3D fibrin gel seem to be a suitable alternative to normal human tissue. To work out the differences between factors released from normal or diseased human cartilage it is crucial to use a suitable cartilage defect model. An ovine model appears to be a good choice and allows realistic conclusions that might be crucial for further progress in cartilage tissue engineering.

2 Aim of the thesis

OA is a painful joint degenerative disease, which is characterized by degradation of articular cartilage. As an alternative treatment for implantation of prosthesis, the use of tissue engineering for cartilage regeneration systems is rising. Scientists are keen on finding a cell-based therapy, which succeeds in production of high quality cartilage like tissue and finding a source that provides a sufficient number of hyaline ECM building cells with a stable articular phenotype including prevention of donor morbidity. To date mainly two cell types are suitable for such a regeneration approach: adult stem cells with chondrogenic differentiation potential and articular chondrocytes. So far, an optimal therapy method was not found though several studies established suitable treatment options **(see 1.1.2).** Clinical and experimental trials with a 3D surrounding provided by a biomaterial in combination with cells gave best results. However, drawbacks like inadequate quality of regeneration tissue, unstable articular phenotype resulting in hypertrophy of BMSC or simply an insufficient number of donor chondrocytes prevented the standard usage of tissue engineering for cartilage defect treatment. Further, also regulatory obstacles in clinical use of BMSC-based treatments are impeding progress of tissue engineering strategies for cartilage repair.

In the present study, the aim was to find answers, why treatment of articular cartilage defects – especially OA defects – with cells embedded in a degradable biomaterial fails or does not develop the supposed hyaline cartilage like tissue.

Therefore, focus of this study is set on the following goals:

- Establishment of a reproducible *in vitro* paracrine coculture model, which allows to investigate the influence of soluble factors from normal and OA-tissue explants on matrix forming capacity of adult ASC, BMSC and articular chondrocytes as well as of mixed cultures (BMSC and chondrocytes in equal ratio).
- Gain of a better understanding of mechanisms for BMSC differentiation and generation of a stable articular phenotype and a better integration and quality of regenerated tissue.
- Examination of the effects induced by articular cartilage or subchondral bone explants on ASC and BMSC differentiation, phenotype of articular chondrocytes and the biochemical and biomechanical properties of their newly formed ECM.
- Analysis of the effects induced by a mixed culture of BMSC and chondrocytes (in equal ratio) in respective explant cocultures on BMSC differentiation, phenotype of articular chondrocytes and the biochemical and biomechanical properties of their newly formed ECM.

- Identification of signaling factors derived from the microenvironment of cartilage or subchondral bone explants (e.g. pro-inflammatory cytokines, growth factors).
- Clarification whether these factors influence chondrogenic differentiation and ECM formation of BMSC and articular chondrocytes (mainly collagen and proteoglycan production and degradation).
- Survey of the inflammatory microenvironment of OA-cartilage and subchondral bone and its influence on chondrogenic differentiation as well as production and deposition of typically articular ECM in comparison with normal (healthy) cartilage and subchondral bone.
- Discovery of differences between OA- stem cells (BMSC) and young normal stem cells (ASC), with respect to chondrogenic differentiation and matrix production.

In summary, aim of this study is to gain insight in chondrogenic differentiation and ECM generation of BMSC under the influence of microenvironmental cues provided by cell-cell and cell-matrix interactions with OA-chondrocytes, OA-cartilage and OA-subchondral bone at cartilage defect sites. Additionally, this study wants to explore differentially expressed or released factors in the OA microenvironment, which probably lead to a higher or diminished ECM quality, to increased or decreased biomechanical properties or alterations in composition of regenerated tissue. A high quality cartilage like matrix and a stable articular phenotype is important when using cell-based therapies considering future therapeutic strategies to halt or even reverse OA progression.

3 Material and methods

3.1 Culture and isolation of articular cartilage, subchondral bone and chondrocytes

Human articular cartilage was collected from surgically removed joints of patients undergoing total knee replacements (TEP) due to OA (**see figure 6**). This had been approved by the local Ethics Committees (IRB: 08/065; Ethikkommission an der Universität Regensburg, email: ethikkommission@klinik.ukr.de) and specimens were taken with patients' written consent. For experiments with subchondral bone, knee joints were obtained from 32 different donors (13 male and 19 female, mean age 67 ± 9).

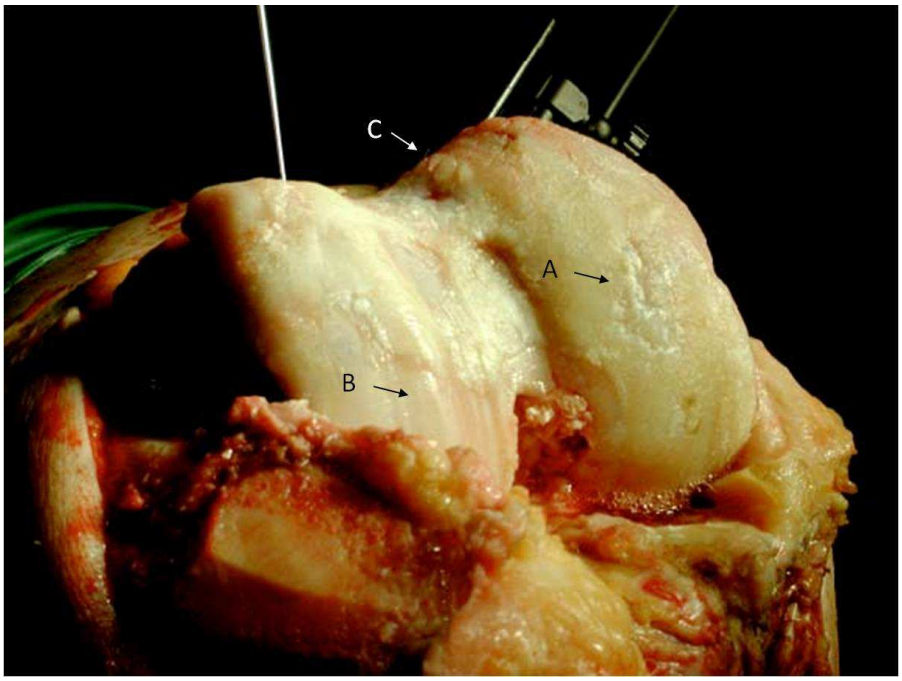


Figure 6: Representative photo of an osteoarthritic (OA) knee joint during knee surgery
Articular cartilage of a joint prepared for knee TEP (total endoprosthesis) surgery showed typical signs for OA: wide areas with cartilage lesions (A), zones with completely denuded bone and groove forming (B) as well as beginning of osteophyte formation (C). (Photo: Johannes Beckmann Asklepios Bad Abbach)

Prior to culture, cartilage tissue was first classified macroscopically as either damaged or intact according to a predefined procedure comprising color, surface integrity and tactile impression tested with a standard scalpel (Geyer, Grassel et al. 2009). Only healthy appearing pieces were used for isolation of chondrocytes and generation of cartilage and subchondral bone explants. OA-subchondral bone and cartilage explants were produced as follows: Surgically removed tissue was thoroughly washed with PBS. Then the cartilage surface was scored like described above and only

justly evaluated pieces were accepted for usage. Cartilage was cut off for isolation of chondrocytes and preparation of cartilage explants. The completely denuded bone was used for generation of explants of 8mm in diameter and 4mm height. Cartilage explants were punched out (8 mm^2 x 2 mm; Biopsy punch, Stiefel GmbH, Munich, Germany) from intact cartilage slices including the superficial zone **(Fig.7)**. Chondrocytes were isolated from cartilage slices upon overnight digestion with collagenase II (PAA, Piscataway/ New Jersey, USA) at 37°C. Isolated cells were resuspended in Dulbecco's modified Eagle´s medium (DMEM, Gibco Invitrogen, UK) containing 10% fetal calf serum (FCS, Sigma-Aldrich, St. Louis/ Missouri, USA) and 1% penicillin/streptomycin (P/S, PAA, Piscataway/ New Jersey, USA). Chondrocytes were kept in monolayer in an initial density of 17.000 cells per cm^2 cultured in a humidified 37°C / 5% CO_2 incubator for 7 to 14 days and were used when confluent (passage 1) (Dreier, Grassel et al. 2004).

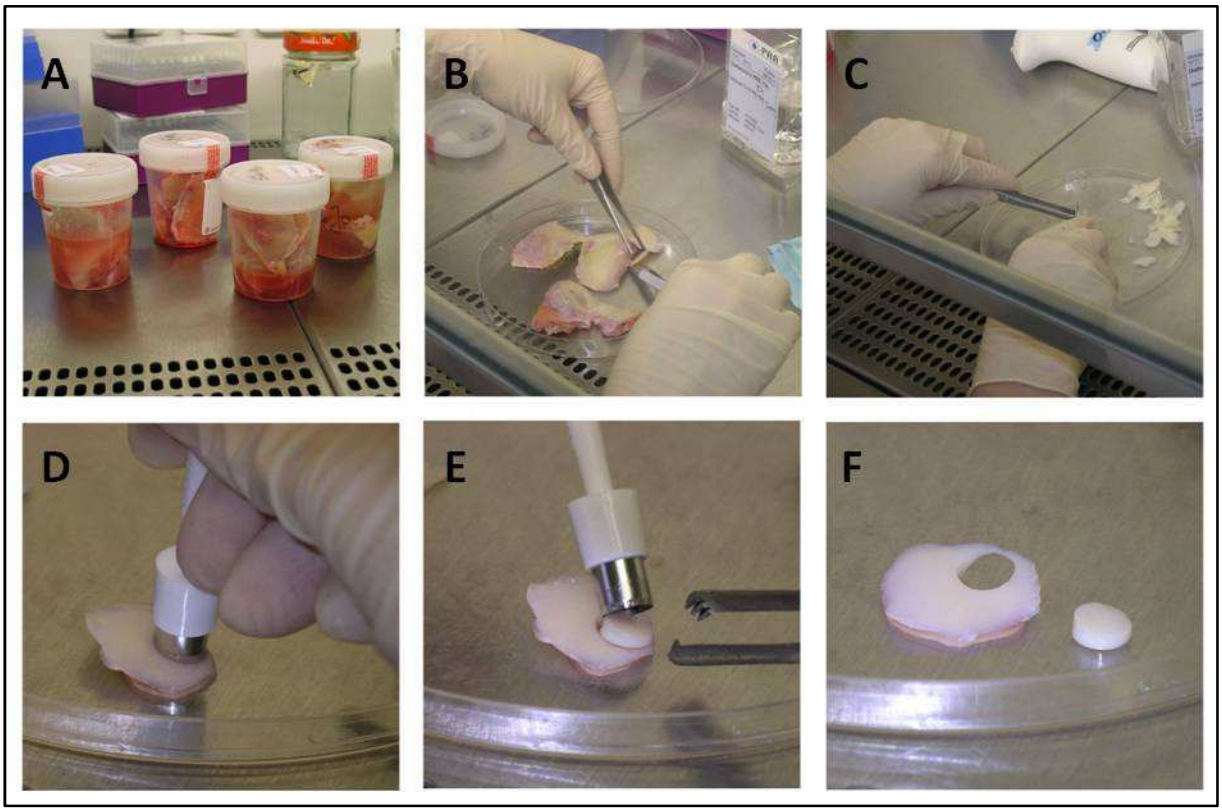


Figure 7: Preparation of cartilage explants from OA-knees
Knee joints were obtained from different donors and taken with patients' written consent (A). Cartilage tissue was classified macroscopically according to a predefined procedure comprising color, surface integrity and tactile impression tested with a standard scalpel (B). Intact cartilage slices including the superficial zone were cut off and denuded subchondral bone was prepared (C) either for cell isolation or for coculture setups. Cartilage explants were punched out from cartilage slices including the superficial zone (D-F).

Normal human cartilage and subchondral bone tissue (2 male and 2 female, mean age 35 ± 11) was received from knee joints of rare trauma affected patients treated for sports accidents. The tissue used in this study was not injured directly but was collected during the surgical treatment from another non-affected region of the same joint and explants were prepared like described above.

Normal ovine articular cartilage was prepared from surgically removed joints of sheep euthanized for unrelated experiments obtained from Dr. med. vet. Uta Delling (University of Leipzig, Germany, Faculty of Veterinary Medicine, Large Animal Clinic for Surgery) or from slaughterhouse waste. Ovine chondrocytes (oCh) and ovine cartilage explants were prepared according to the protocol of human chondrocyte isolation.

3.2 Culture and isolation of mesenchymal stem cells (BMSC) and adipose-derived stem cells (ASC)

Human BMSC used for this study have either been purchased from Lonza (Basel, Switzerland) or isolated from bone marrow aspirates obtained from patients (14 male and 16 female, mean age 61 ± 8) undergoing a hip replacement surgery due to OA. This had been approved by the local Ethics Committee (IRB 08/065; Ethikkommission an der Universität Regensburg, email: ethikkommission@klinik.ukr.de) and specimens were taken with patients' written consent. The bone marrow was centrifuged and cells were fractionated on a density-gradient (Biocoll Separating Solution, Biochrom, Berlin, Germany). The low-density cell-fraction concentrated in the interphase ("buffy coat") was washed, seeded in cell culture flasks supplied with MesenchymStem Medium (PAA, Piscataway/ New Jersey, USA) supplemented with Mycozap (Lonza, Basel, Switzerland) and non-adherent cells were removed after 5 to 7 days. Adherent cells were cultured until they reached approximately 80% confluence. After splitting, BMSC were seeded at a density of 4×10^4 cells per cm^2 and kept in culture for up to three passages before they were used for experiments.

oBMSC were obtained from Dr. med. vet. Uta Delling (University of Leipzig, Germany, Faculty of Veterinary Medicine, Large Animal Clinic for Surgery). Briefly, oBMSC were harvested from ovine bone marrow aspirated from iliac crest. Cells were isolated and cultured as described above followed by cryo-preservation at passage 0. After thawing, cells were seeded at a density of 4×10^4 cells/cm^2 in cell culture flasks, supplied with low glucose DMEM (PAA, Piscataway/ New Jersey, USA) supplemented with 1% P/S (PAA, Piscataway/ New Jersey, USA), 10% FCS (Sigma-Aldrich, St. Louis/ Missouri, USA) and ascorbat 12.5 mg/mL (PAA, Piscataway/ New Jersey, USA) and kept in culture for up to three passages before they were used for experiments.

Adipose-derived stem cells (ASC, isolated as described previously by Gehmert et. al.) (Gehmert, Hidayat et al. 2011) were a friendly gift from the Department of Otorhinolaryngology, Division of Facial Plastic Surgery, University of Regensburg. Five different patients were used for our coculture setup (female, age < 50). Written consent from the local Ethics Committee to harvest ASC from patients was obtained from the Institutional Review Board (IRB) of the University of Regensburg (IRB: 08/117; Ethikkommission an der Universität Regensburg, email: ethikkommission@klinik.ukr.de)

Briefly, the ASC were isolated from subcutaneous fat tissue, which was obtained from patients undergoing elective body-contouring procedures. Fat tissue was washed, minced and digested in serum-free MEM (1 mL/1 g tissue) with LiberaseBlendzyme 3 (2U/1g tissue; Roche Diagnostics, Basel, Switzerland) at 37°C for 45 minutes. The lysate was filtered (100 and 40 µm filters; Fisher Scientific, Schwerte, Germany) and centrifuged at 450*g* for 10 minutes. The cell pellet was washed twice with Hanks' balanced salt solution (Cellgro, Manassas, VA, USA), cells were seeded in culture vials (Greiner Bio-one, Frickenhausen, Germany) and daily washed to remove unwanted red blood cells or non adherent cells. After reaching a confluence of 80%, ASC were seeded at a density of 3000 cells/cm^2, maintained in ASC medium (αMEM containing 20% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin, Sigma, St. Louis, MO, USA) and used for experiments at passage 5.

ASC were characterized in accordance with guidelines of the Declaration of Helsinki for biomedical research from the Applied Stem Cell Research Center of the University of Regensburg.

3.3 Flow cytometric characterization of BMSC and differentiation into different lineages

Plastic adherent BMSC at passage 3 were trypsinized, centrifuged and the cell pellet was resuspended into blocking buffer (PBS with 0.1% sodium azide, 5% FCS and 10%FcR Blocking Reagent) at a concentration of 10^6 cells/mL. After blocking for 15 minutes at 4°C the cell suspension was stained with a conjugated (40% CD19-PE, 10% CD105-FITC), or a unconjugated (2% CD34, 2% CD44) primary antibody or with corresponding isotype controls (40% IgG1-FITC, 40% IgG1-PE, 20% IgG2a and 20% IgG1) in 50 µl blocking buffer **(Table 1)** for 15 minutes on ice in the dark. In case of unconjugated primary antibodies (CD34, CD44, IgG2a and IgG1) the two times washing step with FACS buffer (PBS containing 0.1% sodium azide and 5% BSA, Sigma-Aldrich, St. Louis/ Missouri, USA) was followed by incubation with an immunofluorescent secondary antibody Alexa 568 or Alexa 488 1:400 in blocking buffer for 30 minutes on ice. After washing, the cells were resuspended in 250 µl FACS buffer and analysed by FACScanto® (Becton Dickinson, USA) linked with the CellQuest 3.1 data

acquisition software (Becton Dickinson, Franklin Lakes, New Jersey, USA). Data analysis was performed with FlowJo 7.6.5 software (Tree Star Inc., Ashland, Oregon, USA).

To demonstrate pluripotency of BMSC, 2 x 10^5 BMSC were differentiated in a 6-well in adipogenic or osteogenic medium for 21 days. Adipogenic medium consisted of DMEM F-12 (Gibco Invitrogen, UK) supplemented with 10% FCS (Sigma-Aldrich, St. Louis/ Missouri, USA), 1%P/S (PAA, Piscataway/ New Jersey, USA), 0.1% dexamethasone (Sigma-Aldrich, St. Louis/ Missouri, USA), 0.1% 3-Isobutyl-1-methylxanthin (IBMX, Sigma-Aldrich, St. Louis/ Missouri, USA) and 0.001% insulin (Sigma-Aldrich, St. Louis/ Missouri, USA). Osteogenic medium consisted of DMEM high glucose (Gibco Invitrogen, UK) supplemented with 10% FCS (Sigma-Aldrich, St. Louis/ Missouri, USA), 1% P/S (PAA, Piscataway/ New Jersey, USA), 0.1% Na-β-glycerophosphate (Sigma-Aldrich, St. Louis/ Missouri, USA), 50 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis/ Missouri, USA), and 60 ng/mL dexamethasone (Sigma-Aldrich, St. Louis/ Missouri, USA). Chondrogenic medium consisted of DMEM high glucose (Gibco, Invitrogen, UK) supplemented with 1% P/S (PAA, Piscataway/ New Jersey, USA), 0.1% ITS+ premix (6.25 µg/mL insulin, 6.25 ng/mL selenium acid, 6.25µg/mL transferrin, 1.25 mg/mL BSA and 5.35 µg/mL, linoleic acid, BD Biosciences, USA.), 50 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis/ Missouri, USA), 110 µg/mL pyruvate (PAA, Piscataway/ New Jersey, USA), 40 µg/mL proline (AppliChem, Darmstadt, Germany), 0.6 µg/mL dexamethasone (Sigma-Aldrich, St. Louis/ Missouri, USA) and 10 ng/ml transforming growth factor β-3 (TGFβ-3, R&D Systems, Minneapolis/ Minnesota, USA).

Evaluation of differentiation capacity of isolated BMSC was performed microscopically after Oil Red staining (adipogenic differentiation) and alizarin staining (osteogenic differentiation). Differentiated cells were washed with PBS, fixed for 10 minutes with PFA 4% and stained for 5 minutes with oil-red (Sigma-Aldrich, St. Louis/ Missouri, USA) in 60% isopropanol (Roth, Arlesheim, Germany) or they were fixed for 10 minutes with methanol (Roth, Arlesheim, Germany) and stained for 2 minutes with alizarin staining solution (0.1% alizarin and 2.5% ammonium, Sigma-Aldrich, St. Louis/ Missouri, USA, in H_2O), respectively. Alizarin stained plates were dried and stored, oil-red stained plates were microscoped and photographed with an Olympus BX 61 imaging system and cell^P software (Olympus, Hamburg, Germany) immediately after the staining.

Table 1: Antibodies used for FACS analysis
Isolated plastic adherent BMSC in passage 3 were analyzed by flow cytometry using specific antibodies against the negative markers CD19 and CD34 and the positive markers CD44 and CD 105. Specificity of epitope staining was proofed using isotype controls and an FcR Blocking reagent.

Antibody	Company	Ref. number
CD44	Acris Antibodies, Herford, Germany	BM2431PT
CD34	Acris Antibodies, Herford, Germany	DM3515P
CD19-PE	BD Pharmingen, San Diego, USA	555413
CD105-FITC	BD Pharmingen, San Diego, USA	561443
Alexa Fluor 488 F(ab´)	Invitrogen life technologies, Paisley, UK	A-11017
Alexa Fluor 568 F(ab´)	Invitrogen life technologies, Paisley, UK	A-21124
FITC mouse IgG1k Isotype Control	BD Pharmingen, San Diego, USA	555748
PE mouse IgG1k Isotype Control	BD Pharmingen, San Diego, USA	555749
Mouse IgG1 Isotype Control	MACS Miltenyi Biotec, Bergisch Gladbach, Germany	140-001-521
IgG2a-Isotype Control	Immunotech SAS, Marseille, France	A12689
FcR Blocking Reagent human	MACS Miltenyi Biotec, Bergisch Gladbach, Germany	130-059-901

3.4 Cocultivation of fibrin gel embedded BMSC/chondrocytes during chondrogenic differentiation

A suspension of 10 µl fibrinogen (100 mg/mL, Sigma-Aldrich, St. Louis/ Missouri, USA) and 1 x 10^6 BMSC, 1 x 10^6 ASC, 2 x 10^6 chondrocytes or a mixture of 5 x 10^5 BMSC and 5 x 10^5 chondrocytes was homogenously mixed with 18 µl thrombin (5 U/mL, Baxter, Munich, Germany). Because of the smaller size of chondrocytes, their cell number had to be adjusted to obtain a comparable volume of fibrin gel and to avoid effects due to different medium diffusion capacity resulting in an altered maintenance with nutrients. In case of ovine chondrocytes or ovine BMSC, 100 µg/mL Aprotinin, a protease inhibitor (3000 KIE/mL, Tissuecol immune kit, Baxter, Munich, Germany) was added to the fibrinogen solution. The cell-fibrinogen suspension was either applied on the surface of the superficial zone of articular cartilage explants, on subchondral bone explants (cocultures) or as a droplet on the bottom of a 24-well plate (monocultures). Accordingly, the mixed cell-fibrinogen suspension was applied on respective explants (tricultures) or as a droplet at the 24-well plate bottom (mixed monocultures) **(Fig. 8)**. Notably cartilage, chondrocyte and BMSC donors were not used autologous. Therefore, in the mixed tricultures up to three different patients were merged (one patient for chondrocytes, one for the BMSC and a third patient for the explant). Full polymerization of the suspension was reached after 45 minutes at 37°C and resulted in a stable and clear hydrogel with a pore size of approximately 50 µm. Cell-free cartilage and subchondral bone explants were included to our setups. Mono-, co- and tricultures as well as cell-free explants were kept in

chondrogenic medium in the presence of TGFβ-3 (10 ng/mL, R&D Systems, Minneapolis/ Minnesota, USA) and cultivated for up to 4 weeks (Ahmed, Dreier et al. 2007). For the LC-MS analysis, fibrin gels were supplemented with chondrogenic medium without ITS. After 7 and 28 days a specimen of co- or tricultured fibrin gels was carefully separated from cartilage or subchondral bone explants using a thin spatula. Fibrin gels were processed for histology, immunofluorescence, protein biochemistry, biomechanics or gene expression analysis as described below. Culture supernatants of days 7 and 28 were collected and frozen at -80°C until they were used for ELISA, hydroxyproline-assay, DMMB-assay or collagen preparation.

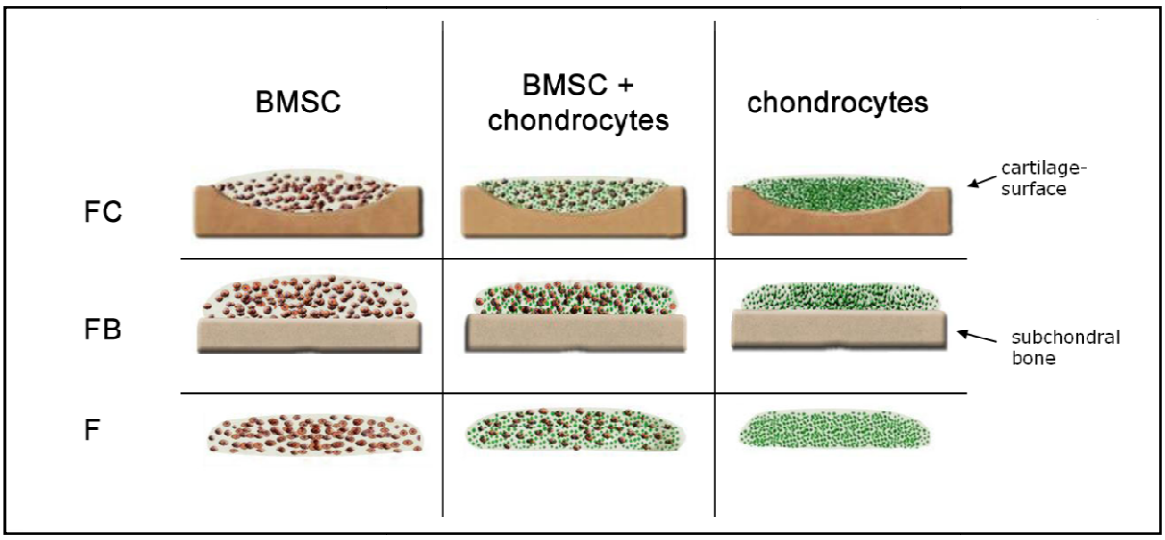


Figure 8: Model of contact co- or triculture between cells and cartilage- or subchondral bone explants
BMSC, mixed cultures (BMSC and chondrocytes in equal ratio) and chondrocytes were embedded in fibrin gel and applied onto the surface of articular cartilage- or subchondral bone explants (co- or tricultures). As control, cells were embedded in fibrin gels and cultured without cartilage or subchondral bone (monocultures). All experimental set ups were kept for up to 28 days in chondrogenic medium. Samples were harvested at days 7 and 28. FC = co- or tricultures with cartilage explants; F = monocultures (without explants) FB = co- or tricultures with subchondral bone explants

3.5 Stimulation of fibrin gel embedded BMSC and/or chondrocytes

Stimulation of BMSC, mixed and chondrocyte monocultures with IL-1β (5 ng/mL, Biomol, Hamburg, Germany), IL-6 (5 ng/mL, RayBiotech, Norcross/ Georgia, USA) or IL-8 (10 ng/mL, RayBiotech, Norcross/ Georgia USA) was performed in chondrogenic medium for the first 7 days. Fibrin gels were harvested after 7 or 28 days and processed for gene expression analysis as described under **3.7**. or for quantification of GAGs and collagens as described under **3.9.** Repetition was in triplicates at least 5 times with cells and explants from different donors.

3.6 Cell vitality and proliferation in fibrin gel cocultures

In order to evaluate vitality of cells in our different culture set ups, lactate dehydrogenase (LDH) concentration in supernatants of BMSC, mixed cultures and chondrocytes was determined in monocultures and co- or tricultures with OA-cartilage or OA-subchondral bone kept in chondrogenic medium. Content of LDH was analyzed at days 7, 14, 21 and 28 with an LDH-based cytotoxicity detection Kit (Roche, Penzberg, Germany) and compared with respective assay controls (high control = all cells in fibrin gels were lysed; low control = spontaneous cell death of an equivalent cell amount in monolayer) according to the manufacturer´s instructions. LDH concentration released from dead cells into supernatant was determined on photometrical basis at absorption of 490 nm (Tecan GENios with Magellan 6.5, Crailsheim, Germany). Due to high inter-experimental variability, presumably caused by personal living conditions and physical activity, medical treatment or general health status of tissue donors, the raw data was calculated as percent of control per individual experiment. Repetition was in triplicates at least 3 times with cells and explants from different donors.

For proliferation tests of monocultures and OA-cartilage cocultures, the fibrin gels were processed as described under **3.8**. Sections were fixed in 4% PFA (Sigma-Aldrich, St. Louis/ Missouri, USA) for 10 minutes and endogen peroxidase was blocked by treatment with 3% H_2O_2 containing 10% EtOH for 5 minutes. This was followed by an epitope demasking by boiling sections with 10mM sodium citrate containing 0.05% Tween for 2 minutes at 650 watt and for another 5 minutes at 350 watt in the microwave. Sections were blocked with 2% goat serum (Dako, Glostrup, Denmark) diluted in PBS for 1 hour and labeled over night at 4°C with an antibody against PCNA (DAKO, Glostrup, Denmark, mouse anti PCNA clone PC10-M0879). For detection, all sections were incubated with a biotinylated secondary antibody (Dako, Glostrup, Denmark) for 1 hour at 37°C followed by an incubation with streptavidin peroxidase in PBS for 1 hour at 37°C. Staining was performed with the liquid substrate system (DAKO Glostrup, Denmark) for 15 minutes. Sections were counterstained with Mayer´s hemalaun solution (Roth, Arlesheim, Germany) for 5 minutes followed by successive dehydration with upraising alcohol concentrations and were mounted with 1-2 drops of Depex (Serva Electrophoresis, Heidelberg, Germany). Stained sections were photographed with Olympus BX 61 imaging system and cell^P software, the number of total nuclei and number of PCNA stained nuclei was counted and the proliferation ratio was calculated.

3.7 Isolation and quantification of messenger RNA

Cell-fibrin gel suspensions suspended in peqGOLD TriFast (PeqLab, Erlangen, Germany) were minced and RNA was isolated according to a Trizol protocol, followed by column purification with the absolutely RNA Microprep Kit (Agilent Technologies Stratagene, Santa Clara/ California, USA) according to the manufacturer´s instructions. cDNA was generated from 500ng of RNA using Affinity Script QPCR cDNA synthesis Kit and oligo(dT) primers (Agilent Technologies Stratagene, Santa Clara/ California, USA) according to the manufacturer´s instructions. Repetition was in triplicates at least 5 times for BMSC, mixed and chondrocyte cocultures with explants from different OA donors and at least 4 times for ASC cultures.

Table 2: qPCR setup

Gene expression level in mono-, co- and tricultures at days 7 and 28 was determined by using 30 µl of cDNA (mRNA equivalent), specific primer pairs and the 2x brilliant II SYBR green qPCR master mix.

Component	Volume in µl
PCR grade water	6.125 µl
2x brilliant II SYBR green qPCR master mix	12.500 µl
Primer forward	0.500 µl
Primer reverse	0.500 µl
cDNA (6ng/µl)	5.000 µl
reference dye (diluted 1:500 in PCR grade water)	0.375 µl
total volume	25.000 µl

Quantitative real-time PCR was performed in triplicate according to **table 2** using 30ng cDNA and qPCR master mix SYBR Green Dye I on MxPro-Mx305P (Agilent, Santa Clara/ California, USA). After an initial denaturation step at 95°C for 10 minutes, the cDNA products were amplified with 40 PCR cycles, consisting of a denaturation step at 95°C for 10 seconds and an extension step at 60°C for 30 seconds.

Two different types of cDNA quantification were used: On the one hand, for quantification of human *COL1A1, COL2A1, COL3A1, COL10A1* and *SOX9* gene expression in cell-fibrin gels of mono-, co- and tricultures, absolute cDNA quantification was used and a plasmid standard curve was included on each PCR plate (Plasmid copy ranges for *COL1A1:* $1x10^6 – 1x10^2$; *COL2A1:* $3.4x10^7 – 3.4x10^3$; *COL3A*1: $1x10^6 – 1x10^2$ and *COL10A1:* $5x10^4 – 5x10^0$). Due to high inter-experimental variability, the raw data was calculated as percentage of highest cDNA copy number per individual experiment.

On the other hand, for quantification of ovine *COL1A1, COL2A1, COL3A1, COL10A1* and *oACAN* in cell-fibrin gels of ovine mono-, co- and tricultures, relative cDNA quantification was used. For evaluation, raw data were normalized with *oGAPDH* and calculated employing the ddCT method using gene expression of oBMSC monolayer cells (in case of oBMSC mono- and cocultures and ovine mixed (oMixed) mono- and tricultures) and gene expression of oCh monolayer cells (in case of oCh mono- and cocultures) as calibrator. For quantification of human *COL1A1, COL2A1, COL3A1, COL10A1, MMP2, MMP3, MMP13* and *hACAN* in IL-1ß, IL-6 or IL-8 stimulated monocultures, relative cDNA quantification was used. For evaluation, raw data were normalized with *hGAPDH* and calculated employing the ddCT method using gene expression of respective unstimulated monocultures (cultured without IL- supplementation) as calibrator.

Data analysis was carried out by using the MxPro QPCR 4.0 software (Agilent Technologies Stratagene, Santa Clara/ California, USA). The forward and reverse primer pairs listed in table 3 were used for gene expression analysis.

Table 3: Oligonucleotides for q-RT-PCR

Human and ovine gene expression was quantified with specific forward and reverse primer pairs using the 2x brilliant II SYBR green qPCR master mix and an MxPro-Mx305P cycler (Agilent Technologies Stratagene, Santa Clara/ California, USA). Primer pairs were used for both species (human and ovine) if not indexed as different. Primer pairs with an initial 'h' were used for quantification of human cDNA and primer pairs with an initial 'o' were used for quantification of ovine cDNA.

GENE	forward primer	reverse primer
COL1A1	5´-AGC TCC TGG TGA AGT TGG TC-3´	5´-ACC AGG GAA GCC TCT CTC TC-3´
COL2A1	5´-TGC TGC CCA GAT GGC TGG AAG A-3´	5´-TGC CTT GAA ATC CTT GAG GCC C-3´
COL3A1	5´-GTC CAT GGA TGG TGG TTT TC-3´	5´-GTG TGT TTC GTG CAA CCA TC-3´
COL10A1	5´-CCC TCT TGT TAG TGC CAA CC-3´	5´-AGA TTC CAG TCC TTG GGT CA-3´
MMP2	5´-GCC AAT GGA GAC TGT CTC AAG A-3´	5´-TTC TAA GGC AGC CAG CAG TGA A-3´
MMP3	5´-AAC CTG TCC CTC CAG AAC CT-3´	5´-GGAAGAGATGGCCAAAATGA-3´
MMP13	5´-CAC CGG CAA AAG CCA CTT-3´	5´-TAGAC TGG TAA TGG CAT CAA GGG A-3´
SOX9	5´-ACA CAC AGC TCA CTC GAC CTT G-3´	5´-AGG GAA TTC TGG TTG GTC CTC T-3´
hGAPDH	5´-ACC CAG AAG ACT GTG GAT GG-3´	5´-TTC TAG ACG GCA GGT CAG GT-3´
hACAN	5´-CTA TAC CCC AGT GGG CAC AT-3´	5´-GGC ACT TCA GTT GCA GAA GG-3´
oGAPDH	5´-CAT CAC TGC CAC CCA GAA GA-3´	5´-CCT GCT TCA CCA CCT TCT TG-3´
oACAN	5´-ACT TCC GCT GGT CAG ATG GA-3´	5´-TCT CGT GCC AGA TCA TCA CC-3´

3.8 Histology and immunofluorescence of fibrin gel embedded cultures

ECM composition was microscopically analyzed with respect to collagens I, II, III and X and GAG expression at the end of coculture time. Therefore, fibrin gels were rinsed with phosphate buffered saline (PBS, PAA, Piscataway/ New Jersey, USA), embedded in TissueTec (Sakura Finetek, Alphen aan den Rijn, the Netherlands), frozen in liquid nitrogen and cross-sectioned (10 µm thick) with a cryo-microtom (Mikrom® HM 500 OM, Walldorf, Germany). For histological evaluation, sections were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis/ Missouri, USA) for 10 minutes at room temperature (RT) and stained with 1% Alcian blue 8GX (Sigma-Aldrich, St. Louis/ Missouri, USA) for GAGs and counterstained with nuclear fast red aluminum sulfate solution (Roth, Arlesheim, Germany). For immunofluorescence analysis, sections were fixed in 4% PFA for 10 minutes at room temperature (RT), treated for 15 minutes at 37°C with pepsin (3 mg/mL in 0.01 M HCl, Sigma-Aldrich, St. Louis/ Missouri, USA) or in case of collagen X with hyaluronidase (0,2% in acetate buffer pH5, Sigma-Aldrich, St. Louis/ Missouri, USA) and were blocked with 3% BSA (Sigma-Aldrich, St. Louis/ Missouri, USA) diluted in PBS for one hour. Sections were labeled over night at 4°C with an antibody against collagen I (C-2456, Sigma-Aldrich, St. Louis/ Missouri, USA), collagen II (CIIC1, DSHB, Iowa City/ Iowa, USA), collagen III (MAB3392, Merck Millipore, Billerica/ Massachusetts, USA) or collagen X (2031501005, Quartett, Berlin, Germany). For fluorescence detection all sections were incubated with an Alexa 488 secondary antibody (Invitrogen, Paisley, UK) for 1 hour at 37°C. Finally, samples were mounted in Vectashield mounting medium with DAPI (1 µg/mL, Vector, Burlingame, Canada) and were analyzed with an Olympus BX 61 imaging system and cell^{P} software (Olympus, Hamburg, Germany). Repetition was in triplicate at least 5 times with cells and explants from different donors and two observers chose representative slides.

3.9 Biochemical analysis of fibrin gel cell lysates

3.9.1 DMMB Assay

Fibrin gels were removed from explants, homogenized and digested with pepsin (1 mg/mL in 0.5 M acetic acid containing 0.4 M NaCl, Sigma-Aldrich, St. Louis/ Missouri, USA) for 48 hours at 4°C and further digested with elastase (1 mg/mL in TBS pH 8, Serva Electrophoresis, Heidelberg, Germany) for 24 hours at 4°C. Samples were stored at -20°C until they were analyzed for GAG and collagen contents. At least seven culture setups (different donors) were analyzed in triplicate.

GAG concentration was measured spectrophotometrically using 25 µl of the digested cell lysates or 25 µl undiluted cell supernatant supplemented with dimethylmethylene blue (DMMB, AppliChem, Darmstadt, Germany) which forms a complex with GAG (Farndale, Buttle et al. 1986). Quantification

was performed in µg per 1 x 10^6 cells with a chondroitin sulfate standard at 525 nm (Tecan GENios with Magellan 6.5, Crailsheim, Germany).

3.9.2 Collagen I and II ELISA

Collagen I and II contents of day 28 cell lysates digested as described above were measured with specific sandwich ELISAs, which recognize the native conformation of collagen I and II chains (Chondrex, Redmond/ Washington, USA) according to the manufacturer´s instructions.

3.9.3 Collagen III Dot-Blot

Human collagen I, II and III protein in digested cell lysates was quantified with a dot blot assay using an aliquot of 1 µl from 500 µl of cell lysate and including a standard curve (range: 100 ng/µl, 50 ng/µl, 25 ng/µl, 12.5 ng/µl, 6.25 ng/µl and 3.125 ng/µl) of recombinant collagen I or collagen II (Chondrex, Redmond/ Washington, USA) or (range: 100 ng/µl, 50 ng/µl, 25 ng/µl, 12.5 ng/µl, 6.25 ng/µl and 3.125 ng/µl) of recombinant collagen III (Abcam, Cambridge, UK). Quantification was performed in µg per 1 x 10^6 cells densitometrically using a Chemi-Smart 500 (PeqLab, Erlangen, Germany) for chemiluminescence detection and the CS4 for Windows software for calculation of luminescence intensities. Repetition was in triplicates at least 5 times with cells and explants from different donors.

3.10 Immunoblotting of Sox9 and phospho Sox9

For immunoblotting of Sox9 or phospho Sox9 (pSox9) total cell proteins were isolated from mono-, co- or tricultures with peqGOLD TriFast (PeqLab, Erlangen, Germany) and total protein content was determined by a bicinchoninic acid (BCA) assay with the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer´s instructions. For Western blotting of pSox9 25 µg of total cell lysate were denaturated with 6x SDS-Buffer containing β-mercaptoethanol for 5 minutes at 95 °C and separated by denaturing sodium dodecyl sulfate polyacrylamide (SDS) gel electrophoresis using 100 V **(Table 4)**. Proteins were blotted to Protran® nitrocellulose transfer membrane for 1 to 2 hours at 120 ampere (Whatman® GE Helthcare, Buckinghamshire, UK) using a tank-blot apparatus (Bio-Rad, Hercules, California, USA). After blocking with 5% non-fat milk for 1 hour the membrane was probed with a specific antibody against pSox9 and β-Actin (ab59252 and ab8227, both abcam, Cambridge, UK) over night at 4°C. After incubation with a peroxidase-coupled goat-anti-mouse secondary antibody (Santa Cruz Biotechnology, Dallas,

Texas, USA) for 1 hour at 37°C **(Table 5)**, bands were visualized using the Pierce ECL Western blotting substrate or the Super Signal Western Femto Maximum Sensitivity Substrate (Thermo Sientific, Waltham, Massachusetts, USA). For detection of total Sox9, the membrane was stripped and probed a second time with a Sox9 specific antibody and β-Actin (AB5535, Millipore, Billerica/ Massachusetts, USA ab8227 abcam, Cambridge, UK) following the same procedure as described above.

Table 4: Buffer concentrations for SDS-PAGE according to Lämmli

Isolated proteins from mono-, co- and tricultures were separated via denaturing SDS gel electrophoresis with a 12.5% SDS-gel. Therefore, 25 µg of total cell lysate were denaturated with 6x SDS-Buffer containing β-mercaptoethanol for 5 minutes at 95°C and loaded into slots of the 12.5% SDS-gel.

Solutions	Concentration of ingredients	Volume	Company
Resolving gel mix	1.5 M Tris/HCl, pH 8.8	1.3 mL	AppliChem, Germany
	10x SDS	50 µL	Roth, Germany
	30% bisacrylamide (Acrylamid)	1.7 mL	Roth, Germany
	1% triethylmethylethylendiamin (TEMED)	5 µL	AppliChem, Germany
	Ammonium-persulfate (0.1 g/mL, APS)	50 µL	Serva Electrophoresis, Germany
	distilled water	1.9 mL	
Stacking gel mix	0.5M Tris/HCl, pH 6.8	625 µL	AppliChem, Germany
	10x SDS	25 µL	Roth, Germany
	30% bisacrylamide (Acrylamid)	325 mL	Roth, Germany
	1% TEMED	3.5 µL	AppliChem, Germany
	APS (0.1 g/mL)	29 µL	Serva Electrophoresis,
	distilled water	1.525 mL	Germany
Sample buffer	0.1 M Tris/HCl, pH 6.8	360.95 µL	AppliChem, Germany
	1% SDS	5 µL	Roth, Germany
	20% glycerol	100 µL	AppliChem, Germany
	5% β-mercaptoethanol	25 µL	Sigma-Aldrich, USA
	0.01% bromphenol blue	0.05 µL	Sigma-Aldrich, USA

Table 5: Antibodies used for immunoblotting

Detection of Sox9 protein level and phosphorylation status in cell lysates of mono-, co- and tricultures, was performed densitometrically using specific antibodies against phospho Sox9, Sox9 or β-Actin. For determination of Sox9 protein activity, Sox9 phosphorylation status was analyzed and the levels of pSox9 were compared with total Sox9.

Antibody	Company	Ref. number
phospho Sox9	Abcam, Cambridge, UK	ab59252
Sox9	Millipore, Billerica, Massachusetts, USA	AB5535
β-Actin	Abcam, Cambridge, UK	ab8227

3.11 Analysis of supernatants (DMMB- und hydroxyproline assay, PTHrP, Fibronectin, bFGF, IL-1β, IL-6 and IL-8 ELISA)

3.11.1 Hydroxyproline assay

The amount of total soluble collagen in culture supernatants was determined by the Total Collagen Hydroxyproline Assay according to the manufacturer´s protocol (QuickZyme, Biosciences, Leiden, the Netherlands). Briefly, 1 mL culture supernatant from mono-, co- and tricultures or cell free explants was harvested after three days and soluble collagens in the supernatant were hydrolyzed into amino acids (12M HCl for 20h at 95°C). Hydroxyproline was stained and color formation was quantified at absorbance maximum at 570 nm (Tecan GENios with Magellan 6.5, Crailsheim, Germany). Repetition was in triplicates at least 5 times with cells and explants from different donors.

3.11.2 PTHrP, fibronectin, bFGF, IL-1ß, IL-6 and IL-8 ELISA

To determine the concentration of specific proteins in the supernatant, human PTHrP ELISA construction kit (Antigenix America Inc., Huntington/ New York, USA), human fibronectin sandwich ELISA kit (Boster Biological Technology Co., LTD, Fremont/ California, USA), human IL-1β sandwich ELISA kit (RayBiotech, Inc., Norcross/ Georgia, USA), IL-6 sandwich ELISA kit (R&D Systems, Minneapolis/ Minnesota, USA), IL-8 ELISA kit (Gen-Probe, Bedford/ Massachusetts, USA) and bFGF sandwich ELISA kit (R&D, Bedford/ Massachusetts, USA) were used according to the manufacturer´s instructions. Repetition was in triplicates at least 6 times for BMSC, mixed cultures and chondrocytes cocultured with bone explants from different OA donors and at least 4 times for cell-free OA- and normal bone explants as well as for ASC cultures.

3.11.3 DMMB assay

GAG concentration was measured spectrophotometrically using 25 µl of the digested cell lysates or undiluted cell supernatant supplemented with dimethylmethylene blue (DMMB, AppliChem, Darmstadt, Germany) which forms a complex with GAG. Quantification was performed in µg per 1 x 10^6 cells with a chondroitin sulfate standard at an absorption maximum at 525 nm (Tecan GENios with Magellan 6.5, Crailsheim, Germany).

3.12 Screening for differentially expressed factors in coculture supernatant with LC-MS

In order to identify differentially expressed proteins in mono- and coculture with OA-cartilage, a SDS-PAGE/LC-MS approach was performed in cooperation with Prof. Dr. R. Deutzmann (Department of Biochemistry I, University of Regensburg) as described previously (Hamperl, Brown et al. 2014). Supernatants of BMSC mono- and cocultures with OA-cartilage explants and of cartilage explants with cell-free fibrin gel were precipitated and separated by SDS-PAGE. The gel was stained with Coomassie Blue and the pattern of protein bands between lines with proteins from different coculture setups was compared. For analysis, differentially expressed bands were identified and either single bands or whole lines were cut out. Lanes of the different culture setups were divided in three different areas (high > 70kd, medium 70-35kd and low < 35kd). Proteins were isolated, digested with trypsin and identified by MALDI-TOF mass spectrometry using standard protocols. Briefly, LC-MS analyses were operated in positive ion reflector mode on an Applied Biosystems 4700 or 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer and evaluated by searching the NCBI protein sequence database with the Mascot search engine (Matrix Science) implemented in the GPS Explorer software (Applied Biosystems, Darmstadt, Germany). Intensity of the laser was adjusted due to sample concentration and laser condition. This approach was performed in collaboration with the AG from Prof. Deutzmann (Inst. Biochemistry, Microbiology and Genetics; University of Regensburg).

3.13 Biomechanical testing

Fibrin gel constructs were removed from respective explants and mono- co- and tricultures were adapted to pieces of same size by punching out constructs with an outer diameter of 2.6 mm (Biopsy punch, Stiefel GmbH, Munich, Germany). All biomechanical tests were carried out in a standard material testing machine (Z010; Zwick GmbH, Ulm, Germany) using a 40N load cell **(Fig. 9).** The initial sample height (h_0) was measured under a preload of 0.1N using a laser displacement transducer (optoNCDT 2200-20, Micro-Epsilon GmbH & Co. KG, 0.3 µm resolution, ±0.03% accuracy).

An unconfined compression test was performed by placing the samples in a cell culture dish filled with 0.9% NaCl and loading it by a flat ended cylinder at a strain rate of 100% h_0/minutes until 50% strain was reached. In order to measure the stiffness of the new generated ECM in our constructs, the Young´s modulus was graphically determined from the related stress-strain diagrams. Therefore, two typical regions were evaluated: the progressive region at 0-10% strain and the linear region at 40-50% strain.

After adequate relaxation time of 24 hours an additional relaxation test was performed under confined compression conditions. The samples were placed in a confining chamber of 2.6 mm diameter filled with 0.9% NaCl and loaded by a flat ended porous ceramic cylinder (Al_2O_3) allowing for fluid flow. After application of 50% strain at a loading rate of 100% h_0/minutes the strain was held constant for 10 minutes until the equilibrium state was reached.

Based on this data hydraulic permeability (k) was calculated referred to a given diffusion equation (Mow, Kuei et al. 1980; Frank and Hochmuth 1987) using Formula 1 and the aggregate modulus (HA) at equilibrium state with 50% strain was assessed using Formula 2 considered that $\Delta l/h_0$ is the applied strain, H the modulus, σ the stress and σ_∞ the stress at equilibrium state with 50% strain. Repetition was for 4 times with cells and explants from different donors.

Formula 1 $$\sigma_t = \sigma_\infty + 2\,H * \frac{\Delta l}{h_0} * e^{\left(\left(\frac{\pi}{h_0}\right)^2 * H*K*t\right)}$$

Formula 2 $$H_A = \frac{\sigma_\infty}{e_{50\%}}$$

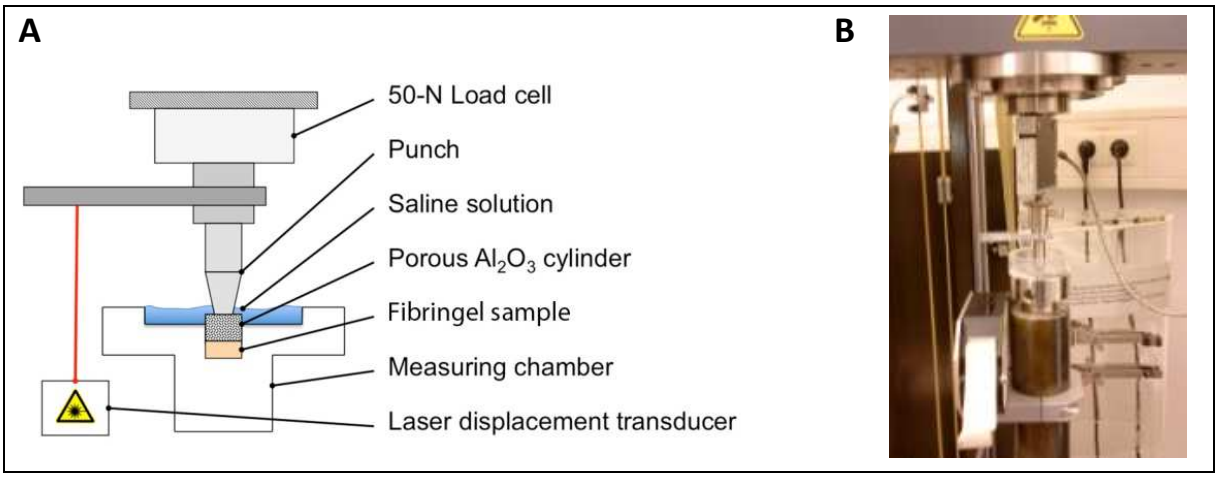


Figure 9: Setup for biomechanical tests
The fibrin gel samples were tested in a standard material testing machine for their biomechanical properties (A and B). The initial height was measured under a preload of 0.1 N using a laser displacement transducer (unpublished Figure provided by Andreas Seitz, University of Ulm).

3.14 Statistical analysis

All results were calculated for an initial cell number of 1 x 10^6 cells. The mean and SD values were calculated for all variants. The nonparametric Wilcoxon test (for paired analyses) or the Mann-Whitney test (unpaired analyses) was applied to analyze differences between time points and between culture conditions. All experiments were performed in triplicates and repeated at least four times with cells from different donors. $P < 0.05$ values were considered to indicate statistically significant differences. Due to the limited sample number provided for the biomechanical tests, these data were analyzed descriptively. Data analysis and graphing were performed with GraphPad for Windows version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

4 Results

4.1 Part I: Microenvironment of cartilage influences chondrogenic differentiation and ECM production of BMSC and chondrocytes

4.1.1 Bone marrow derived BMSC can be differentiated into different lineages

To prove stemness of isolated plastic adherent bone marrow cells **(see 3.2.)** the expression of a selection of surface markers was analyzed by FACS. BMSC showed a typical expression pattern of characteristic surface markers and were shown to be positive for the expression of CD44 and CD105 ($CD44^{pos}$ and $CD105^{pos}$) and negative for the expression of CD19 and CD34 ($CD19^{neg}$ and $CD34^{neg}$) **(Fig. 10A).** A further criteria for human BMSC is that they are capable to differentiate into osteogenic and adipogenic lineages additionally to chondrogenic lineage (Dominici, Le Blanc et al. 2006). All of the tested BMSC specimens were shown to reveal adipogenic differentiation, proofed by numerous oil-red stained oil drops in differentiated cells and osteogenic differentiation, which was confirmed by alizarin staining of mineralized bone matrix **(Fig. 10B).**

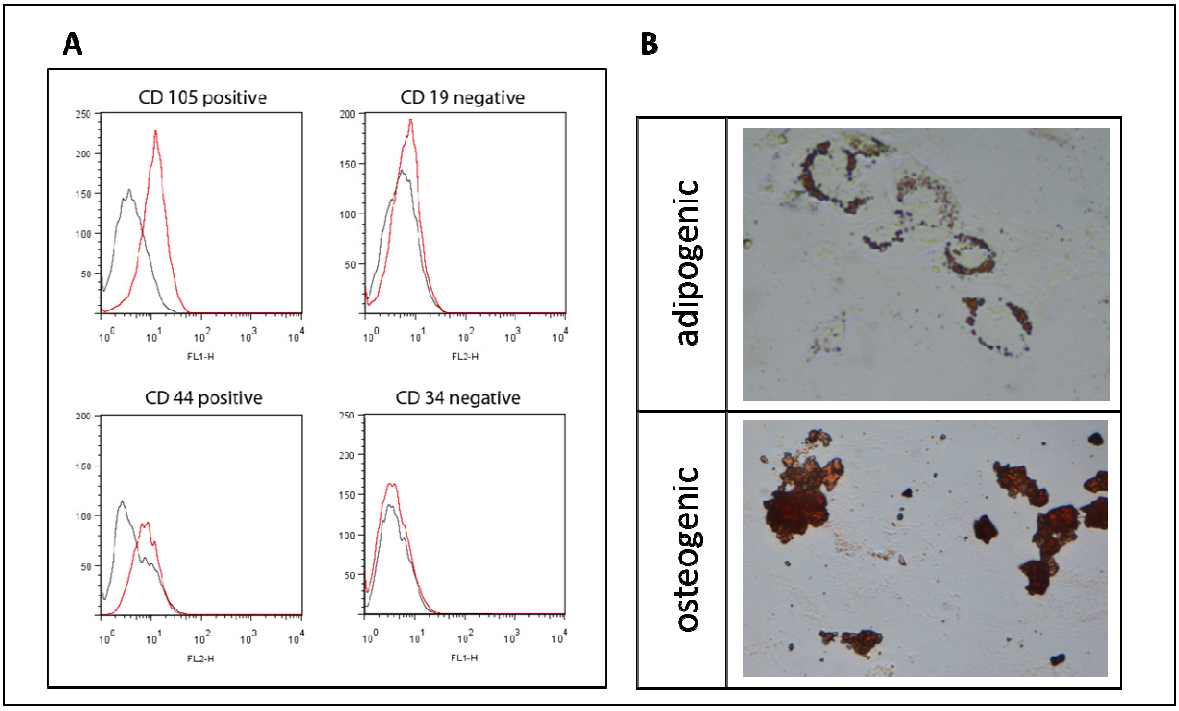


Figure 10: FACS analysis of BMSC surface markers and staining of adipogenic and osteogenic differentiation
(A) Isolated plastic adherent BMSC in passage 3 were analyzed by flow cytometry using specific antibodies against BMSC surface markers $CD19^{neg}$, $CD34^{neg}$, $CD44^{pos}$ and $CD105^{pos}$ (red line). Specificity of epitope staining was proofed using an isotype control (black line). (B) BMSC were stained with oil red for oil drops incorporated during adipogenic differentiation (upper row) and with alizarin red for matrix mineralization during osteogenic differentiation (lower row).

4.1.2 Cells embedded in fibrin gel co- or tricultured with OA-cartilage remain vital and proliferative for up to 28 days

In order to evaluate if the fibrin gel system affects the vitality of cells in our different culture setups, LDH concentration in culture supernatants was analyzed and compared with respective monolayer controls.

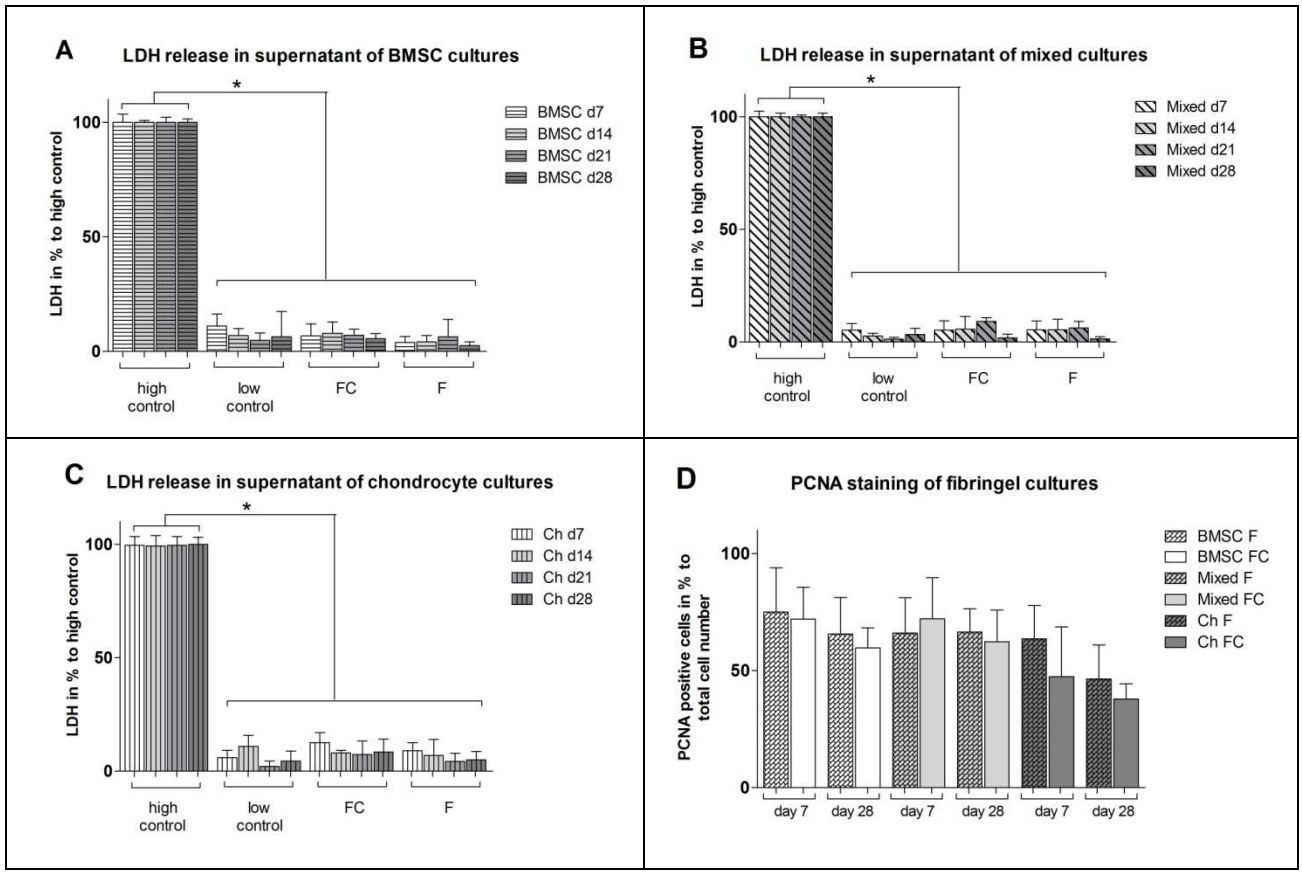


Figure 11: Vitality and percentage of proliferating cell nuclear antigen (PCNA) positive cells of mono-, co-, and triculture setups

Vitality of (A) BMSC, (B) mixed cultures (BMSC and chondrocytes in equal ratio) and (C) chondrocytes (Ch) was determined in monocultures (F) and cocultures with articular OA-cartilage (FC) kept in chondrogenic medium. Content of LDH was quantified in the supernatant of days 7, 14, 21 and 28 and compared with controls (high control = all cells in a fibrin gel were lysed; low control = spontaneous cell death of an equivalent cell amount in monolayer). Due to high inter-experimental variability, the raw data was calculated as percent of control per individual experiment. (D) Mitotic activity of BMSC (white bars), mixed cultures (light grey bars) and chondrocytes (dark grey bars) was determined in monocultures (F) and cocultures with articular OA-cartilage (FC) kept in chondrogenic medium. PCNA positive stained cell nuclei were counted at days 7 and 28 and percentage of positive cells to total cell number was calculated. Results are presented as mean with standard deviation (SD). * $p<0.05$; (A-C): n=4; (D): n=5;

The cytotoxicity detection test demonstrated that chondrocytes, BMSC and mixed cultures embedded in a fibrin gel cocultured or kept as monocultures showed no enhanced LDH release compared with assay controls. Consequently, cells were assumed – despite to their normal death rate during chondrogenesis – to be not influenced in their vitality or overall metabolic activity by fibrin gel components or OA-cartilage explants during a culture time period of 28 days **(Fig. 11A-C)**. Cartilage explants cultured without cells embedded in fibrin gel showed no enhanced LDH release compared with assay controls and revealed a LDH level somewhat lower than respective co- or tricultures (data not shown). Fibrin gel embedded OA-cartilage cocultures were processed, stained with PCNA and counterstained with haematoxylin in order to determine if cells proliferate. In all culture conditions, positive PCNA staining was detected - indicating mitotic activity. Evaluation of PCNA stained cell nuclei revealed neither significant differences in mitotic activity of cells placed onto cartilage explants compared with monocultured controls during the entire culture period nor of cells on day 7 compared with day 28 **(Fig. 11D)**.

Coculture conditions with cartilage explants and fibrin gel components did not affect mitotic activity or vitality of BMSC, mixed cultures or chondrocytes.

4.1.3 mRNA expression of differentiation/dedifferentiation markers is altered in cartilage cocultures

To determine whether OA-cartilage explants affect gene expression of ECM components in BMSC and chondrocytes, mRNA expression of *COL1A1* (de-differentiation marker), *COL2A1* (chondrogenic differentiation marker), *COL3A1* (mesenchymal cell marker) and *COL10A1* (hypertrophic chondrocyte marker) were analyzed at days 7 and 28 by using quantitative PCR.

At day 7, a significant inhibition of *COL1A1* gene expression in all cocultured regimens in comparison with monocultures was observed. An increase of *COL1A1* gene expression was detected in mixed and chondrocyte cocultures from day 7 to day 28 **(Fig. 12A).** *COL2A1* gene expression was significantly decreased on day 28 in BMSC cocultures and on day 7 in mixed tri- and chondrocyte cocultures compared with monocultures. In BMSC mono- and cocultures a significant upregulation of *COL2A1* gene expression from day 7 to day 28 was detected, suggesting induction of chondrogenic differentiation. *COL2A1* gene expression was significantly decreased in chondrocyte cocultures from day 7 to day 28 **(Fig. 12B).** *COL3A1* gene expression was significantly reduced in all three coculture conditions at day 7 and in mixed tricultures also at day 28. A significant downregulation of *COL3A1* gene expression from day 7 to day 28 was detected in chondrocyte monocultures **(Fig. 12C).** *COL10A1* gene expression was significantly inhibited in cocultures of BMSC and chondrocytes at day

7 and at day 28 in BMSC and mixed co- and tricultures. In BMSC and mixed mono- and cocultures, a significant upregulation of *COL10A1* gene expression from day 7 to 28 was observed **(Figure 12D)**.

In general, collagen gene expression of BMSC, mixed and chondrocytes co- and tricultures with OA-articular cartilage was significantly reduced compared with monocultures.

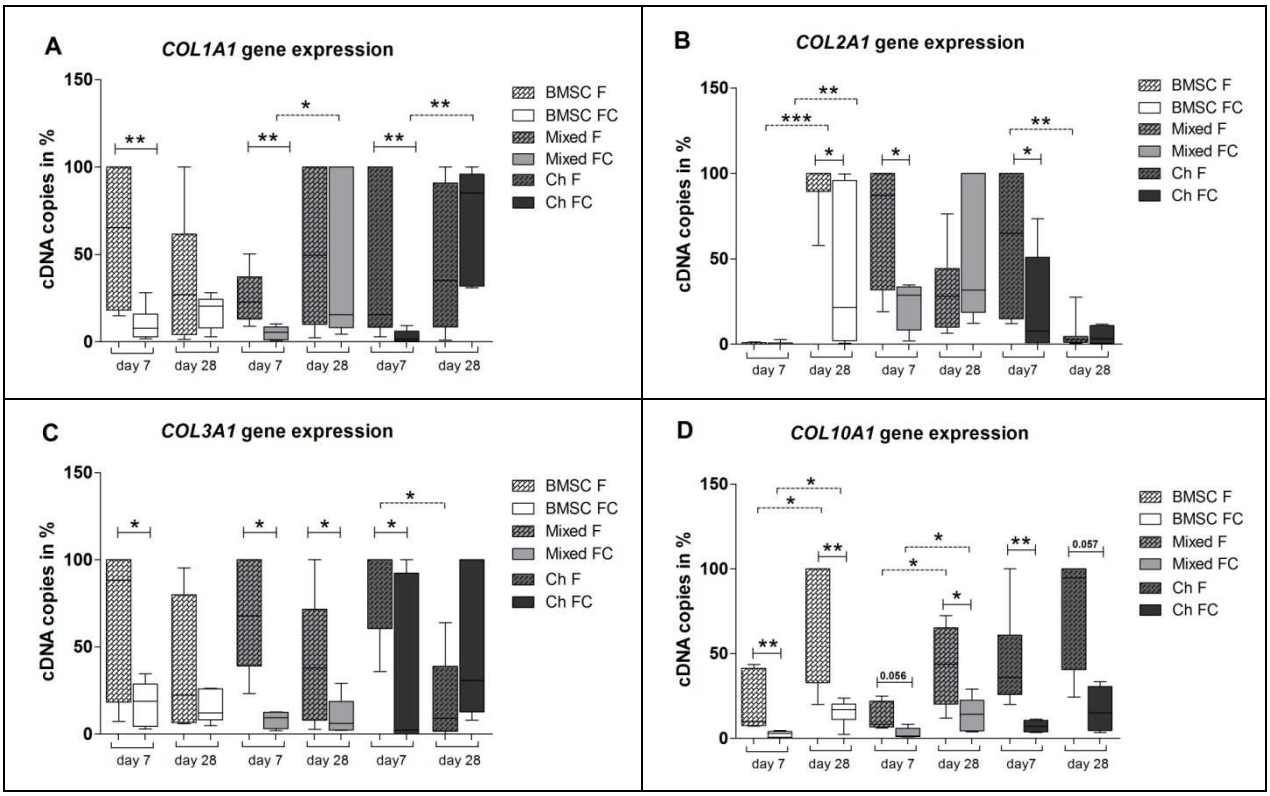


Figure 12: Quantification of gene expression ratio with qPCR
Gene expression levels of (A) *COL1A1,* (B) *COL2A1,* (C) *COL3A1* and (D) *COL10A1* were determined in mono-, co- and tricultures by using standard curves. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (Ch, dark grey bars) were kept as monocultures (F, bars with pattern) or as co- and tricultures with OA-cartilage explants (FC, solid bars) in chondrogenic medium. Due to high inter-experimental variability, the raw data was calculated as percentage of highest cDNA copy number per individual experiment. Results are presented as mean with SD. * p<0.05; ** p < 0.01; ***p<0.001; n=6;

4.1.4 Microenvironment of cartilage alters immunofluorescent staining pattern of collagens

After seeding, the fibrin gels were clear and had a soft consistence. During chondrogenic differentiation, production and deposition of ECM was observed in the fibrin gels, by a change of their color from clear to milky opaque. Additionally, fibrin gels kept their lens-shape during the entire culture period and no shrinking of the construct was observed **(Fig.13)**.

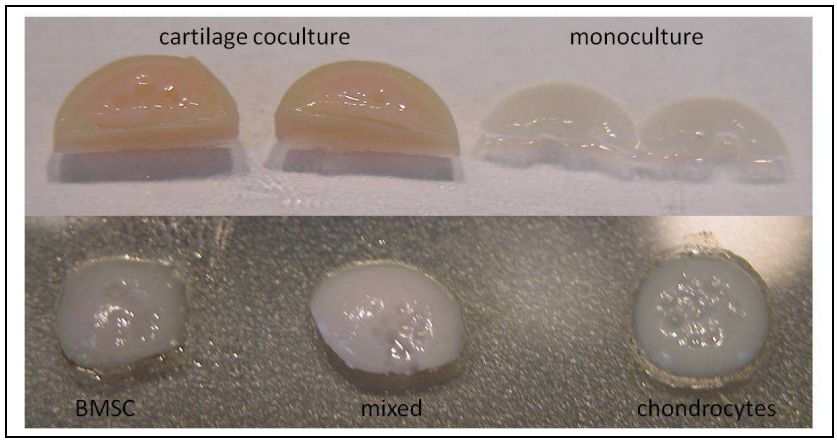


Figure 13: Representative specimen of different culture conditions
After 28 days of chondrogenic differentiation in both culture setups ECM deposition was visible and no shrinking of fibrin gels was observed. In the upper part a representative profile of a cartilage coculture and a monoculture fibrin gel is shown. In the lower part representative fibrin gel monocultures of BMSC, mixed cultures and chondrocytes were photographed.

The collagen production and ECM deposition of BMSC, mixed and chondrocytes in mono-, co- and triculture conditions was evaluated by immunofluorescent staining of cryosections at day 28. As shown in **figure 9A** monocultured BMSC and mixed cultures embedded in fibrin gel deposited a higher amount of collagen I into their ECM compared with co- and tricultures with articular cartilage explants. Chondrocytes revealed only little collagen I staining in both culture conditions **(Fig. 14A).** The presence of collagen II was demonstrated in BMSC monocultures but no signals were detected in cocultures of BMSC with OA-cartilage. The mixed cultures showed similar collagen II staining in both culture conditions. Chondrocyte monocultures exhibited a strong collagen II signal in their ECM comparable to BMSC monocultures with somewhat reduced staining in cocultures **(Fig. 14B)**. Collagen III deposition in BMSC and chondrocytes was modest and remained unchanged by coculture with cartilage. In contrast, monocultures of mixed cells revealed a stronger collagen III signal compared with tricultures **(Fig. 14C)**. Deposition of collagen X was clearly enhanced in BMSC monocultures in comparison with cartilage cocultures. Interestingly, there was only poor collagen X staining in chondrocyte and mixed cultures and there was no visible difference between mono-, co- and tricultures **(Fig. 14D)**. Alcian blue staining was used to detect proteoglycan (aggrecan) deposition. At day 28 all culture conditions were stained blue and exhibited no differences between culture regimens. Additionally, the fibrin gels were equally stained and no gradient originating from the cartilage explants was observed **(Fig. 14E)**.

Immunofluorescent staining of collagens I, II, III and X revealed in general an inhibition of collagen production in cartilage co- and tricultures.

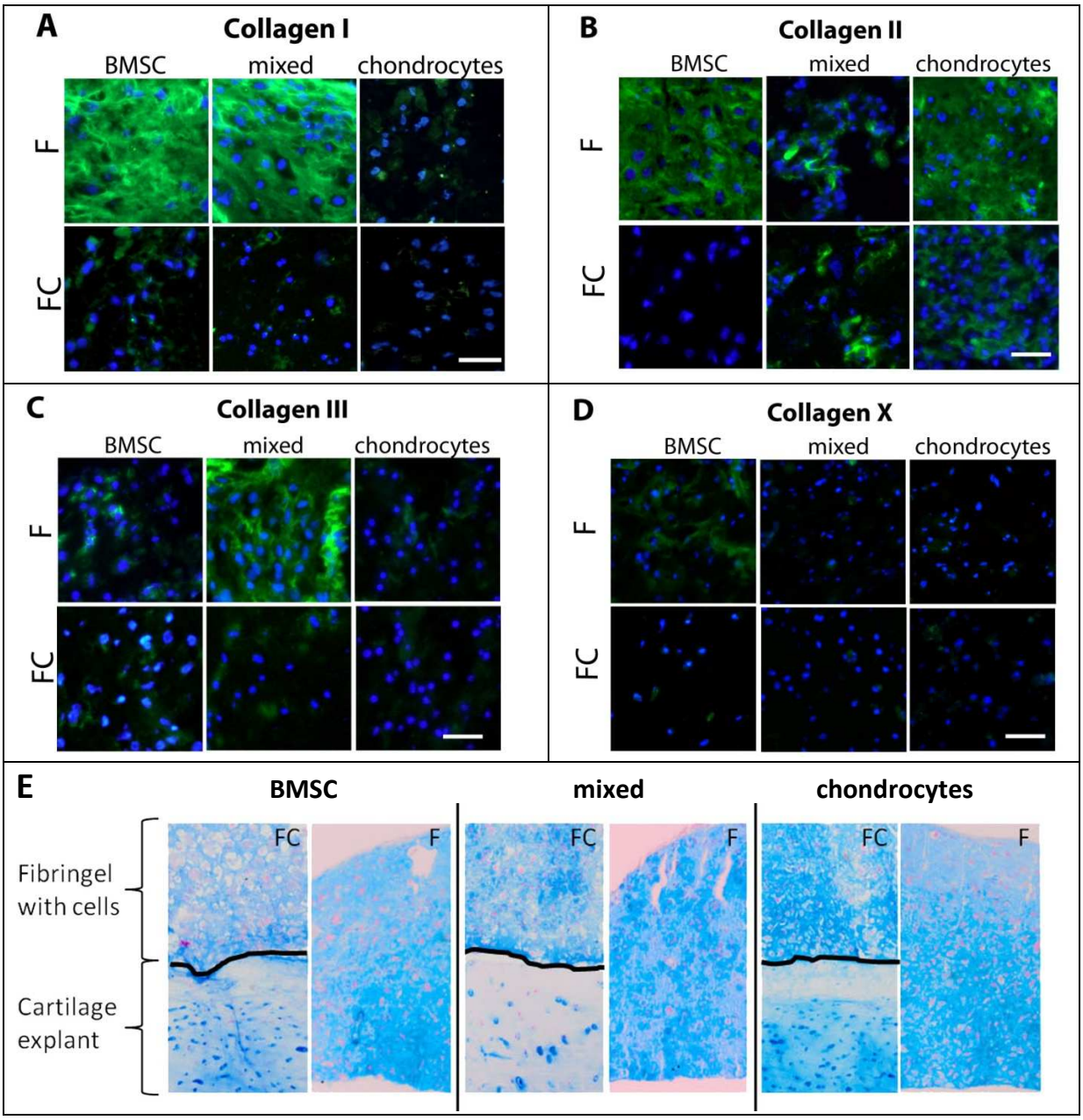


Figure 14: Immunofluorescent staining of collagens and histological staining of GAGs
Representative overview of immunofluorescence staining images of collagens I (A), II (B), III (C), X (D) and alcian blue staining of GAGs (E) of cryosections after 28 days of culture. BMSC, mixed cultures (BMSC and chondrocytes in equal ratio) and chondrocytes were monocultured (F), co- and tricultured (FC) with OA-cartilage. (A-D): Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue color). Scale bar is 100 µm. n=5

4.1.5 Collagen I, II and III and GAG contents in fibrin gel cell lysates of mono-, co- and tricultures

In further experiments, the influence of cartilage explants on collagen and proteoglycan synthesis or degradation, respectively, was studied. Therefore, fibrin gels were separated from cartilage explants and were digested with pepsin and elastase. A collagen I specific ELISA revealed significantly reduced

collagen protein content for BMSC and mixed co- and tricultures in comparison with monocultures. Collagen I protein was hardly present in chondrocyte mono- and cocultures **(Fig. 15A)**. A collagen II specific ELISA revealed that collagen II synthesis in BMSC was clearly inhibited by cartilage explant coculture in comparison with monoculture. For mixed tricultures and chondrocyte cocultures, no significant differences between both culture conditions were detectable **(Fig. 15B)**. Dot-blot analysis of collagen III protein content demonstrated a significant reduction in mixed tricultures, whereas no significant differences were detectable in BMSC or chondrocyte cocultures versus monocultures **(Fig. 15C)**. In contrast to the collagenous ECM components, GAG content of BMSC, mixed culture and chondrocyte cell lysates was not affected by coculture with OA-cartilage explants **(Fig. 15D)**.

The generally diminished collagen production and deposition in the fibrin gels was confirmed on protein level while an inhibitory effect for GAGs was excluded.

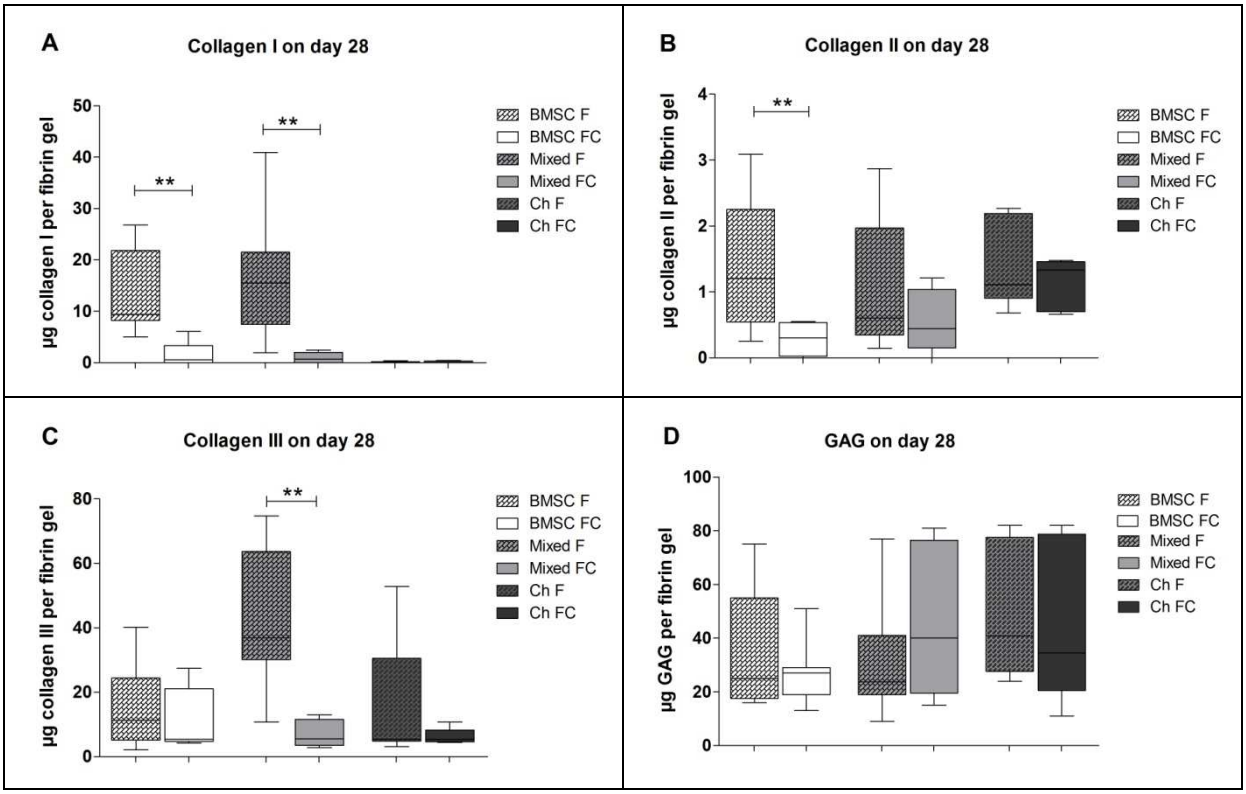


Figure 15: Quantification of collagens I, II, III and GAGs in fibrin gel cell lysates
Protein level of collagens I (A), II (B) and III (C) and GAGs (D) in cell lysates after 28 days of culture. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (Ch, dark grey bars) were kept as monocultures (F, bars with pattern) or as co- and tricultures with OA-cartilage explants (FC, blank bars) in chondrogenic medium. (A, B) Collagens I and II were quantified with ELISA. (C) Collagen III was evaluated densitometrically by dot-blot analysis containing a recombinant collagen III standard curve. (D) GAG concentration in fibrin gel lysates was quantified by a dimethylmethylene blue (DMMB)-assay including a chondroitin sulfate standard curve. Results are presented as mean with SD. ** $p < 0.01$; n=7

4.1.6 Sox9 is not influenced by OA-cartilage

To determine whether OA-cartilage explants affect expression of the chondrogenic differentiation marker Sox9, which is an important transcription factor during chondrogenesis, protein expression, phosphorylation status and mRNA expression of human Sox9 was analyzed in mono-, co- and tricultures with OA-cartilage at days 7 and 28. In addition, ovine *SOX9* mRNA status of normal ovine cells in mono-, co- and tricultures with normal articular cartilage was examined.

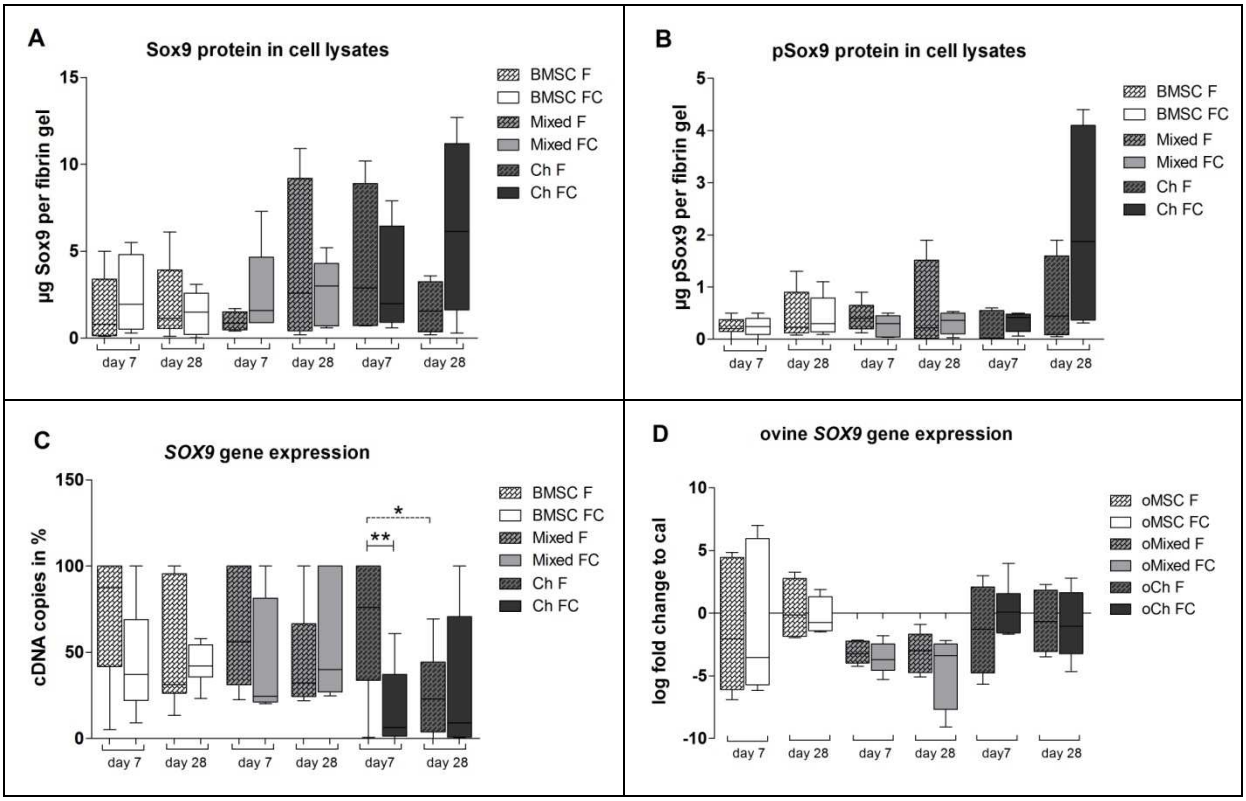


Figure 16: Sox9 pattern of fibrin gel cocultures with articular cartilage

Protein content of Sox9 (A) or phospho Sox9 (B) was quantified densitometrically after immunoblotting of total cell lysates. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (Ch, dark grey bars) were kept as monocultures (F, bars with pattern) or as cocultures with OA-cartilage explants (FC, solid bars) in chondrogenic medium. Gene expression level of human *SOX9* (C) was determined in mono-, co- and tricultures of human BMSC, chondrocytes (Ch) and mixed cultures (Mixed) with OA-cartilage explants by absolute cDNA quantification using a plasmid standard curve. Due to high inter-experimental variability the raw data was calculated as percentage of highest cDNA copy number per individual experiment. Gene expression of ovine *SOX9* (D) was determined in mono-, co- and tricultures of ovine BMSC (oBMSC), ovine chondrocytes (oCh) and mixed cultures (oBMSC and oCh in equal ratio) with normal ovine articular cartilage explants by relative cDNA quantification. Gene expression is shown as log fold change to a calibrator, which was gene expression of oBMSC monolayer cells (in case of oBMSC and oMixed mono-, co- and tricultures) or gene expression of oCh monolayer cells (in case of oCh mono-and cocultures). Raw data was calculated with the ddCT method and is shown as log fold change to calibrator which is represented by the zero-line. Results are presented as mean with SD. * $p<0.05$; ** $p < 0.01$; n=5

No significant differences in total Sox9 protein or phospho Sox9 protein expression in all coculture regimens compared with monocultures were observed **(Fig. 16A and B)**. Moreover, no differences in *SOX9* gene expression of BMSC and mixed culture conditions was found, but a significant upregulation of *SOX9* gene expression in chondrocyte monocultures in comparison with cartilage cocultures on day 7. *SOX9* gene expression in chondrocyte monocultures was significantly downregulated from day 7 to day 28 **(Fig. 16C)**. *SOX9* gene expression of normal oBMSC, oMixed and oCh mono-, co- or tricultures with normal ovine cartilage revealed no differences between culture conditions **(Fig. 16D)**.

In general, neither coculture with OA-cartilage nor with normal ovine cartilage had an effect on Sox9 gene or protein expression or phosphorylation status.

4.1.7 Comparison of soluble ECM fragments, PTHrP, bFGF and cytokines in supernatants of mono-, co- and tricultures

To quantify pro-inflammatory cytokines released from cell-free cartilage explants into culture supernatants, ELISAs for IL-1β, IL-6 and IL-8 were performed. IL-1β concentration in supernatants of normal and OA-cartilage explants cultured without cells showed no differences. Additionally, no temporary differences were detectable for cell-free normal cartilage explants, whereas cell-free OA-cartilage explants showed a significant increase of IL-1ß level from day 7 (1.1pg/mL +/- 0.7) to day 28 (3.0pg +/- 0.7) **(Fig. 17A)**. IL-6 was only detected at day 7 in supernatant of cell-free normal cartilage (103pg +/-292), whereas it was not detectable at day 28 or in cell-free OA-cartilage explants at days 7 and 28 **(Fig. 17B)**. Concentration of IL-8 in supernatant of normal cell-free cartilage explants was significantly higher at day 7 (4347pg/mL +/-1604) compared with day 28 (527pg +/-667). Similarly, concentration of IL-8 in supernatants of cell-free OA-cartilage explants was significantly higher at day 7 (2905pg/mL +/-1953) and was not detectable at day 28. Additionally, IL-8 concentration in supernatant of normal cartilage explants was significantly higher than in OA-explants at day 28 **(Fig. 17C)**. In cell-free normal and OA-cartilage explant supernatants soluble GAG concentration (43µg/mL +/- 23) was comparable to soluble GAG measured in coculture supernatants **(Fig. 17D)**.

ELISAs were performed to quantify the representative pro-inflammatory cytokines IL-1β, IL-6 and IL-8, which are released into culture supernatants of mono-, co- and tricultures. Results revealed that BMSC (day 28), mixed cultures (days 7 and 28) and chondrocytes (day 28) cultured with cartilage explants secrete significant higher IL-1β levels at day 28 than monocultures. Monocultured chondrocytes significantly reduced release of IL-1β from day 7 to day 28 **(Fig. 18A)**. Furthermore, cocultured BMSC, mixed cultures and chondrocytes had significantly elevated IL-6 and IL-8 levels at

day 7 compared with monocultures. Over the culture time, IL-6 and IL-8 levels decreased significantly from day 7 to day 28 in supernatants of BMSC, mixed and chondrocyte co- and tricultures and also of chondrocyte monocultures **(Fig. 18B and C)**. The concentration of bFGF revealed no differences between BMSC culture conditions, but showed a significant upregulation in mixed tricultures and a trend towards upregulation (p=0.057) in chondrocyte cocultures **(Fig. 18D)**. However, a significant upregulation of PTHrP in all culture regimens from day 7 to day 28 was detectable **(Fig. 18E)**. In contrast, the concentration of fibronectin fragments **(Fig. 18F)** and total soluble collagens **(Fig. 18H)** in culture supernatants were not altered in the presence of cartilage explants. Only a time dependent decrease of fibronectin was observed for OA-cartilage cocultured BMSC and mixed cultures from day 7 to day 28 **(Fig. 18F)** as well as a time dependent increase of hydroxyproline in cocultured BMSC **(Fig. 18H)**. Notably, soluble GAG content of supernatants in all co- and triculture conditions was significantly elevated in comparison with monocultures at days 7 and 28 **(Fig. 18G)**. IL-10 and TNF-alpha was also quantified at days 7 and 28 in supernatants of mono-, co- and tricultures, but their concentration was close to the ELISA detection limit, which prevented detection of putative differences between mono-, co- and tricultures (data not shown). No differences in PTHrP concentration between culture conditions of all cell types were observed.

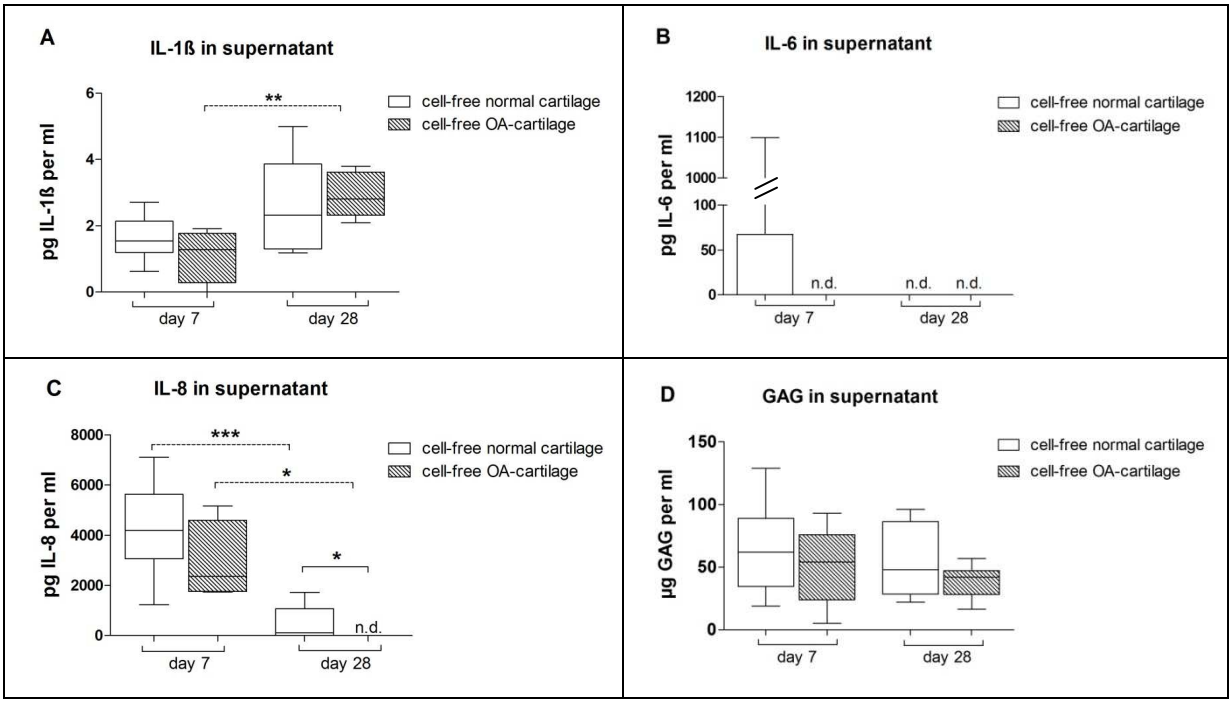


Figure 17: Analysis of cell-free OA- and normal cartilage supernatants
Supernatants of normal (blank bars) and OA- (striped bars) cartilage explants cultured without cells were analyzed at days 7 and 28. Total amount of cytokines (A) IL-1β, (B) IL-6 and (C) IL-8 released into the supernatant were quantified by antigen-specific ELISAs. (D) Soluble GAG in the supernatant was determined by a DMMB-assay. Results are presented as mean with SD. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; n= 6

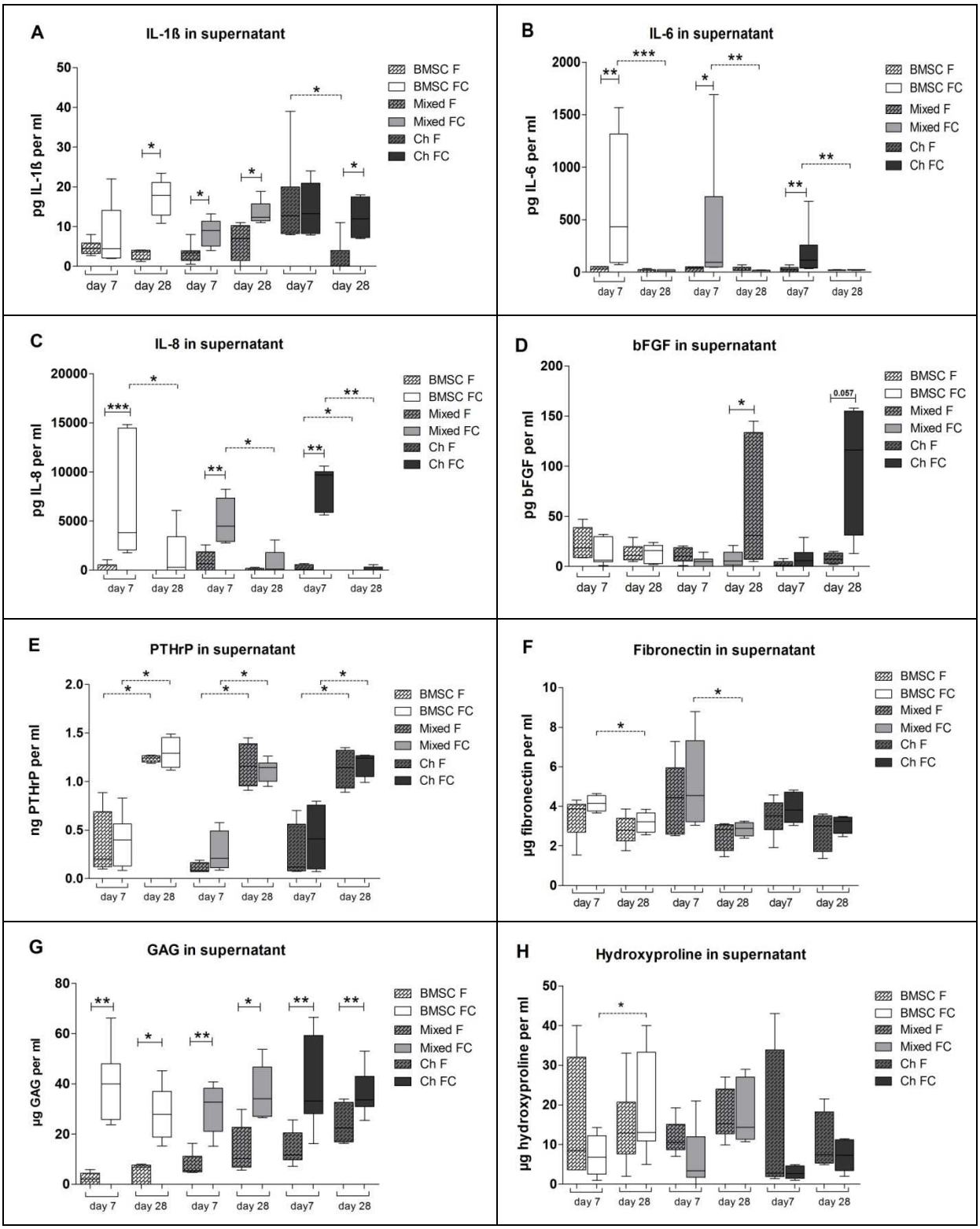


Figure 18: Analysis of culture supernatants

Supernatants of BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) monocultured (F, bars with pattern), co- or tricultured with OA-cartilage (FC, blank bars) were analyzed at days 7 and 28. Total amount of cytokines (A) IL-1β, (B) IL-6 and (C) IL-8 as well as of (D) PTHrP, (E) bFGF and (F) fibronectin released into the supernatant were quantified by antigen-specific ELISAs. (G) Soluble GAG in the supernatant was determined by a DMMB-assay and (H) total soluble collagen in the supernatant was determined by using a hydroxyproline assay. Results are presented as mean with SD. * $p<0.05$; ** $p < 0.01$; *** $p<0.001$; n= 6

Concentrations of pro-inflammatory cytokines like IL-1β, IL-6 and IL-8 and soluble GAG in supernatants of co- and tricultures were higher than in supernatants of monocultures. In contrast, concentrations of IL-10 and TNF-alpha were not detectable and hydroxyproline, fibronectin and PTHrP concentrations were similar in mono-, co- and tricultures.

4.1.8 LC-MS analysis revealed differentially expressed factors in BMSC cartilage coculture supernatants

A SDS-PAGE approach was performed in order to identify differentially expressed proteins in BMSC OA-cartilage coculture compared with monocultures and cell-free cartilage explants **(Fig. 19)**. Focus was on differentially expressed single bands or on whole lines of the different culture setups (BMSC monocultures, BMSC cocultures and cell-free OA-cartilage explants with fibrin) divided in three different areas (high molecular, medium and low). This work was performed in cooperation with AG Prof. Deutzmann were the subsequent MALDI-TOF based identification of selected CooBlue stained bands have revealed several differentially secreted proteins in mono- versus cocultures (see **table 7)**. Some of these proteins are known to be involved in cartilage and OA regulation and part of these data are a base for further experiments and assays.

Table 7: Factors identified by LC-SM analysis of supernatant

List of differentially secreted proteins in supernatants of BMSC coculture with OA-cartilage compared with BMSC monocultures and cell-free OA cartilage. Proteins were identified subsequently by a SDS-PAGE followed by LC-MS. Scores > 100 were considered significant.

Protein
Ribonuclease 4
MMP1
Prolargin
Serum Amyloid
Lysozym C
Titin
Extracellular superoxid dismutase
Chondroadherin
Fibronectin
IL-8
Stromelysin (MMP3)

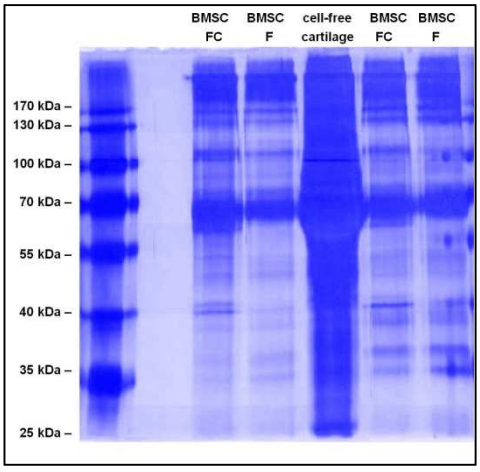


Figure 19: Representative SDS gel

Proteins in the supernatants of BMSC monocultures (F), cocultures with OA-cartilage (FC) or cartilage explants with cell-free fibrin gel were precipitated and separated by SDS-PAGE. The SDS gel was stained with Coomassie Blue and the pattern of protein bands between lines with proteins from different coculture setups was compared.

4.1.9 Microenvironment of OA-cartilage alters biomechanical properties of new extracellular matrix

Mechanical property and load capacity of newly generated ECM was determined. Unconfined mechanical testing indicated that BMSC, chondrocytes or a mixture of both cell types, cultured together with OA-cartilage, exhibited a decrease in Young´s modulus (0 to 10% strain) in 3 out of 4 samples of each cell type **(Fig. 20A)** and a decrease in all samples (BMSC) or 3 out of 4 samples (mixed cultures) at 40 to 50% strain. In cocultured chondrocytes, Young´s modulus under 40 to 50% strain tended to be higher in 3 out of 4 samples **(Fig. 20B)**. Aggregate modulus at equilibrium was lowered in almost any case for all co- and triculture samples **(Fig. 20C)**. Hydraulic permeability of all cartilage co- and tricultures was increased in 3 out of 4 samples in comparison with monocultures **(Fig. 20D)**.

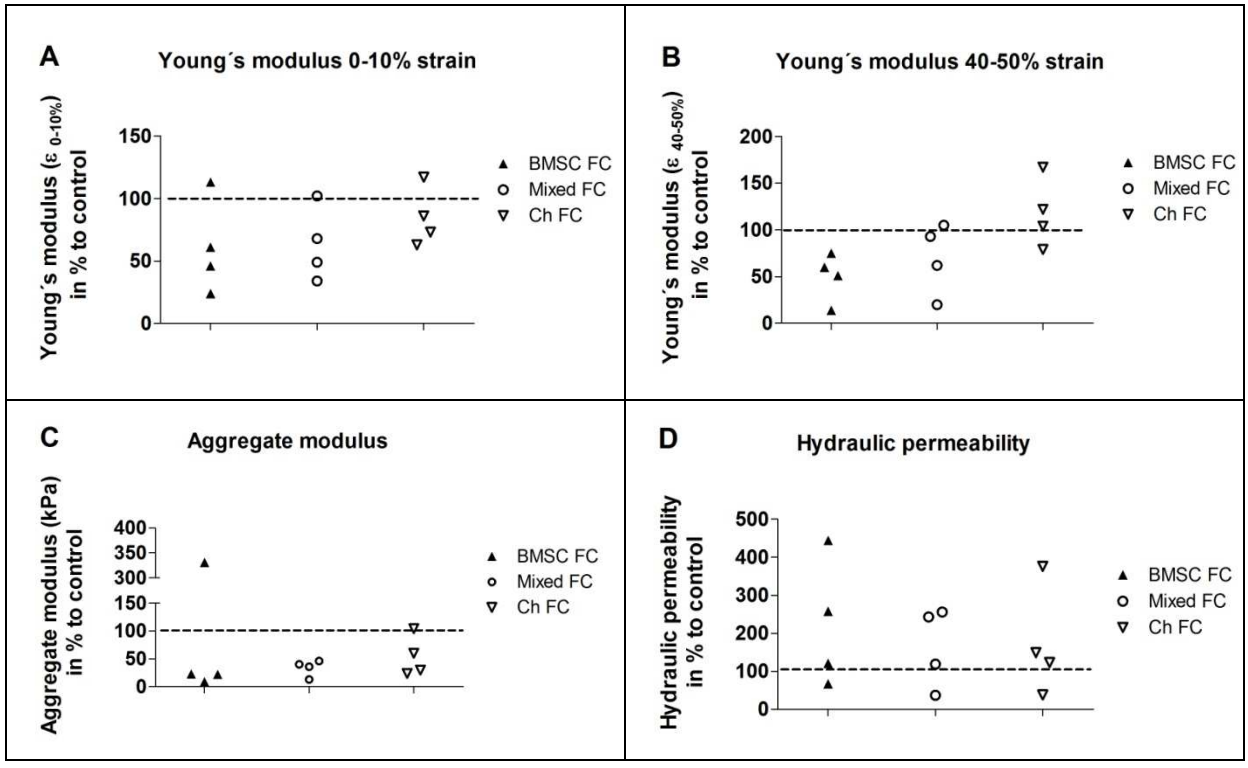


Figure 20: Determination of biomechanical properties

Biomechanical properties of the newly formed ECM at day 28 of BMSC, mixed cultures or chondrocytes (Ch) co- and tricultured with OA-cartilage (FC) were analyzed and calculated in relation to monocultured controls (dotted lines at 100% represent monoculture values). Young´s modulus of (A) 0-10% and (B) 40-50% strain was determined by using unconfined compression. (C) Aggregate modulus at equilibrium and hydraulic permeability (D) were determined by using confined compression performed at 50% compressive strain. n=4

Overall, biomechanical tests showed that newly formed ECM in BMSC and mixed co- or tricultures had lower Young´s and aggregate modulus, and increased hydraulic permeability compared with monocultures. Mechanical parameters in chondrocyte cocultures were lower in case of Young´s modulus (0-10%) and aggregate modulus and higher in case of Young´s modulus (40-50%) compared with monocultures.

4.2 Part II: Microenvironment of subchondral bone influences chondrogenic differentiation and ECM production of BMSC and chondrocytes

4.2.1 Cells embedded in fibrin gel and co- or tricultured with subchondral bone remain vital for up to 28 days

In order to evaluate if the fibrin gel system affects vitality of cells in our different culture set ups, LDH concentration in culture supernatants was analyzed and compared with respective monolayer controls. The cytotoxicity detection tests demonstrated that chondrocytes, BMSC and mixed cultures embedded in a fibrin gel cocultured on subchondral bone explants or kept as monocultures showed no enhanced LDH release compared with monolayer assay controls. Subchondral bone explants cultured without cells in fibrin gel showed no enhanced LDH release compared with assay controls and revealed a LDH level somewhat lower than respective co- or tricultures (data not shown). Therefore, cells are assumed to be not influenced in their vitality or overall metabolic activity by fibrin gel components or OA-subchondral bone explants during a culture time period **(Fig. 21 A, B and C)**.

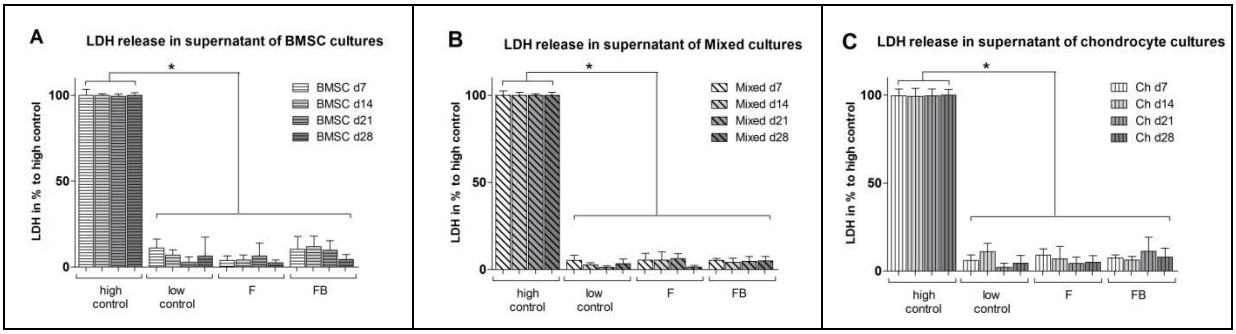


Figure 21: Vitality of mono- co- and triculture setups
Vitality of (A) BMSC, (B) mixed cultures (BMSC and chondrocytes in equal ratio) and (C) chondrocytes was determined in monocultures (F) and co- or tricultures with subchondral bone (FB) kept in chondrogenic medium. Content of LDH was quantified in the supernatant of days 7, 14, 21 and 28 and compared with controls (high control = all cells in a fibrin gel were lysed; low control = spontaneous cell death in monolayer). Due to high inter-experimental variability, the raw data were calculated as percent of control per individual experiment. Results are mean with SD. * $p<0.05$; n=4

4.2.2 mRNA expression of differentiation/dedifferentiation markers is altered in subchondral bone co- and tricultures

To determine whether the microenvironment of OA-subchondral bone affects matrix production or chondrogenic differentiation of BMSC and chondrogenic phenotype of chondrocytes, mRNA expression of *COL1A1* (de-differentiation marker), *COL2A1* (marker for chondrogenic differentiation),

COL3A1 (mesenchymal marker) and *COL10A1* (marker for chondrocyte hypertrophy) was analyzed at days 7 and 28.

At day 7, a significant inhibition of *COL1A1* gene expression was observed for BMSC cocultured with OA-subchondral bone compared to monocultures, whereas BMSC at day 28 and mixed and chondrocyte cultures remained unaffected all over the culture time **(Fig. 22A)**. Analyses of *COL2A1* gene expression in OA-subchondral bone cocultures revealed a significant downregulation in BMSC cocultures compared to monocultures at day 28. *COL2A1* gene expression in all other culture regimen remained unaltered. Additionally, *COL2A1* gene expression significantly increased from day 7 to day 28 in BMSC monocultures, whereas it significantly decreased from day 7 to day 28 in chondrocyte mono- and cocultures with subchondral bone **(Fig. 22B)**. In all co- and triculture conditions *COL3A1* gene expression was significantly downregulated at day 7 and in mixed tricultures and chondrocyte cocultures also at day 28 compared to monocultures. A time dependent downregulation of *COL3A1* gene expression was observed in chondrocyte mono- and cocultures **(Fig. 22C)**. *COL10A1* gene expression was reduced in OA-subchondral bone cocultures with BMSC at day 7 and in BMSC and chondrocyte cocultures at day 28 compared to respective monocultures. Further, *COL10A1* gene expression was significantly induced in both BMSC conditions and significantly reduced in chondrocyte cocultures from day 7 to day 28 **(Fig. 22D)**.

Gene expression pattern in ASC cocultured with normal or OA-subchondral bone was analyzed at days 7 and 28. A significant inhibition of *COL1A1* gene expression was observed at day 7 in ASC cocultured with OA-subchondral bone, whereas ASC cocultured with normal subchondral bone remained unaffected. A time dependent upregulation of *COL1A1* in ASC cocultures with OA-subchondral bone conditions was detected from day 7 to 28 **(Fig. 23A)**. Analyses of *COL2A1* gene expression in OA-subchondral bone cocultures revealed a significant upregulation in ASC cocultures compared to respective monocultures at day 28. No significant differences in *COL2A1* gene expression were detectable between normal subchondral bone and OA-subchondral bone cocultures or monocultures. Gene expression of *COL2A1* was upregulated in all culture conditions from day7 to day 28 **(Fig. 23B)**. In ASC cocultures with normal or OA-subchondral bone gene expression of *COL3A1* was significantly downregulated at day 7 compared to monocultures. *COL3A1* gene expression of ASC cocultures with OA-subchondral bone was significantly upregulated from day 7 to day 28 **(Fig. 23C)**. *COL10A1* gene expression was reduced in ASC cocultures with OA-subchondral bone at days 7 and 28 and in cocultures with normal subchondral bone at day 28 compared to respective monocultures. In ASC mono- and cocultures with OA-subchondral bone gene expression of *COL10A1* was significantly upregulated from day 7 to day 28 **(Fig. 23D)**.

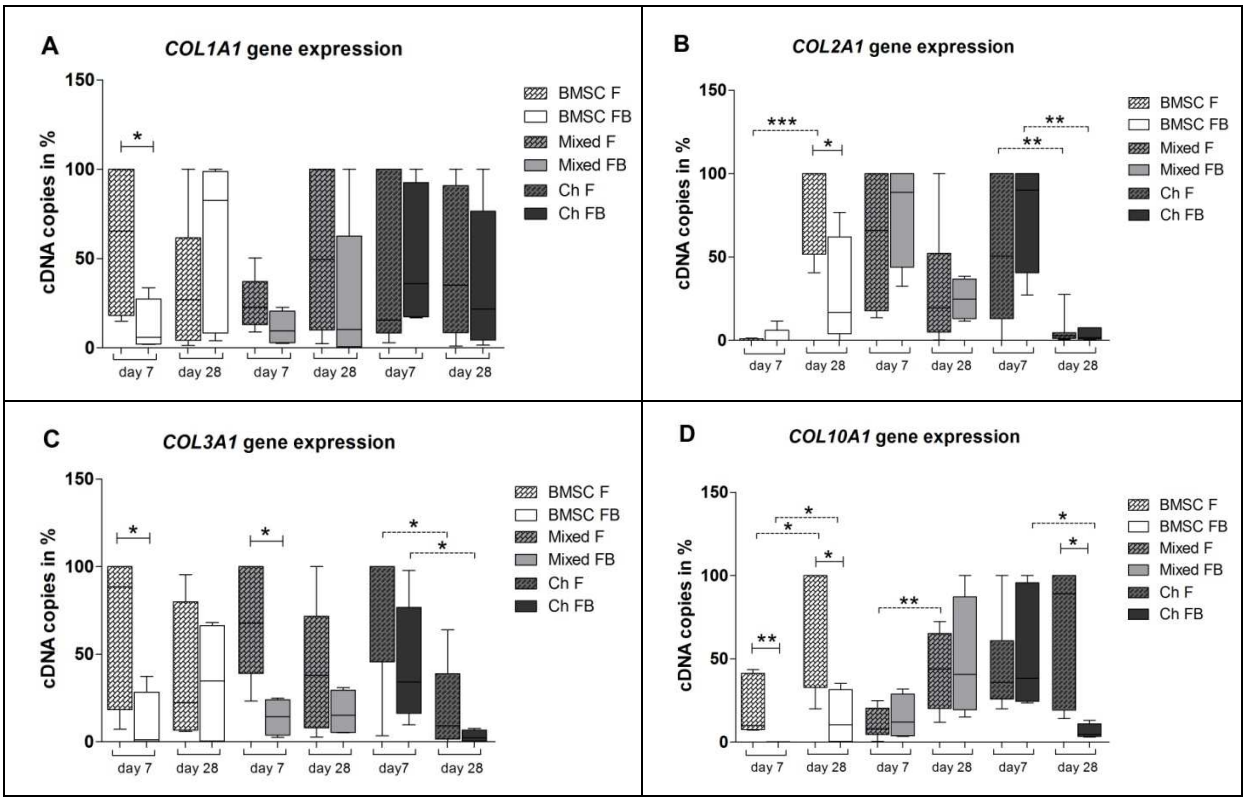


Figure 22: Quantification of absolute gene expression ratio in OA-subchondral bone cocultures

Gene expression levels of (A) *COL1A1*, (B) *COL2A1*, (C) *COL3A1* and (D) *COL10A1* were determined in mono-, co- and tricultures. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) were embedded in fibrin gel and were kept in monoculture (F, bars with pattern) or as cocultures with OA-subchondral bone explants (FB, solid bars) in chondrogenic medium. Due to high inter-experimental variability, raw data were calculated as percent of highest copy number per individual experiment. Gene expression is shown as log of cDNA copies. Solid lines indicate significant differences between culture conditions, dotted lines indicate significant differences between culture time points (days 7 and 28). Results are mean with SD. * $p<0.05$; ** $p < 0.01$; ***$p<0.001$; n=5

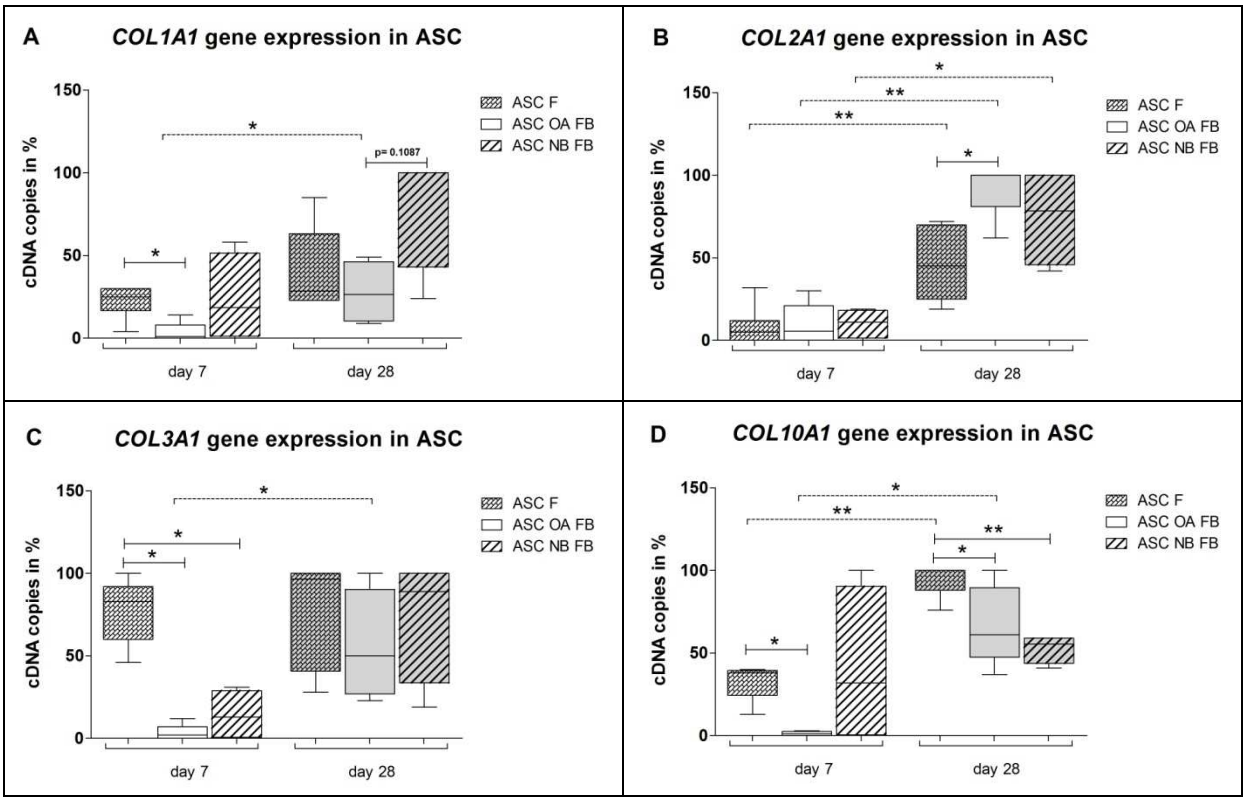


Figure 23: Quantification of gene expression ratio in ASC cocultures with OA- or normal subchondral bone
Absolute gene expression levels of (A) *COL1A1*, (B) *COL2A1*, (C) *COL3A1* and (D) *COL10A1* were determined in mono- and cocultures. ASC were embedded in fibrin gel and were kept in monoculture (F, bars with pattern) or as cocultures with either OA- (OA FB, solid bars) or normal subchondral bone explants (NB FB, bars with stripes) in chondrogenic medium. Due to high inter-experimental variability raw data were calculated as percent of highest copy number per individual experiment. Gene expression is shown as log of cDNA copies. Solid lines indicate significant differences between culture conditions, dotted lines indicate significant differences between culture time points (days 7 and 28). Results are mean with SD. * p<0.05; ** p < 0.01; ***p<0.001; n=5

4.2.3 Microenvironment of subchondral bone alters immunofluorescent staining pattern of collagens

Collagen deposition into the ECM of BMSC, mixed and chondrocyte mono-, co- and tricultures was evaluated by immunofluorescence of cryosections at day 28.

BMSC and mixed monocultures embedded in fibrin gel deposited a higher amount of collagen I into their ECM than those co- or tricultured with OA-subchondral bone explants. Staining for collagen I in chondrocytes was very low in both culture conditions **(Fig. 24A)**. Staining for collagen II was detected in all monoculture conditions but not in co- and tricultures with OA-subchondral bone explants **(Fig. 23B)**. Similar collagen III staining in BMSC, mixed and chondrocyte monocultures but only little or no staining was observed in the co- and tricultures **(Fig. 24C)**. A similar trend was detected for collagen X that was positively stained in BMSC and mixed monocultures but only weak in

chondrocyte monocultures and co- and tricultures **(Fig. 24D)**. Alcian blue staining was used to detect the proteoglycan/aggrecan deposition in the fibrin constructs. All culture conditions were uniformly stained blue and showed no differences between monocultures and subchondral bone co- and tricultures **(Fig. 24 E)**. Overall, reduced collagen deposition into the ECM in co- and tricultured fibrin gels compared to monocultures was observed.

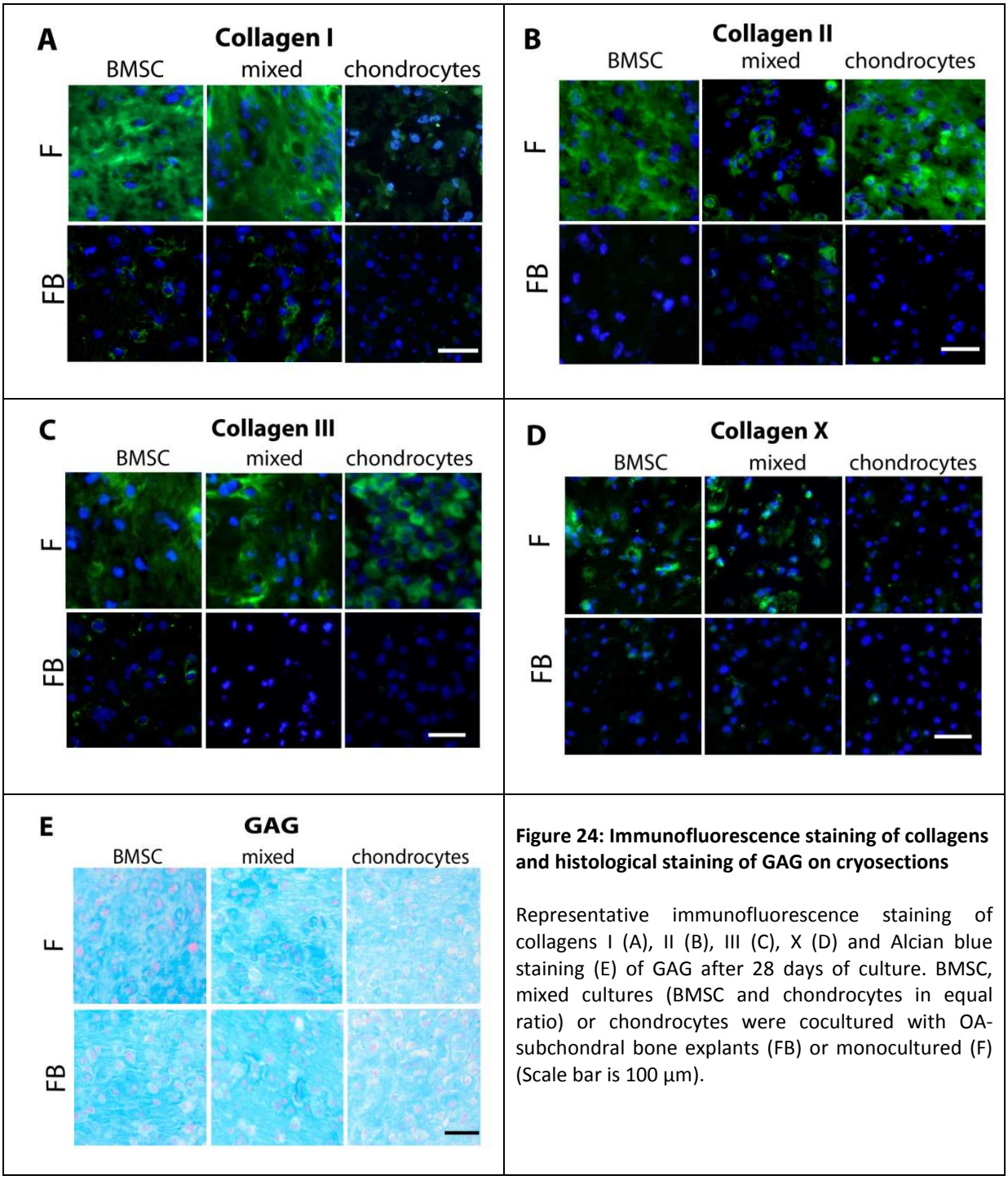


Figure 24: Immunofluorescence staining of collagens and histological staining of GAG on cryosections

Representative immunofluorescence staining of collagens I (A), II (B), III (C), X (D) and Alcian blue staining (E) of GAG after 28 days of culture. BMSC, mixed cultures (BMSC and chondrocytes in equal ratio) or chondrocytes were cocultured with OA-subchondral bone explants (FB) or monocultured (F) (Scale bar is 100 µm).

4.2.4 Fibrin gel cell lysates reveal different GAG, collagen I, II and III content in mono-, co- and tricultures

In further experiments, the influence of OA-subchondral bone explants on collagen and proteoglycan synthesis and degradation was studied. A collagen I specific ELISA revealed a significantly reduced collagen I production in BMSC cocultures and mixed tricultures in comparison with monocultures.

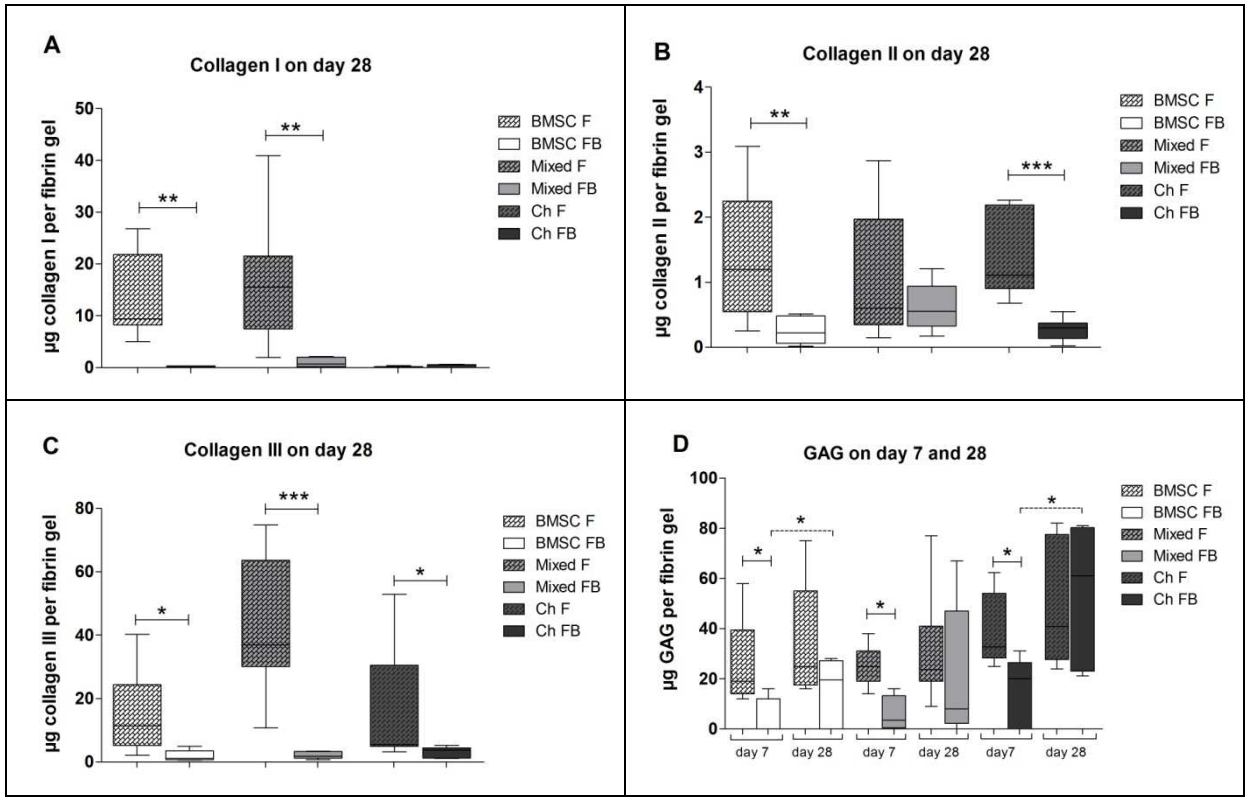


Figure 25: Quantification of collagens I, II, III on protein level and GAGs in cell lysates
Protein synthesis of collagens I (A), II (B) and III (C) in cell lysates after 28 days of culture in fibrin gels and GAG (D) in cell lysates on days 7 and 28. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) were embedded in fibrin gel and kept in monoculture (F, bars with pattern) or in coculture with OA-subchondral bone explants (FB, blank bars) in chondrogenic medium. (A, B) Collagens I and II were quantified with ELISA, or (C) by densitometrically evaluated dot-blot analysis containing a recombinant collagen III standard curve. (D) GAG concentration in cell lysates was quantified by a DMMB-assay including a chondroitinsulfate standard curve. Results are mean with SD. *p<0.05; **p<0.01; ***p<0.001; n=7.

Notably, collagen I content in chondrocytes was below assay detection limits in both culture conditions **(Fig. 25A)**. A collagen II specific ELISA revealed that collagen II production in BMSC and chondrocytes was significantly reduced in OA-subchondral bone coculture compared with monocultures while mixed tricultures remained unaffected **(Fig. 25B)**. A semi-quantitative dot blot analysis was performed to quantify the collagen III protein and found that collagen III production in all co- and tricultures was significantly reduced **(Fig. 25C)**. A DMMB-assay demonstrated that all co-

and tricultures had a significantly decreased GAG content on day 7**.** In BMSC and chondrocyte cocultures a significant induction of GAG synthesis was observed from day 7 to day 28 **(Fig. 25D)**.

4.2.5 Sox9 in BMSC is not influenced by subchondral bone

To determine whether OA-subchondral bone co- or tricultures affect expression of Sox9, protein expression and phosphorylation status in BMSC, mixed and chondrocyte mono-, co- and tricultures were analyzed. mRNA expression of *SOX9* was analyzed in BMSC, mixed and chondrocyte cultures, at days 7 and 28 using quantitative PCR and additionally *SOX9* gene expression status of normal ASC cocultured with normal or OA-subchondral bone explants was analyzed.

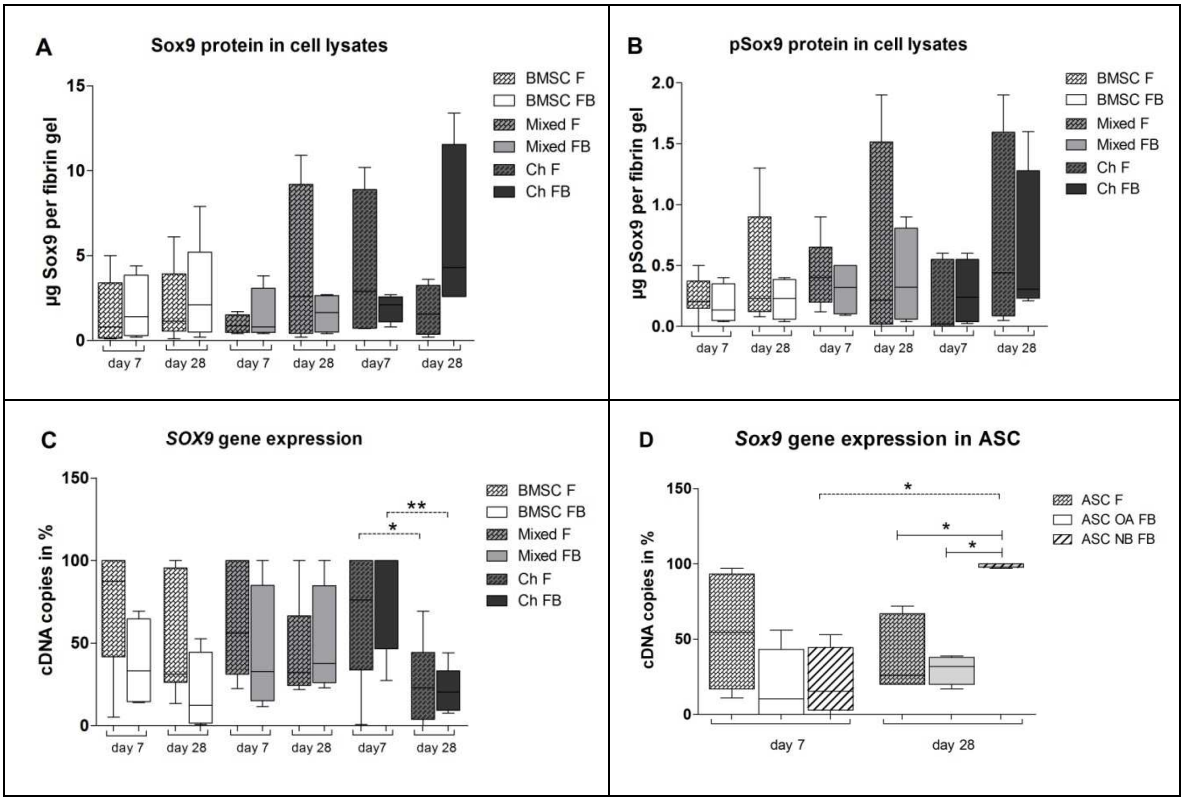
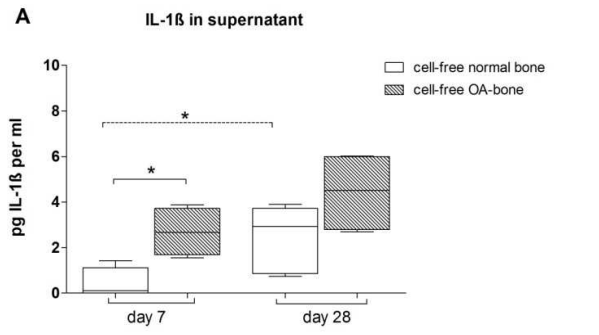


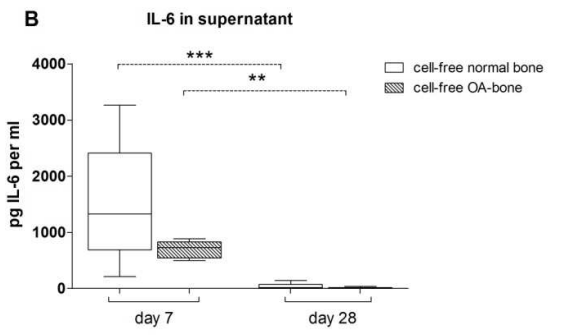
Figure 26: Sox9 gene and protein expression pattern of mono-, co- and tricultures with subchondral bone
Protein contents of (A) Sox9 or (B) phospho Sox9 were quantified densitometrically after immunoblotting of total cell lysates. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) were kept as monocultures (F, bars with pattern) or as cocultures with OA-subchondral bone explants (FC, solid bars) in chondrogenic medium. Gene expression level of *SOX9* (C) was determined in BMSC mono-, co- and tricultured with OA-subchondral bone (C) and ASC mono- or cocultured with normal (NB FB) or OA-subchondral bone (OA FB, D). Due to high inter-experimental variability the raw data was calculated as percent of highest cDNA copy number per individual experiment. Results are mean with SD. * $p<0.05$; ** $p < 0.01$; n=5

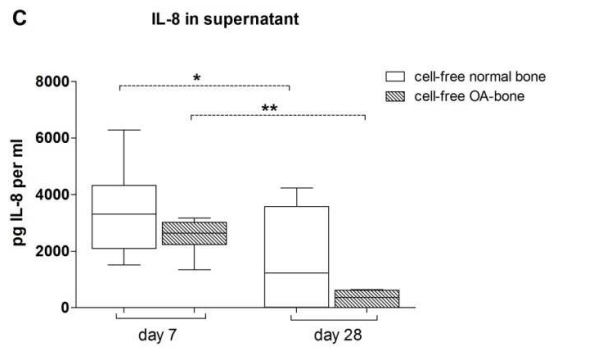
No significant differences in Sox9 protein in all co- and triculture regimens in comparison with monocultures were observed **(Fig. 26A)**. In addition, protein expression of phospho Sox9 revealed no significant differences between culture conditions **(Fig. 26B)**. Moreover, gene expression of *SOX9* in BMSC and mixed cultures was not affected by co- or triculture with OA-subchondral bone. A time dependent significant downregulation of *SOX9* gene expression in chondrocyte mono- and cocultures from day 7 to day 28 was detected **(Fig. 26C)**. In contrast, coculture of ASC with normal subchondral bone resulted in a strong upregulation of *SOX9* in comparison to ASC monocultures and ASC cocultures with OA-subchondral bone at day 28. Additionally *SOX9* gene expression increased with culture time from day 7 to day 28 **(Fig. 26D)**.

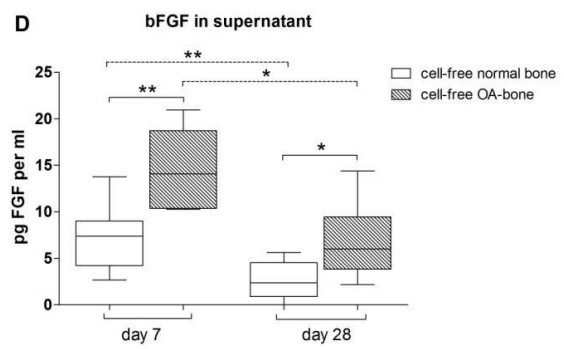
4.2.6 Comparison of soluble ECM molecule fragments, PTHrP and cytokines in supernatants of mono-, co- and tricultures

To determine differentially released factors in culture supernatants of cell-free normal and OA-subchondral bone explants, cytokines/chemokines, PTHrP and soluble ECM fragments were quantified. In supernatants of cell-free normal subchondral bone explants at day 7 IL-1β concentration was significantly reduced compared to OA- subchondral bone explants. Concentration of IL-1β was increased in normal subchondral bone explant supernatants during culture time **(Fig. 27A)**. There was no difference in concentration of IL-6 or IL-8 between cell-free OA- and normal subchondral bone explants. However, a time dependent decrease of both cytokines from day 7 to day 28 was detected in supernatants of cell-free OA- and normal subchondral bone explants **(Fig 27B and 27C)**. Concentration of bFGF was significantly higher in supernatants of cell-free OA-subchondral bone explants than in normal subchondral bone explants at both time points. Additionally, bFGF concentration significantly decreased in supernatants of both subchondral bone explants from day 7 to day 28 **(Fig. 27D)**. Cell-free subchondral bone explants revealed at both time points a significant higher level of soluble GAG in supernatants of normal subchondral bone explants compared to OA-subchondral bone explants **(Fig. 27E)**.









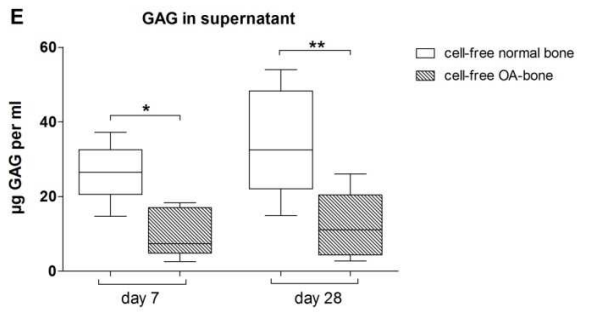


Figure 27: Analysis of cell-free OA- and normal subchondral bone supernatants

Supernatants of normal (blank bars) and OA- (striped bars) subchondral bone explants cultured without cells were analyzed at days 7 and 28. Total amount of cytokines (A) IL-1β, (B) IL-6 and (C) IL-8 released into the supernatant were quantified by antigen-specific ELISAs. (D) Soluble GAG in the supernatant was determined by a DMMB-assay. Results are presented as mean with SD. * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; n= 6

In supernatants of ASC, BMSC, mixed and chondrocyte co- or tricultures with OA-subchondral bone explants, significant more IL-1β was detected at day 28 compared to monocultures whereas coculture of ASC with normal subchondral bone did not affect IL-1β release. In addition, mixed tricultures and chondrocyte cocultures with OA-subchondral bone released more IL-1β into the culture supernatant than ASC normal subchondral bone cocultures **(Fig. 28A)**. All coculture regimen (except for ASC OA-subchondral bone coculture) released more IL-6 into the culture supernatants at day 7 than respective monocultures. In addition, a significantly higher IL-6 level was detected in normal subchondral bone ASC cocultures compared to all other OA-subchondral bone co- and triculture regimen at day 7 **(Fig. 28B)**. IL-8 concentrations in supernatant of normal subchondral bone cocultures of ASC and OA-subchondral bone cocultures of BMSC and chondrocytes at day 7 were significantly elevated compared with respective monocultures. IL-8 levels of mixed cultures remained unchanged. Moreover, IL-8 level in ASC cocultures with OA-subchondral bone was significantly lower

than in ASC cocultured with normal subchondral bone **(Fig. 28C)**. ASC, BMSC and chondrocyte co- and tricultures released significantly more bFGF into supernatants in comparison with respective monocultures while bFGF levels of mixed cultures remained unaffected by culture condition. In supernatants of ASC cocultures with normal subchondral bone a significant higher level of bFGF was detected than in supernatants of BMSC cocultures with OA-subchondral bone **(Fig. 28D)**. Furthermore, PTHrP concentration was not altered in BMSC, mixed and chondrocyte co- or tricultures with OA-subchondral bone in comparison with respective monocultures at days 7 and 28. However, a significant upregulation of PTHrP was observed in all samples from day 7 to day 28 **(Fig. 28E)**. Analysis of supernatants for soluble fibronectin content did not reveal significant differences **(Fig. 28F)**. A significantly higher soluble GAG level was observed in supernatants of all co- and tricultures at day 7 and of BMSC cocultures at day 28 **(Fig. 28G)**. However, a hydroxyproline assay revealed no significant differences in total soluble collagen content between all culture conditions **(Fig. 28H).** As IL-10 and TNF-α were both at assay detection limits, no differences were detected between co- and tricultures compared to respective monocultures (data not shown).

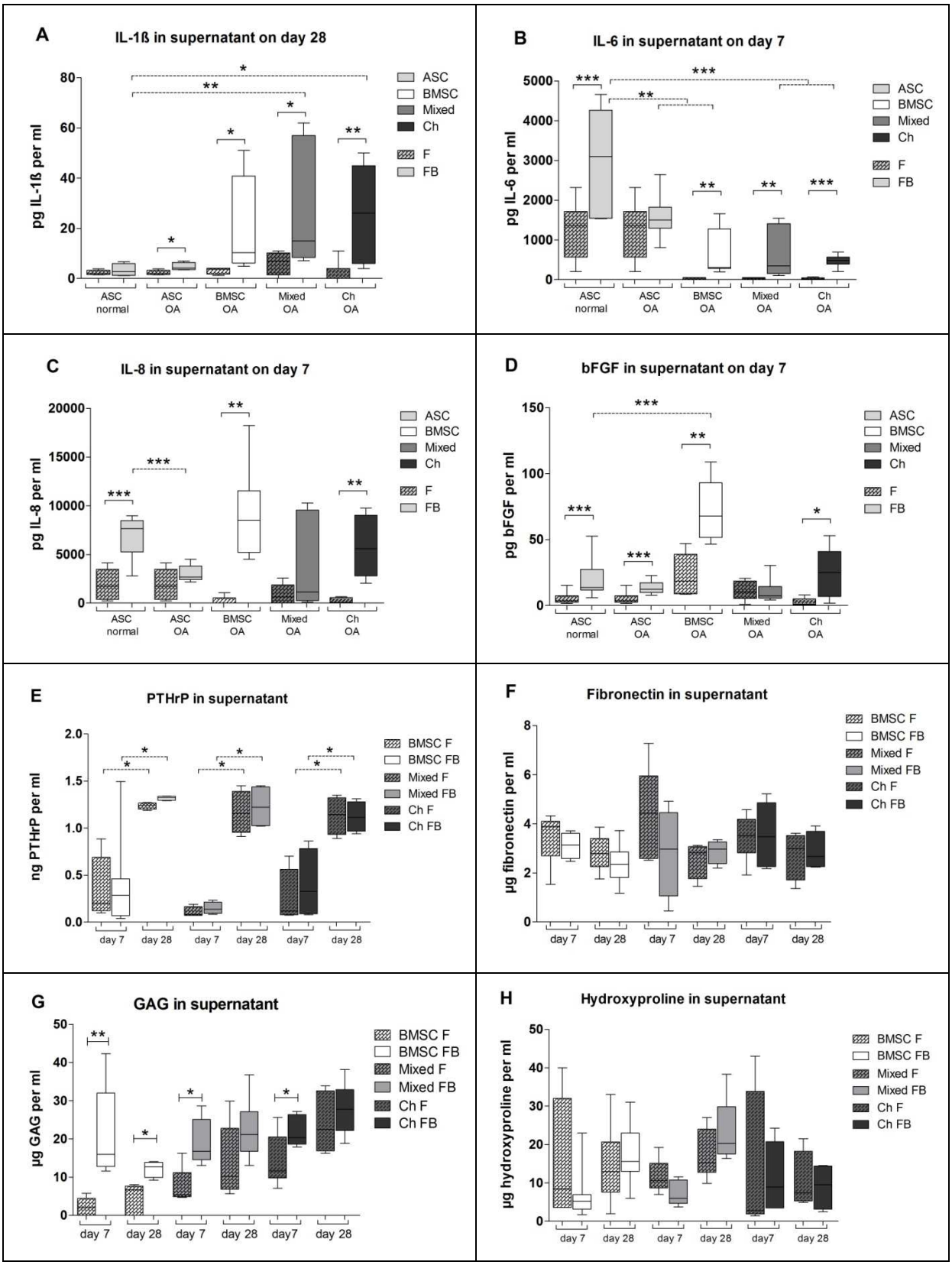

A
IL-1ß in supernatant on day 28
pg IL-1ß per ml
ASC
BMSC
Mixed
Ch
F
FB
ASC normal
ASC OA
BMSC OA
Mixed OA
Ch OA
B
IL-6 in supernatant on day 7
pg IL-6 per ml
C
IL-8 in supernatant on day 7
pg IL-8 per ml
D
bFGF in supernatant on day 7
pg bFGF per ml
E
PTHrP in supernatant
ng PTHrP per ml
BMSC F
BMSC FB
Mixed F
Mixed FB
Ch F
Ch FB
day 7
day 28
day7
F
Fibronectin in supernatant
µg fibronectin per ml
G
GAG in supernatant
µg GAG per ml
H
Hydroxyproline in supernatant
µg hydroxyproline per ml

Figure 28: Quantification of cytokines, bFGF, PTHrP and GAGs in culture supernatants
Analysis of supernatants at days 7 and 28 of ASC (light grey bars), BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, medium grey bars) or chondrocytes (dark grey bars) monocultured (F, bars with pattern) or cocultured with OA-subchondral bone explants (FB, blank bars). Total amount of cytokines (A) IL-1β, (B) IL-6, (C) IL-8, (D) PTHrP, bFGF (E) and fibronectin (G) released into the supernatant were quantified by antigen-specific ELISAs. Analysis of soluble GAG was carried out using a DMMB-assay containing a chondroitin sulfate standard curve for quantification (F). Total soluble collagen was quantified using a hydroxyproline assay (H). Solid lines indicate significant differences between culture conditions, (A-D): dotted lines indicate significant differences between normal and OA- cartilage co- and tricultures, (E): dotted lines indicate significant differences between culture time points (days 7 and 28). Results are mean with SD. *p<0.05, **p<0.01, ***p<0.001; n= 7

Cell-free normal or OA-subchondral bone explants released IL-1β, IL-6, IL-8, bFGF and GAG into the supernatant indicating an additive effect of bone tissue and co- or tricultured cells. Additionally, differences in supernatants of normal bone cultures compared to OA-subchondral bone cultures were detected for IL-1β, bFGF and soluble GAG concentrations. Overall, an increased level of IL-1β, IL-6, IL-8, bFGF and GAG was observed in supernatants of co- and tricultures compared to respective monocultures.

4.2.7 Microenvironment of OA-subchondral bone alters biomechanical properties of newly regenerated cartilage

The quality and load capacity of newly generated cartilage-like tissue was determined by measuring its biomechanical properties. Unconfined mechanical testing indicated that OA-subchondral bone co- and tricultures with BMSC, chondrocytes or a mixture of both cell types (in equal ratio) embedded in fibrin gel exhibited a decrease in Young´s modulus 0-10% strain in 4 of 4 samples (BMSC) and 3 of 4 samples (mixed and chondrocytes, **Fig. 29A)**. Young´s modulus at 40-50% strain showed a decrease in BMSC and mixed OA-subchondral bone co- and tricultures (4 of 4) while Young´s modulus of chondrocytes (3 of 4) was increased **(Fig. 29B).** Aggregate modulus at equilibrium was reduced in all OA-subchondral bone co- and tricultures compared to monocultures **(Fig. 29C).** Further, 3 of 4 BMSC OA-subchondral bone coculture samples had a more than threefold higher hydraulic permeability compared with monocultures, while no clear differences in hydraulic permeability were detected for mixed and chondrocyte co- and tricultures compared with monocultures **(Fig. 29D).**

Taken together, our results suggest that co- and triculture of BMSC, mixed culture or chondrocytes with OA-subchondral bone explants leads to changes in biomechanical properties of newly regenerated cartilage like tissues.

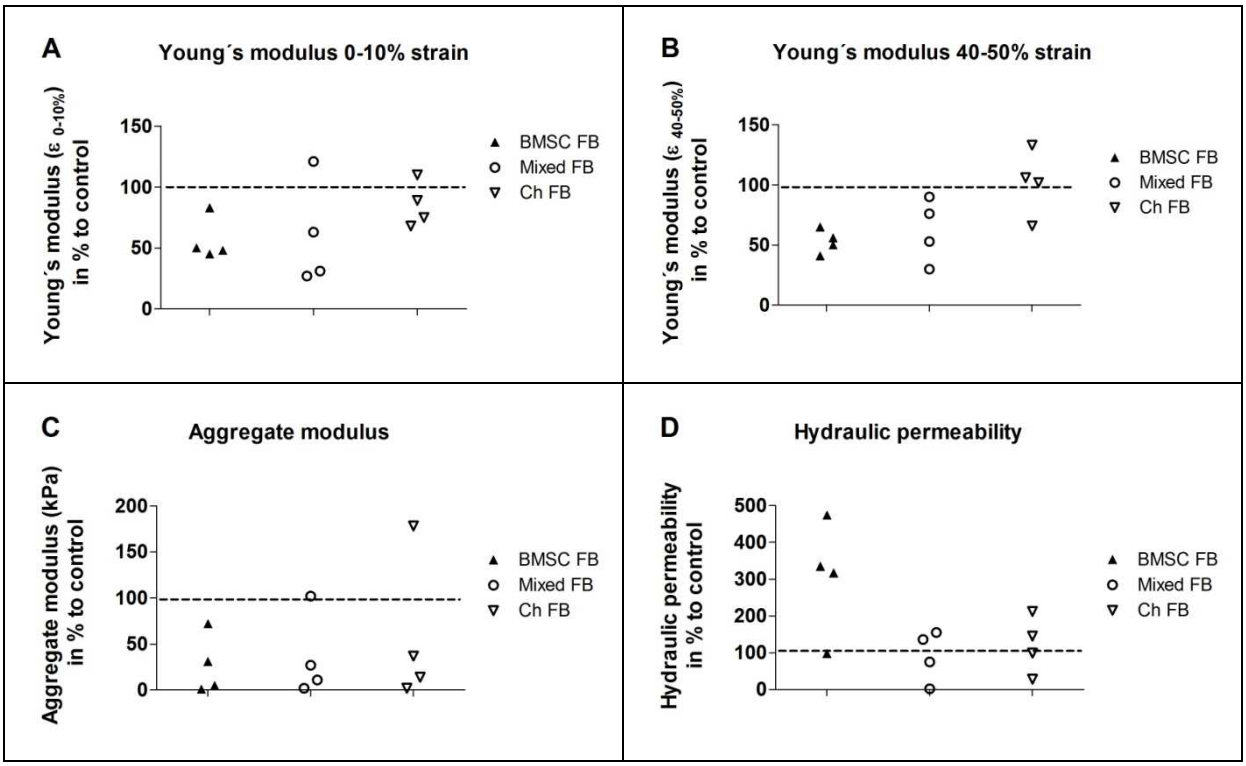


Figure 29: Determination of differences in biomechanical properties

Biomechanical properties of the newly formed ECM at day 28 of BMSC, mixed or chondrocyte co- or tricultures with OA-subchondral bone explants were analyzed and calculated to 100% of monoculture controls (dotted lines). (A) Young´s modulus of 0-10% and (B) 40-50% strain were determined using unconfined compression test. (C) Aggregation modulus and (D) hydraulic permeability were determined using confined tests performed at 50% compression. n=4

4.3 Part III: Effect of IL-1β, IL-6 or IL-8 stimulation on chondrogenic differentiation and ECM production of BMSC and chondrocytes

4.3.1 mRNA expression of (de)-differentiation markers is altered in cytokine stimulated fibrin gels

4.3.1.1 Influence of cytokine stimulation on collagen gene expression

To determine whether stimulation of monocultures with IL-1β, IL-6 or IL-8 affects gene expression in a way similar to stimulation observed in explant co- or tricultures, mRNA expression of *COL1A1, COL2A1, COL3A1* and *COL10A1* was analyzed at days 7 and 28. A significant inhibition of *COL1A1* gene expression was observed in IL-1β stimulated BMSC and in mixed cultures (day 7) and additionally a significant upregulation of *COL1A1* in chondrocytes (day 28) in comparison with unstimulated controls. A decrease of *COL2A1* gene expression was detected in IL-1β stimulated BMSC (days 7 and 28) and mixed cultures (day 7). In chondrocytes (day 28) a significant upregulation of *COL2A1* gene expression compared with unstimulated controls was observed. In BMSC cultures, a significant upregulation of *COL3A1* gene expression was detected at day 7. In contrast, in mixed cultures (days 7 and 28) and in chondrocytes (day 7) gene expression of *COL3A1* was significantly reduced. A decrease of *COL10A1* gene expression was detected in IL-1β stimulated BMSC (day 7) and mixed cultures (days 7 and 28). In chondrocytes (day 28) a significant upregulation of *COL10A1* gene expression compared with unstimulated controls was observed **(Fig. 30A)**.

IL-6 stimulation of BMSC (day 7) and mixed cultures (days 7 and 28) leads to downregulation of *COL1A1* gene expression compared with controls. In contrast, BMSC on day 28 revealed a significant upregulation of *COL1A1* mRNA level while chondrocytes were not affected. *COL2A1* gene expression in all three culture conditions at day 7 was significantly downregulated by stimulation with IL-6. *COL3A1* expression was downregulated in BMSC (day 28), mixed cultures (day 7) and chondrocytes (day 7) in comparison with respective unstimulated controls. Gene expression of *COL10A1* was affected only in chondrocytes where it was significantly lower at day 7 and significantly higher at day 28 in comparison with the unstimulated control **(Fig. 30B)**.

Stimulation with IL-8 induced a decrease in *COL1A1* and *COL2A1* gene expression of BMSC on day 28, while *COL3A1* gene expression was significantly reduced at day 7. IL-8 stimulated mixed cultures and chondrocytes remained unchanged. An upregulation of *COL10A1* gene expression in comparison with controls was observed at day 7 in mixed cultures **(Fig. 30C)**.

In general, inhibitory effects of IL-1β and IL-6 on collagen production of BMSC and mixed cultures were confirmed, whereas collagen gene expression of chondrocytes was partly upregulated at the end of culture. IL-8 stimulation showed only little effects.

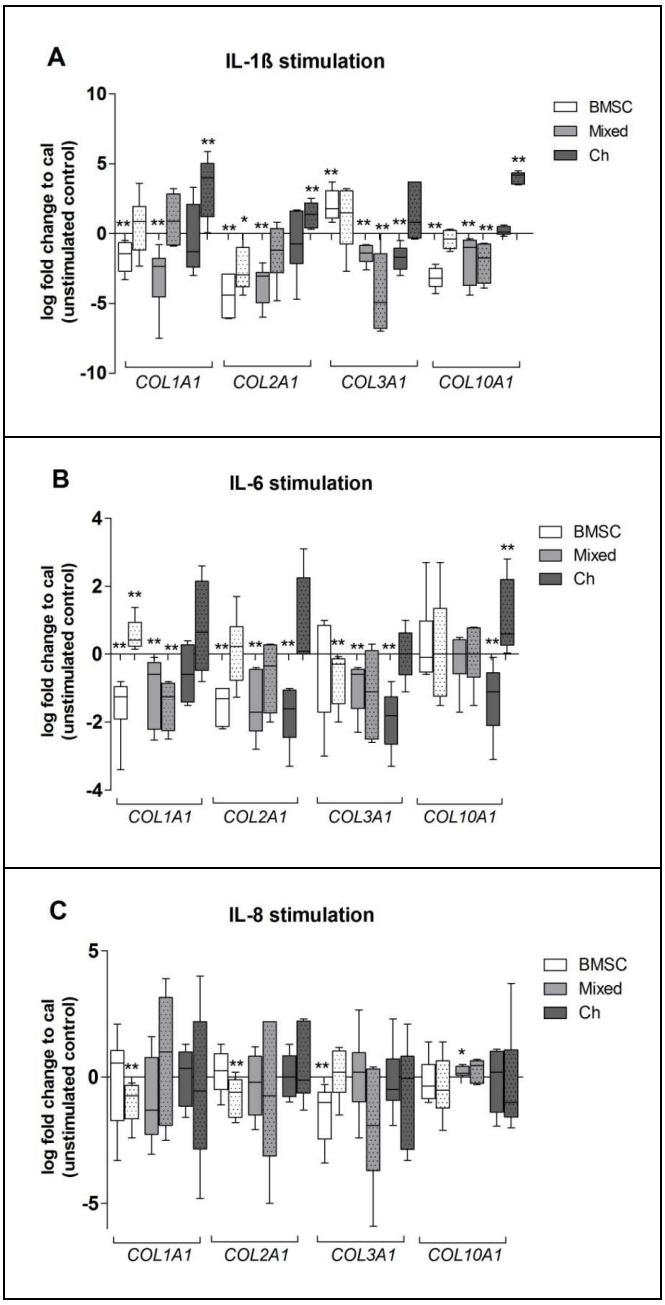


Figure 30: Stimulation of fibrin gel mono-cultures with IL-1β, IL-6 or IL-8

Gene expression of (A) IL-1β, (B) IL-6 and (C) IL-8 stimulated monocultured cells. BMSC (white bars), mixed cultures (BMSC plus chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) were embedded in fibrin gel and were stimulated with 5ng/mL IL-1β, 5ng/mL IL-6 or 10ng/mL IL-8 daily for 7 days in chondrogenic medium. Cultures were analyzed for gene expression of *COL1A1, COL2A1, COL3A1* and *COL10A1* at day 7 (blank bars) and day 28 (bars with dots). The unstimulated respective control served as calibrator, which is represented by the zero-line. Results are mean with SD. * $p<0.05$; ** $p < 0.01$; n=7.

4.3.1.2 Influence of cytokine stimulation on *ACAN*, *MMP2*, *MMP3* and *MMP13* gene expression

To determine whether IL-1β, IL-6 or IL-8 are responsible for the in part I and II observed inhibitory effects of OA-cartilage and subchondral bone on gene expression in co- and tricultures, BMSC, mixed- and chondrocyte monocultures were stimulated with these cytokines and mRNA expression of *ACAN*, *MMP2, MMP3* and *MMP13* was determined at days 7 and 28.

A significant inhibition of *ACAN* gene expression was observed in IL-1β stimulated BMSC (days 7 and 28) and in mixed cultures (day 7) while *ACAN* was upregulated in chondrocytes (day 28) in comparison with unstimulated controls. An increase of *MMP2* gene expression was detected in IL-1β stimulated BMSC (days 7 and 28), mixed cultures (day 7) and chondrocytes (day 28) compared with unstimulated controls. *MMP3* gene expression was upregulated in IL-1β stimulated BMSC (days 7 and 28) and in mixed and chondrocytes (day 7). In contrast, *MMP3* gene expression was significantly inhibited on day 28 in chondrocytes. A significant induction of *MMP13* gene expression was observed in IL-1β stimulated BMSC and chondrocytes (days 7 and 28), while *MMP13* was downregulated in mixed cultures (days 7 and 28) in comparison to unstimulated controls **(Fig. 31A).**

IL-6 stimulation reduced *ACAN* gene expression in BMSC (day 28) and chondrocyte (day 7) monocultures compared with unstimulated controls. *MMP2* gene expression in all three culture conditions remained unchanged. In contrast, *MMP3* expression was downregulated in BMSC (day 28), mixed cultures (day 7) and chondrocytes (day 7) and significantly upregulated in mixed cultures at day 28 in comparison with unstimulated monocultures. IL-6 stimulation reduced *MMP13* gene expression in BMSC (days 7 and 28) and mixed (day 28) monocultures compared to unstimulated controls. Chondrocytes remained unaltered by stimulation with IL-6 **(Fig. 31B)**.

Stimulation with IL-8 induced *ACAN* gene expression in BMSC on day 7 and decreased it on day 28 while mixed and chondrocyte monocultures remained unchanged. *MMP2* gene expression was significantly reduced in BMSC (day 28) and increased in mixed cultures (day 7). A significant upregulation of *MMP3* gene expression in comparison with controls was observed at day 28 in mixed cultures while all other culture conditions remained unaffected. Stimulation with IL-8 inhibited *MMP13* gene expression in BMSC and mixed cultures on day 28 and increased *MMP13* gene expression in mixed cultures on day 7. Chondrocyte monocultures remained unaffected **(Fig. 31C)**.

Overall, a mostly inhibitory effect of IL-1β and IL-6 on gene expression of *ACAN* was observed. *MMP2, MMP3* and *MMP13* gene expression was induced mainly by IL-1β while IL-6 had no effect on *MMP2* expression and rather downregulated *MMP3* and *MMP13* gene expression. IL-8 stimulation had only little effects on gene expression and showed no clear tendency.

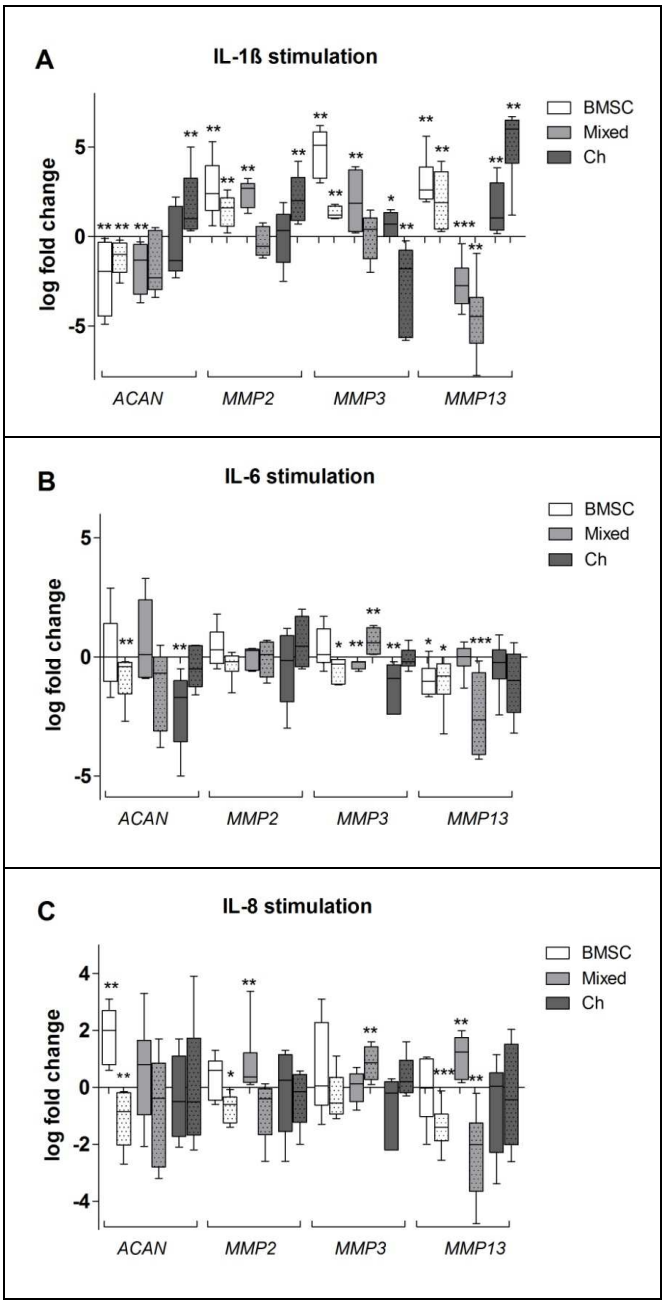


Figure 31: Stimulation of fibrin gel mono-cultures with IL-1β, IL-6 or IL-8

Gene expression of *ACAN*, *MMP2*, *MMP3* and *MMP13* of (A) IL-1β, (B) IL-6 and (C) IL-8 stimulated monocultures. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) embedded in fibrin gel were stimulated for 7 days with 5ng/mL IL-1β, 5ng/mL IL-6 or 10ng/mL IL-8 in chondrogenic medium containing dexamethasone and TGF-β3. Specimens were analyzed for gene expression of *ACAN*, *MMP2*, *MMP3* and *MMP13* at day 7 (blank bars) and day 28 (bars with dots). The unstimulated respective control served as calibrator, which is represented by the zero-line. Results are mean with SD. *p<0.05; **p<0.01; ***p<0.001; n= 6

4.3.2 Stimulation with cytokines revealed different GAG, collagen I, II and III content in fibrin gel lysates

To determine whether stimulation of monocultures with IL-1β, IL-6 or IL-8 affects ECM molecule expression, GAG content and protein expression of collagens I, II and III were analyzed in all lysates at day 28. A significant inhibition of GAG production in IL-1β stimulated BMSC, mixed and chondrocyte cultures was observed compared with unstimulated controls and a significant inhibition of collagen II protein expression was shown in IL-1β stimulated mixed and chondrocyte cultures but not in BMSC cultures **(Fig. 32A)**. No significant effects on collagens I or III protein expression were determined for IL-1β stimulations. Despite to gene expression results, IL-6 and IL-8 stimulation setups had no effects on the evaluated ECM components **(Fig. 32B-C)**.

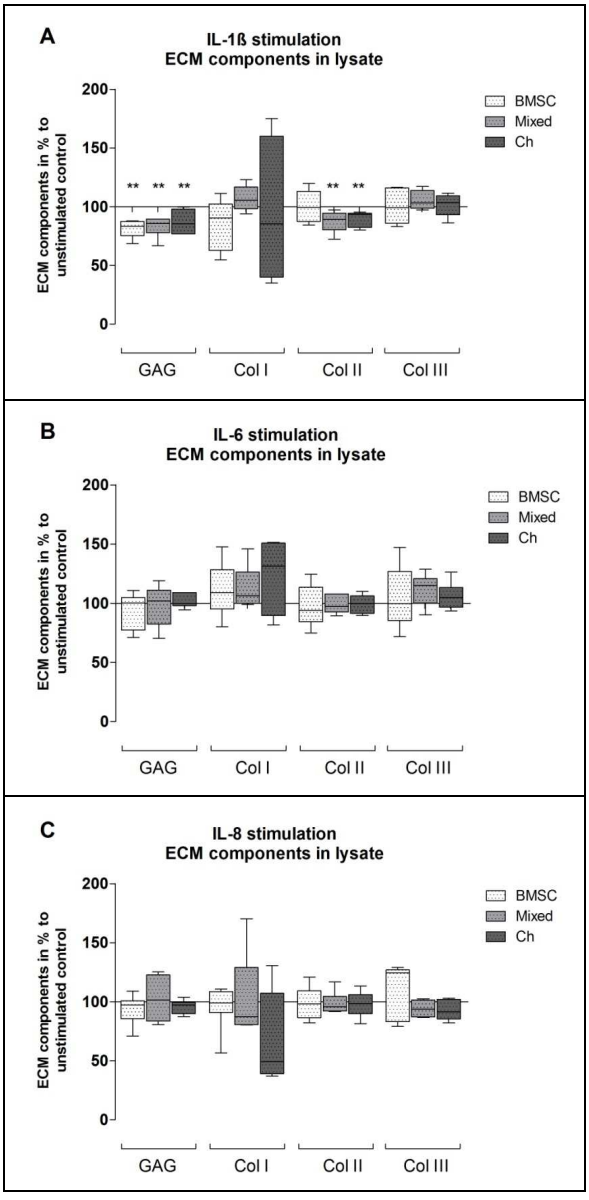


Figure 32: Quantification of ECM molecule expression in newly regenerated cartilage tissue

GAG, collagen I, II and III expression of (A) IL-1β, (B) IL-6 and (C) IL-8 stimulated fibrin gels were determined in lysates of BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars). Cells were embedded in fibrin gel and were stimulated in chondrogenic medium containing TGF-β. Protein expression of collagens and GAG was determined densitometrically using dot-blot analysis. Due to high inter-experimental variability the raw data were calculated as percent of control per individual experiment. Results are mean with SD. **p<0.01; n=6

4.4 Part IV: Influence of normal ovine cartilage on mRNA expression of ovine BMSC, mixed and chondrocyte cultures

To gain insight into cartilage regeneration of normal tissue, an ovine coculture model according to the established human protocol described under **3.4** was used**.**

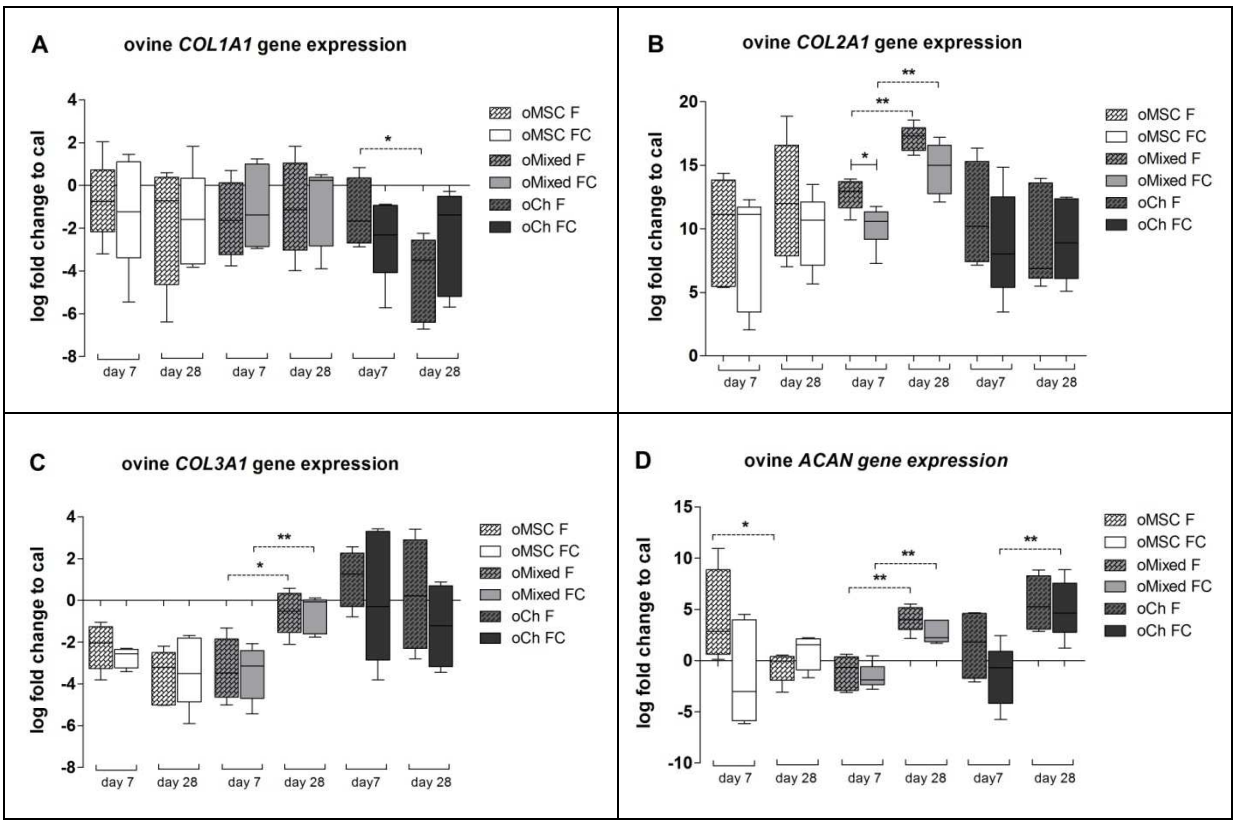


Figure 33: Quantification of ovine ECM gene expression ratio with qPCR

Gene expression level of (A) *Col1A1*, (B) *Col2A1*, (C) *Col3A1* and (D) *ACAN* were determined in co- and monocultures. BMSC (white bars), mixed cultures (BMSC and chondrocytes in equal ratio, light grey bars) or chondrocytes (dark grey bars) were embedded in fibrin gel and were kept in monoculture (F, bars with pattern) or co- and triculture with ovine articular cartilage explants (FC, solid bars) in chondrogenic medium. Gene expression is shown as log fold change to a calibrator, which was gene expression of oBMSC monolayer cells (in case of oBMSC and oMixed mono-, co- and tricultures) or gene expression of oCh monolayer cells (in case of oCh mono- and cocultures). Solid lines indicate significant differences between culture conditions, dotted lines indicate significant differences between culture time points (days 7 and 28). Results are mean with SD. *p<0.05, **p<0.01; n=5

For *COL1A1* gene expression in oBMSC, mixed (oMixed) and chondrocyte (oCh) cultures no differences between monoculture and co- or triculture with normal ovine cartilage was detected. Therefore, a time dependent significant downregulation of *COL1A1* gene expression was found in chondrocyte monocultures from day 7 to day 28 **(Fig. 33A)**.Gene expression of *COL2A1* and *COL3A1* in oBMSC and oCh showed no significant differences between culture conditions. In oMixed cultures,

COL2A1 was significantly reduced in ovine cartilage tricultures at day 7 compared with monocultures. Additionally, there was a significant upregulation of *COL2A1* and *COL3A1* in both oMixed culture conditions from day 7 to day 28. Gene expression of *ACAN* was downregulated in oBMSC monocultures and upregulated in both oMixed cultures and oCh co- and tricultures from day 7 to 28 **(Fig. 33B-D).**

Overall, gene expression pattern of oBMSC, oMixed and oCh co- and tricultured with normal ovine articular cartilage revealed no differences to respective monocultures.

5 Discussion

5.1 General discussion

Since western population gets continuously older, the number of degenerative joint diseases like osteoarthritis is steadily increasing (Hunter and Felson 2006). Various options for treatment of symptoms are available, however there is only little chance to repair cartilage defects. Modern medicine has opened BMSC as a potential source for tissue regeneration and plenty of new high-tech biomaterials have been established in the last few years. Despite of numerous cell-based tissue-engineering attempts, stem cell-based therapies are not jet venturous and artificial cartilage reconstruction still needs improvement (Steinert, Ghivizzani et al. 2007; Grassel and Lorenz 2014). Evidently, more intensive research in the field of stem cell-based OA reconstruction is needed. Consequently, the focus of this study was on the usage of fibrin gel embedded BMSC and chondrocytes, and the mixture of both cell types in equal ratio for cartilage regeneration in the presence of OA-cartilage or subchondral bone.

BMSC are predicted to have a promising future in the field of tissue engineering, because they easily can be isolated and expanded in order to be differentiated into repair cells and implanted in respective sites of injury (Sylvester and Longaker 2004). In this study, stemness and differentiation potential of isolated plastic adherent bone marrow cells was proven by flow cytometry with respect to typical expression patterns of characteristic BMSC surface markers namely $CD105^{pos}$, $CD44^{pos}$, $CD34^{neg}$ and $CD19^{neg}$. As second criteria for their differentiation potential, BMSC were shown to differentiate into chondrogenic, osteogenic and adipogenic lineages (Dominici, Le Blanc et al. 2006). Even though these BMSC were obtained from OA-patients, they seemed not to be affected in their differentiation capacity. These findings provide evidence to suggest that autologous obtained BMSC from OA-patients contain the potential for cartilage tissue repair.

In order to discover differences between BMSC from normal and OA-donors, normal adipose derived stem cells (ASC) were used for experiments with OA- or normal cartilage- and bone explants. These ASC were previously shown by Schreml et al. to be capable for adipogenic, chondrogenic and osteogenic differentiation and additionally expression pattern of characteristic BMSC surface markers were analyzed by FACS (Schreml, Babilas et al. 2009). Therefore, ASC were a suitable alternative cell source for verification of coculture results obtained with BMSC.

Human articular chondrocytes were isolated from different patients undergoing total knee replacements due to OA. Despite critical selection of cartilage explants with similar OA grade, inter-patient variations in cellular responses were not eliminated and resulted in a high variance of data values. Not only different influences like genetics and previous drug therapy are affecting expression

patterns – also the position of excised cartilage varies between patients. Symptoms of terminal stage OA are heterogeneous in phenotype and underlying molecular mechanisms, in consequence differences in key pathways or gene expression could be masked or misrepresented (Snelling, Rout et al. 2014). As isolated chondrocytes were cultured up to 14 days in monolayer until they were confluent, donors of chondrocytes and cartilage- or bone explants were not autologous. Additionally, allogeneic BMSC were used for mixed coculture setups. Up to three different patients per approach were combined resulting in even higher inter-patient variations, which impede generation of statistical significances or trends. This phenomenon is widely known by usage of primarily patient cells and can be countervailed by a high number of samples (Snelling, Rout et al. 2014).

Several studies indicate that BMSC alter the cytokine secretion profile of different cocultured immune cells into a more anti-inflammatory or more tolerant phenotype (Aggarwal and Pittenger 2005). BMSC provide anti-inflammatory factors, which might interfere with inflammatory factors released from OA-cartilage explants leading to a positive effect on differentiation and ECM production. However, until now, immunosuppressive properties were not reported for chondrocytes and thus explant cocultivation with chondrocytes was an important part for verification of BMSC modulated effects.

To establish a reproducible coculture model for investigation of specifics in OA-cartilage repair, it was necessary to verify survival and proliferation of cells in this setup. Analysis of supernatants for LDH, a marker for cell-death, showed that neither cultivation of cells embedded in fibrin gel nor coculture with cartilage or subchondral bone explants diminished cell vitality of BMSC, mixed or chondrocyte cultures compared with respective control cells (cells differentiated in monolayer). This vitality test revealed that local cells in OA-cartilage and OA-subchondral bone explants had a similar spontaneous death rate than cells cultured in monolayer. Additionally, these cells are still metabolically active and potentially paracrine regulative.

An important premise for chondrogenic differentiation is a moderate proliferative activity. Thereto staining of PCNA, a marker for cell proliferation, in cryo sections of cell-fibrin gels was positive in some nuclei equally distributed over the whole slice suggesting proliferation of a subpopulation of fibrin gel embedded cells. Furthermore, no differences between monoculture and OA-cartilage co- or triculture were observed indicating that fibrin gel or OA-cartilage had no influence on cell proliferation in this culture set up.

Taken together, microenvironment of OA-cartilage or subchondral bone has no cytotoxic or cytostatic features. Moreover, results from vitality tests with explants cultured without cell-fibrin gels suggested that local cells in the explants are vital and metabolically active. Thus, it is likely, that

these local cells in the explant release similar factors into the culture supernatant, as they would do *in vivo*. In this study, usage of OA-tissues suggests mainly the release of pro-inflammatory cytokines and degradation products of ECM molecules.

In detail, the coculture setups of this study combined two different conditions. At first, cells were embedded in a fibrin gel matrix to provide a 3D chondrogenic surrounding which enhances chondrogenic differentiation of BMSC, maintenance of articular phenotype and production of a cartilage specific ECM (Lutolf and Hubbell 2005). Fibrin is the perfect biomaterial for this cast because it occurs in the natural human healing system. In many studies it was successfully used together with stem cells or chondrocytes for cartilage repair and negative influences on chondrogenic differentiation are not known (Fussenegger, Meinhart et al. 2003; Park, Yang et al. 2009). Chondrogenic differentiation auxiliary was induced by chondrogenic medium containing TGF-ß3 and dexamethasone (Ahmed, Dreier et al. 2007). Interestingly, dexamethasone is reported to act chondroprotective and to decrease joint inflammation as well as joint tissue degradation (Huebner, Shrive et al. 2014). A chondrogenic phenotype and matrix production was detected in all monoculture conditions, with respect to present data. A second aspect of our setup was combination of this chondrogenesis supporting fibrin gel and medium with OA-cartilage or subchondral bone explants providing a diseased and inflamed microenvironment like in OA-patients (Tchetina, Squires et al. 2005). Nevertheless, changes in the differentiation pattern of BMSC with respect to *COL2A1* and *COL10A1* expression as well as alterations in ECM production and composition in newly generated cartilage like tissue were postulated by coculture with diseased OA-explants (Ahmed, Dreier et al. 2007).

Readout parameters of this study were gene expression of typical marker genes namely *COL1A1*, a de-differentiation marker (Marlovits, Hombauer et al. 2004; Cheng, Maddox et al. 2012), *COL2A1*, a chondrogenic differentiation marker (Mendler, Eich-Bender et al. 1989; Bruckner and van der Rest 1994)*, COL3A1*, a mesenchymal cell marker (Ku, Johnson et al. 2006; Juncosa-Melvin, Matlin et al. 2007)), *COL10A1* (hypertrophic chondrocyte marker (Schmid and Linsenmayer 1985), and *SOX9*, master transcription factor during chondrogenesis (de Crombrugghe, Lefebvre et al. 2000; Hattori, Muller et al. 2010).

These marker genes indicate the functional status of BMSC and chondrocytes like de-differentiation and chondrogenic- or terminal differentiation. Further production and functionality of newly generated ECM was assayed by biochemical and biomechanical tests. Additionally, supernatants of cocultures were analyzed. These supernatants are partly representing the unique OA-microenvironment, which is characterized by soluble ECM fragments, newly generated ECM precursor molecules and typical factors like pro-inflammatory cytokines, MMPs or proteases. These

typical factors are either newly produced by local cells in the explant or released from the OA-cartilage ECM to which they were previously bound. In this study, focus of research was restricted on soluble molecules mainly components of the ECM as collagens, GAGs and fibronectin as well as proinflammatory factors like cytokines and chemokines, which are known to be involved in cell differentiation and alterations of ECM homeostasis. In addition, screening of supernatants for yet unknown factors relevant for regulatory mechanisms during OA progression was performed via LC-MS analysis to discover shifts in the secretome of BMSC mono- and cocultures with cartilage explants.

Apparently, up to now, no other groups have analyzed the *in vitro* influence of OA-tissue on cartilage regeneration with BMSC, chondrocytes or an equal mixture of both cell types embedded in fibrin gel. This novel and innovative study gives new important insights in mechanisms, which occur during BMSC differentiation and ECM production influenced by OA-tissue. In future, this knowledge could be used for treatment of patients suffering from OA.

5.2 Part I: Microenvironment of cartilage coculture influences BMSC differentiation and ECM production

Traumatic focal cartilage defects do not heal spontaneously, consequently stable long-term repair and regeneration of destroyed articular cartilage needs innovative therapy strategies like cell-based tissue engineering. At the site of injury, chondrocytes and in few cases BMSC were implanted, but not much attention was paid to effects of neighboring cells and microenvironment provided by remaining cartilage tissue. Treatment of BMSC with growth factors like TGF-ß resulted in induction of mRNA and protein expression of several chondrogenic markers (Diederichs, Baral et al. 2012). Similar reactions were observed for cocultures of BMSC with normal articular rat cartilage or human OA-chondrocytes, which are known to induce chondrogenic differentiation by release of paracrine factors (Ahmed, Dreier et al. 2007; Aung, Gupta et al. 2011). However, reports of proper OA-cartilage regeneration resulting in an ECM with high biomechanical properties and a stable articular phenotype are scarce.

Although many studies have examined effects of culture medium supplemented with well-defined chondrogenic factors on differentiation capacity of BMSC, only few have addressed the influence of OA-affected cartilage ECM or OA-chondrocytes on formation of matrix in cocultured cells and on differentiation of BMSC. In order to identify culture conditions favoring proper matrix production, chondrogenic differentiation and phenotype stability, a novel co- and triculture system was established. BMSC, OA-chondrocytes and mixed cultures were embedded in a fibrin gel bio-matrix providing a 3D environment, which mimics the natural habitat of chondrocytes and promotes chondrogenic differentiation of BMSC (Lutolf and Hubbell 2005; Lee, Yu et al. 2008). To mimic an OA-microenvironment, cell-fibrin gels were cocultured on top of OA-cartilage explants.

Achievement of the first part of this thesis was the realization that coculture with OA-cartilage explants influenced gene expression and biosynthesis of collagens I, II, III and X in all co- and triculture regimens and altered biomechanical properties presumable because of released regulatory factors (Leyh, Seitz et al. 2014 a).

In detail, *COL1A1,* a marker for dedifferentiation was significantly reduced in all coculture conditions compared with monoculture at day 7 possibly representing an initial diminished dedifferentiation potential of cells cultured together with cartilage. Similar observations were made for *COL3A1,* a mesenchymal collagen highly expressed in undifferentiated BMSC, which was also significantly reduced in all cartilage co- and tricultures. A downregulation of *COL3A1* in cocultured fibrin gels during chondrogenic differentiation thus might denote a higher percentage of chondrogenic

differentiated BMSC in presence of cartilage. With respect to chondrogenic differentiation of BMSC suppression of collagen I and III gene expression via cartilage derived factors might be positive.

However, significant upregulation of *COL2A1,* a positive chondrogenic differentiation marker, over time indicated positive chondrogenic differentiation in both mono- and coculture conditions of BMSC, but cocultured BMSC exhibited significant less upregulation of *COL2A1* than cells in monoculture. In addition, *COL2A1* gene expression of mixed- and chondrocyte cultures in presence of OA-cartilage was significantly reduced at day 7, which could indicate a diminished or at least delayed initiation of chondrogenic differentiation or collagen II production. Besides, mixed cultures showed no significant upregulation of *COL2A1* gene expression during culture time. Presumably, chondrocytes produced large amounts of cartilage specific mRNA, mainly *COL2A1,* from the beginning, which obscures the rising mRNA levels of BMSC in the mixed culture. A time dependent downregulation of *COL2A1* in chondrocyte monocultures from day 7 to day 28 might be a hint to inhibition of matrix production induced in chondrocytes.

In this study, gene expression of the hypertrophic marker *COL10A1* was strongly downregulated in the presence of OA cartilage explants in all co- and triculture regimens. This inhibitory effect appears not to be limited to OA-cartilage since it was previously described for normal BMSC-cartilage cocultures (Ahmed, Dreier et al. 2007). One major problem in using BMSC for cell-based cartilage defect repair is the instability of the chondrogenic phenotype, which tends to progress to a hypertrophic phenotype with subsequent entering the endochondral ossification pathway. With respect to *COL10A1* gene expression coculture with cartilage could provide a protective effect, and could promote stability of the chondrogenic versus hypertrophic phenotype.

Taken together, collagen gene expression of BMSC cocultures was inhibited on day 28 indicating an inhibitory effect of OA-cartilage. In contrast, OA-chondrocytes showed signs of inhibition only for the first days of OA-cartilage coculture. Furthermore, a time dependent downregulation of *COL2A1* and *COL3A1* was observed for chondrocytes in monocultures. It seems that monocultured chondrocytes start to produce plenty of ECM proteins (with respect to collagen mRNA) as soon as they are unhinged from matrix, but once they are again trapped in the newly synthesized ECM gene expression decreases. In addition, gene expression of collagens was significantly inhibited in chondrocyte cocultures in comparison to monocultures of day 7, whereas at day 28 the ECM gene expression pattern of chondrocytes in monoculture, which at that time were embedded by newly generated matrix, is similar to chondrocytes in cartilage coculture. These results might be a hint to soluble cartilage derived inhibitory factors which production is induced in chondrocytes by cell-ECM contact to newly generated ECM (Leyh, Seitz et al. 2014 a).

In concert with results from qPCR analysis, fibrin gels indicated matrix deposition in both culture conditions, which was demonstrated by color changes of the fibrin gel from translucent to opaque and also sensed by an explicit rise of stiffness. Analysis of biochemical composition of the newly generated ECM via ELISA and immunofluorescent staining reassured in general inhibition of collagen protein production by OA-microenvironment. In detail, BMSC revealed significant less collagen I and II deposition in coculture with cartilage explants and mixed cocultures revealed significantly reduced collagen I and III deposition. In contrast, no corresponding collagen X immunostaining in all chondrocyte and mixed cultures and only little reactivity in BMSC monocultures was detectable. Several studies showed hypertrophy-like changes in chondrocytes during OA (van der Kraan and van den Berg 2012) and enhancement of hypertrophy induced in BMSC during chondrogenic differentiation (Fischer, Aulmann et al. 2014). However, results of the present study indicated that there is no increased hypertrophic activity in mono-, co- or tricultures after 28 days of differentiation with respect to collagen X production.

Interestingly, inhibition of collagen protein biosynthesis was cell type dependent as chondrocytes showed no significant inhibition of collagens on protein level approving that mono- and cocultured chondrocytes have similar collagen expression patterns. In this line, Fan et al. showed that OA-chondrocytes in 3D culture mimicked OA-aspects even after 3 weeks of culture in comparison with normal chondrocytes (Fan, Bau et al. 2005). Probably because of this severely altered metabolism of OA-chondrocytes, coculture of OA-chondrocytes with OA-cartilage might not induce additional effects and supports the thesis that they are partly unresponsive to OA-cartilage derived factors. Since a majority of cocultured chondrocytes has - at least at the beginning of culture time - no contact to ECM molecules, soluble OA-cartilage released factor(s), possibly including GAG fragments, are suggested to contribute to a reduced chondrogenic differentiation capacity of BMSC while chondrocytes as fully differentiated cells are poorly responsive to these instructions.

Nevertheless, histochemical staining with alcian blue revealed uniform distribution of GAGs in all fibrin gels of mono-, co- and tricultures and no sign for an influence of OA-cartilage explants like a gradient in GAG staining was observed. Additionally, there was no hint to an induction of proteoglycan degradation, since GAG content in fibrin gels of mono- and co- or tricultures of chondrocytes and/or BMSC with OA-cartilage revealed no significant differences at the end of culture, whereas at the beginning GAG production was reduced in cocultures.

In addition to GAG deposition also content of soluble GAG fragments was analyzed. All supernatants of OA-cartilage cocultures showed significantly increased soluble GAG concentrations throughout the culture time. Moreover, experiments with cell-free normal cartilage explants compared with cell-free OA-cartilage explants showed no differences in soluble GAG release and revealed similar GAG levels

like in co- and tricultures. GAGs were released from the explants into supernatant in a constant manner over culture time, what might indicate physiological degradation of proteoglycans, especially aggrecan with respect to recent literature (Little, Flannery et al. 1999; Kobayashi, Squires et al. 2005). As a result, enhanced levels of soluble GAG fragments detected in the supernatant likely are released by OA-cartilage explants and by co- or tricultured cells in an additive way (Leyh, Seitz et al. 2014 a). Nonetheless, these soluble GAG fragments might stimulate degradation of other ECM components, because GAG fragments released from articular cartilage are known to enhance catabolism of collagen II (Aigner and McKenna 2002).

Overall, an inhibition of production of all collagens was investigated in every culture regimen in the presence of OA-cartilage explants suggesting that no collagen type specific factor is responsible for inhibition of collagen expression on gene and protein level. More likely, the effect must have some specificity on inhibition of collagens in general and not on ECM production or deposition as GAG production and deposition was mainly unaltered in OA-cartilage co- and tricultures compared with monocultures.

In the present culture setup no differences in total soluble collagen concentration in supernatants were found with respect to hydroxyproline, which is representative for all soluble collagens in supernatants of cartilage mono-, co- and tricultures. This might be because collagen II degradation products were not analyzed in particular and no distinction between newly synthesized or degraded soluble collagens was made. It is also possible that other collagens like collagen I or III mask the OA-typical collagen II catabolism. A specific collagen type II degradation product ELISA would be indicated to clarify composition of total soluble collagens (Dahlberg, Billinghurst et al. 2000). Assumedly, collagen contents in fibrin gel lysates are not reduced due to increased degradation but presumably *a priori* by a decreased biosynthesis.

Alterations in ECM metabolism, for example an imbalance of proteoglycan and collagen II production or degradation, occur during the progression of OA. The homeostasis of extracellular matrix underlies a delicate balance of turnover and new formation of matrix in parallel (Tchetina 2011). During OA-progression this balanced homeostasis is considerably disturbed towards a dramatically enhanced degradation of articular ECM resulting in elevated contents of soluble ECM fragments, which can serve as regulatory factors (Sofat 2009). Special attention was given to the question whether degradation processes in the presence of cartilage explants contribute to reduction of collagen contents in cells and ECM.

In order to study ECM turnover in fibrin gel cultures, analysis of soluble ECM components released into the supernatants was performed. For that reason in addition to soluble GAG fragments,

concentration of soluble collagens was analyzed via hydroxyproline-assay and soluble fibronectin fragments in supernatants of mono-, co- and tricultures were determined via ELISA. OA-cartilage explant culture setups showed no significant differences in concentration of soluble fibronectin fragments between mono-, co- and tricultures. In contrast to soluble GAG, release of soluble fibronectin fragments revealed no additive effect of OA-cartilage explants and fibrin gel embedded cells. However, a decrease in fibronectin fragment concentration was observed for BMSC and mixed cocultures during culture time. Since both culture conditions revealed similar fibronectin levels in supernatants, soluble fragments have to be newly synthesized and released mainly by fibrin gel embedded cells and not by OA-cartilage explants.

Sox9 is a major chondrogenic transcription factor (de Crombrugghe, Lefebvre et al. 2000; Hattori, Muller et al. 2010), which is known to promote *COL2A1* gene expression and thus chondrogenic differentiation of BMSC during limb bud development. Furthermore, Sox9 is expressed in differentiated chondrocytes where it maintains the articular phenotype (Lefebvre, Behringer et al. 2001; Akiyama, Chaboissier et al. 2002). Therefore, Sox9 gene and protein expression as well as activity, with respect to phosphorylation status, were analyzed in mono-, co- and triculture conditions. In general, co- and tricultures with OA-cartilage explants were without effect on Sox9 gene and protein expression or phosphorylation status. Because of this, a Sox9 independent signaling system might be responsible for inhibitory effects on collagens and alterations in differentiation of cocultured BMSC.

PTHrP was another important soluble factor, which was identified to be involved in chondrogenic differentiation processes, especially in regulation of *COL10A1*. Fischer et al. could show that supplementation of chondrogenic medium with PTHrP significantly decreased the *COL10A1* mRNA level in MSC pellets during chondrogenesis compared to pellets without supplementation (Fischer, Dickhut et al. 2010). Consequently, in the present study PTHrP level was determined by ELISA and was found to be strongly upregulated in the supernatant of all mono-, co- and tricultures during culture time. Notably, co- and triculture conditions with OA-cartilage explants had no effect on PTHrP compared with respective monocultures. Likewise, mixed cultures of BMSC and chondrocytes revealed no differences in PTHrP level. This suggests, that in the present study *COL10A1* gene expression was regulated in a PTHrP-independent way mediated by other cartilage-released factors. Possible candidates are growth factors (bFGF, IGF-1, PDGF) (Mastrogiacomo, Cancedda et al. 2001) members of the CCN family (CYR61, CTGF, WISP-2 and -3) (Bohme, Conscience-Egli et al. 1992) or hormones (thyroxin) (Schutze, Noth et al. 2005) that are also likely involved and that control chondrocyte differentiation through an independent pathway in parallel to PTHrP.

Another soluble factor analyzed in this study was bFGF, which revealed no differences between mono- and cocultures of BMSC or chondrocytes and an upregulation in mixed tricultures for day 28 only. Thus, we suggest no crucial role of bFGF in OA-cartilage controlled regulation of chondrogenic differentiation.

Screening for still unknown factors in the supernatant via LC-MS covers the whole secretome of cells and opens a broad spectrum of possibilities for detection of factors responsible for alternative regulation mechanisms involved in OA-progression and regulation. Consequently, analysis of supernatants from BMSC mono- and cocultures and cell-free OA-cartilage with or without fibrin was performed by LC-MS. Differentially expressed factors from coculture supernatants like ribonuclease 4, MMP1, stromelysin (MMP3) (Murab, Chameettachal et al. 2013), serum amyloid (de Seny, Cobraiville et al. 2013), lysozym C, titin, extracellular superoxid dismutase (Regan, Flannelly et al. 2005), chondroadherin, fibronectin, prolargin and IL-8 were identified (Rosenthal, Gohr et al. 2011; Ikeda, Ageta et al. 2013). All of these factors were already known to be present in OA-diseased microenvironment and some of them are reported to be involved in regulation of cartilage homeostasis. These factors amongst others in the supernatant of OA-cartilage cocultures possibly modulate chondrogenic differentiation of BMSC as well as ECM degradation. To clear questions like why cartilage has no intrinsic healing capacity or why BMSC become hypertrophic in the end of chondrogenic differentiation, several of the mentioned proteins were further analyzed in this study like fibronectin (via ELISA of supernatants see **3.1.7**), IL-8 (via ELISA of supernatants see **3.1.7** and stimulation with IL-8 see **4.3**) and MMP3 (gene expression after stimulation of monocultures see **4.3.1.2**).

To gain insight into the microenvironment created *in vitro* by OA-cartilage explants, composition of supernatants was analyzed for factors well known to be produced by OA-cartilage, such as proinflammatory cytokines and chemokines. Additionally to ECM degradation products IL-1ß, IL-6 and IL-8 were inspected, which have been shown to be secreted by OA-chondrocytes and which are considered to contribute to OA-pathogenesis (Goldring 2000).

OA-cartilage clearly increased the level of proinflammatory cytokines IL-1ß, IL-6 and IL-8 in supernatants of all co- and triculture regimens. BMSC and mixed cultures revealed a significant higher IL-1ß level in co- and tricultures with OA-cartilage as monocultures. Initial IL-1ß concentration in supernatants of chondrocyte cultures was high in both culture conditions and exhibited a significant downregulation only in monocultures. IL-1ß seemed to be either induced or produced by OA-chondrocytes. To clarify the point whether cytokines were produced by the cells embedded in fibrin gel or by local chondrocytes in the explants, analysis of supernatants from cell-free cartilage explants were performed. Supernatants of cell-free normal and OA-cartilage revealed only low levels

of IL-1ß, which however significantly increased in OA-cartilage supernatants during culture time. Thus, a major part of IL-1ß must be either produced by co- and tricultured cells in the fibrin gel or induced in resident cells of the explant. As IL-1ß is a known suppressor of *COL2A1* gene expression in OA-cartilage, it might be responsible for reduced *COL2A1* gene expression in OA-cartilage co- and tricultures (Fernandes, Martel-Pelletier et al. 2002).

Further, IL-6 and IL-8 were significantly increased at day 7 in all cartilage explant co- and tricultures compared with monocultures. With exception of normal cartilage at day 7, IL-6 was not detectable in cell-free cartilage explants. As a result, high IL-6 levels were either produced by co- and tricultured cells in the fibrin gel or induced in local cells of OA-cartilage explants. It is known that traumatic injury of joints causes an abrupt release of proinflammatory cytokines (for example IL-1ß, IL-6 and IL-8) into the synovial fluid and increases the risk of developing osteoarthritis (Cameron, Buchgraber et al. 1997; Irie, Uchiyama et al. 2003). In line with these reports, it is likely that IL-6 serves as a mediator which coordinates responses to cartilage injury. For that reason, IL-6 may be beneficial in the early phase of osteoarthritis, because it reduces proteoglycan and collagen loss in cartilage and may support cartilage repair (van de Loo, Kuiper et al. 1997).

Initially, IL-8 was profoundly increased in supernatants of co- and tricultures and it was also secreted in both cell-free explant cultures. During culture time, IL-8 secretion was reduced in all co- and tricultures and cell-free OA-explants. In contrast, concentration of IL-8 in supernatants of cell-free normal cartilage was significantly higher at day 28 compared to cell-free OA-cartilage. Nevertheless, since IL-8 concentration was profoundly elevated in supernatants of co- and tricultures compared to supernatants of cell-free explant cultures, IL-8 content in co- and triculture supernatants has to be from explants and cocultured cells in an additive manner. This could be interpreted as a trial for cartilage repair and suggests, that IL-8 is required to initiate tissue repair *in vivo* (Mishima and Lotz 2008). IL-8 is a known chemotactic chemokine, which induces metabolic activities in cells, and initiates cell migration in chondrocytes and MSC. In addition to IL-6, IL-8 induces release of IL-1ß (Yu, Sun et al. 1994), which might be in part the cause of rising IL-1ß concentrations in the supernatant of cocultures during the culture time line. Thus, IL-6 and IL-8 were possibly replaced by IL-1ß during establishment of chronic inflammation in OA.

In summary, OA-cartilage clearly increased the level of pro-inflammatory cytokines like IL-1ß, IL-6 and IL-8 in supernatants of all culture regimens while IL-10 and TNF-α were not detectable. High IL-1ß, IL-6 and IL-8 amounts were either produced by co- and tricultured cells embedded in fibrin gel or induced in local cells of OA-cartilage explants. Further, levels of IL-1ß, IL-6 and IL-8 in cell-free normal compared with OA-cartilage supernatants revealed no differences at the beginning of culture time. This could hint to an OA-independent rise of pro-inflammatory cytokines induced by injury of the

cartilage by punching the explants. Increase of IL-1ß in cell-free OA-cartilage explant supernatants during culture time might be due to an altered metabolism of resident OA-chondrocytes, which might mimic chronically inflammation *in vitro*. Additionally, alterations in IL-6 and IL-8 concentrations might be one reason for diminished cartilage regeneration potential during OA (Tsuchida, Beekhuizen et al. 2012). However, the underlying mechanisms, which induce increased release of cytokines, are still unknown and further analysis is necessary to explain the influence of OA-cartilage on chondrogenesis.

Despite to chondrogenesis inhibiting cytokines, it is theoretically possible that TGF-ß3, which is an important initiator of chondrogenic differentiation in this experimental setup, is bound to soluble ECM molecule fragments – especially GAGs – and thus is not or only in low amounts available for co- and tricultured cells (Albro, Nims et al. 2013). To measure the concentration of TGF-ß3, a modification of the present coculture model would be necessary. Chondrogenic medium without TGF-ß3 could be analyzed for newly produced or released TGF-ß3 in supernatants via ELISA, keeping in mind, removing of TGF-ß3 would severely change differentiation of BMSC. Therefore, TGF-ß1, which is known to be released by chondrocytes, could be measured in supernatants of mono-, co and tricultures. This approach would elucidate if TGF-ß is actively bound to soluble ECM molecule fragments leading to a growth factor deficit, which influences differentiation of co- and tricultured cells (Grassel, Rickert et al. 2010).

Biomechanical properties of the newly formed tissue are most important for successful cartilage repair because abnormal mechanical loading is a major factor leading to alterations in chondrocyte metabolism and OA promotion (Guilak 2011). OA-cartilage is even suggested to have a different sensing of the mechanical environment compared with normal cartilage (Salter, Millward-Sadler et al. 2002). Additionally, an inappropriate response to mechanical stress was shown by resident cell populations what might be essential in disease progression (Millward-Sadler, Wright et al. 2000).

Several studies proved that collagen content of ECM determines biomechanical properties of cartilage tissue. In this line, influence of OA-cartilage coculture on biomechanical properties of newly generated matrix tissue was tested. Overall, the ECM of BMSC and mixed co- and tricultures with articular OA-cartilage seemed to be of minor quality assigned by a diminished loading capacity of matrix to withstand mechanical stress, i.e. load, increased porosity and fluid exchange as well as stiffness. This was indicated by decreased Young´s modulus and Aggregate modulus at equilibrium particularly in cocultures of BMSC and tricultures of mixed cells compared with respective controls. Results of hydraulic permeability allowed hardly a conclusion suggesting that influence of OA-cartilage coculture plays only a minor role on porosity. Viscoelastic properties are necessary for an efficient and equal load distribution on hyaline cartilage and are provided by the very specific

mixture of fluid (water) and ECM (collagens and proteoglycans). Under stress, load is carried by interstitial fluid, which is then displaced from the matrix, which carries the load after water is gone from the tissue. Water content and its retention in the matrix are physiologically important together with the stability and integrity of the matrix. Fluid and ECM together determine biomechanical properties of articular cartilage, as a result cartilage is best seen as a biphasic structure (Pearle, Warren et al. 2005).

Cocultured chondrocytes showed increased Young´s modulus at 40% to 50% strain indicating a matrix with better quality compared with BMSC and tricultures. In summary, coculture seemed to produce a highly porous matrix, which is less resistant to load and had diminished biomechanical and biochemical properties. Cartilage derived factors seemed to inhibit in particular the *de novo* collagen protein expression in OA-cartilage cocultures, whereas GAG deposition remained unchanged.

OA-chondrocytes and OA-cartilage release a variety of regulatory growth and signaling molecules, which create a specific hyaline OA-cartilage microenvironment that influences ECM production of chondrocytes and might alter differentiation of BMSC (Grassel and Ahmed 2007). As results of this study are not significantly pointing to clear effects on biomechanical properties induced by cartilage coculture, more biomechanical tests are mandatory. Presumably, alterations in biomechanical properties are mostly due to reduced biosynthesis of collagens, which impairs the formation of proper interconnected fibrillar collagen networks and has a profound effect on the structural integrity of the ECM (Leyh, Seitz et al. 2014 a).

Further it was of interest, whether differentiated chondrocytes in direct cell-to-cell contact with undifferentiated BMSC improve or enhance effects of OA-cartilage explants on BMSC. In a mixed cell population, BMSC contribute positive to total cell number and thus less chondrocytes are necessary, what prevents de-differentiation of chondrocytes due to elongated expansion. Additionally, several studies already reported a synergistic effect of mixed BMSC and chondrocyte cultures concerning induction of differentiation, prevention of hypertrophy, reduction of fibrosis and anti-inflammatory features (Bian, Zhai et al. 2011). A mixed OA-cartilage regimen (triculture) was thus included to the present study to determine whether OA-chondrocytes residing in their native environment have different effects than chondrocytes, which were isolated from their pathological environment (Aung, Gupta et al. 2011). In general, collagen gene and protein expression as well as biomechanical properties of tricultured mixed populations were more similar to cocultured BMSC than to chondrocytes (Leyh, Seitz et al. 2014 a). It seemed that chondrocytes in the mixed tricultures with OA-cartilage explants do not seriously alter BMSC metabolism. Therefore, it is suggested that tricultures with OA-cartilage explants resemble rather the phenotype of cocultured BMSC than that of cocultured chondrocytes.

Results of the present study hint to soluble factors, present in the microenvironment of OA-cartilage explants produced by resident chondrocytes and probably as well from fibrin gel embedded chondrocytes in mixed tricultures, which influence matrix gene and protein expression. It was not possible to determine whether secreted factors, or newly synthesized ECM components detected in mixed culture setups were released by BMSC or chondrocytes. Anyhow, both cell types can function as trophic sources what suggests that chondrocyte derived factors together with factors from BMSC influence differentiation and matrix composition in mixed mono- and tricultures synergistically.

In summary, mRNA and ECM production was inhibited in co- and tricultures with OA-cartilage compared with monocultures. Reduction of biochemical qualities resulted in diminished biomechanical properties (like stiffness or elasticity) of the newly generated matrix. Screening with LC-MS and analysis of supernatants confirmed alterations in concentration of cytokines/chemokines and soluble ECM fragments. A profound upregulation of IL-1ß, IL-6 and IL-8 was detected in co-and tricultures.

Though the inflammatory catabolic effects of OA-cartilage are prevalent, there might be some benefits, like inhibition of collagens I, III and X with respect to the influence of cartilage on BMSC metabolism.

5.3 Part II: Microenvironment of subchondral bone coculture influences BMSC differentiation and ECM production

Until now, it is not comprehensively understood why cell-based therapies especially in OA-joints have only poor healing and repair outcome. Responsible are presumably altered microenvironmental cues from surrounding cartilage and underlying subchondral bone tissue. Because of increased cartilage loss during OA-progression denuded bone is exposed to factors from synovial fluid. So far, there is only scarce knowledge about the impact of OA-subchondral bone on chondrogenic differentiation of BMSC and their matrix forming capacity. On that account, the present study examined the impact of subchondral bone from OA affected joints on BMSC and chondrocytes assuming that factors secreted by OA-osteoblasts, osteocytes or osteoclasts modulate metabolic properties. Chondrogenic differentiation potential and matrix forming capacity might be affected by these modulating factors. To gain a detailed insight in mechanisms taking place when BMSC are implanted into a full thickness osteochondral defect, a reproducible coculture model with subchondral bone was established.

Achievement of the second part of this thesis was that OA-subchondral bone influences gene expression and protein production of collagens I, II, III and X in nearly all co- and triculture set ups and alters biomechanical properties presumable because of released regulatory factors (Leyh, Seitz et al. 2014 b).

In detail, gene expression of *COL1A1* and *COL2A1* was significantly reduced in BMSC cocultured with OA-subchondral bone. Additionally gene expression of *COL2A1* was significantly upregulated during culture time in BMSC monocultures but not in cocultures with OA-subchondral bone, which could be interpreted as an inhibition of chondrogenic differentiation of BMSC induced by OA-subchondral bone explants. Further gene expression of *COL3A1* was significantly reduced in BMSC and mixed co- and tricultures with OA-subchondral bone explants compared to monocultures. With respect to chondrogenic differentiation of BMSC, suppression of collagen I and III gene expression might be positive. In addition, a specific effect on collagen X was observed as coculture of BMSC and chondrocytes with subchondral bone significantly inhibits gene expression of *COL10A1* compared to monocultures. Anyhow, coculture with OA-subchondral bone did not prevent *COL10A1* increase in BMSC during culture time. Interestingly, chondrocyte mono- and cocultures showed a significant downregulation of *COL2A1, COL3A1* and *COL10A1* (in coculture only) during culture time, indicating a general downregulation of collagen gene expression.

As a key experiment, gene expression studies were repeated with normal ASC cocultured with OA- and normal subchondral bone explants. Alterations in gene expression of collagens were confirmed for ASC cocultured with OA-subchondral bone, where *COL1A1, COL3A1* and *COL10A1* gene

expression was significantly inhibited compared to monocultures. Coculture experiments with normal subchondral bone explants revealed an inhibition of *COL3A1* and *COL10A1* gene expression in ASC. *COL1A1* and *COL2A1* gene expression of ASC cocultures with normal subchondral bone was similar to ASC monocultures. This indicates that inhibition of *COL1A1* and *COL2A1* gene expression in ASC cocultures is either related to factors specific for OA or to variations due to the age or trauma of subchondral bone explant donors. In contrast, gene expression of *COL10A1* was decreased in OA- and normal subchondral bone co- and tricultures compared to monocultures, suggesting an OA independent regulatory mechanism initiated by subchondral bone in general. Interestingly, *COL10A1* gene expression was not induced in ASC cocultured with normal subchondral bone during culture time. Since it is known that paracrine coculture of BMSC with normal articular cartilage results in a specific collagen X suppression (Ahmed, Dreier et al. 2007) it appears that this effect is caused by yet unidentified soluble factors, which are in common to both tissue types - cartilage and subchondral bone.

Corresponding to gene expression, analysis of biochemical composition of newly generated ECM confirmed inhibition of collagen protein production. In detail, synthesis of all investigated collagens was suppressed in BMSC cocultures, collagen I and III in mixed and collagens II and III in chondrocyte co- or tricultures with OA-subchondral bone. Notably, collagen X production was not quantified but investigated via immunofluorescent staining and revealed only low staining in all culture conditions.

Taken together, these results hint to a general effect of subchondral bone co- and tricultures on matrix macromolecule synthesis and not specifically on chondrogenic differentiation of BMSC.

The interaction between subchondral bone and cartilage as well as the interaction between osteoblasts and chondrocytes is not completely understood until now. However, there are likely important regulatory events during cartilage regeneration initiated by osteoblasts. Jiang et al. revealed a suppression of certain specific markers in cocultures of chondrocytes and osteoblasts like diminished GAG and collagen II expression, deposition or mineralization of ECM (Jiang, Nicoll et al. 2005). Since collagens are known to exhibit a half-life of more than 100 years *in vivo* (Verzijl, DeGroot et al. 2000), the discovered decrease of deposited collagens is not likely due to enhanced degradation of mature collagens. This was supported in the present study by a hydroxyproline assay, which did not reveal increased levels of soluble collagen in culture supernatants. Thus, a reduced synthesis was presumably the major factor for reduced collagen deposition and diminished accumulation in the ECM during co- and triculture time. Collagen mRNA has a half-life of usually only several hours (Dozin, Quarto et al. 1990) hence, collagen protein levels especially at the end of differentiation time are different from mRNA level and not mandatory mirrored at the same time.

Additionally to collagens, GAG content in lysates was determined. All three OA-subchondral bone conditions revealed a significantly diminished GAG content at day 7 while at the end of culture time no differences between mono and co- or tricultures were detectable. It is known that coculture of OA-chondrocytes with OA-osteoblasts induces inhibition of aggrecan production and increases significantly synthesis of MMP3 and MMP13 (Sanchez, Deberg et al. 2005). This might be an explanation for the observed differences in GAG content of coculture lysates at day7. Anyhow, the initial differences between mono- and co- or tricultures were compensated until day 28 where no differences were detectable. This finding was assured by alcian blue histology, which did not reveal differences between culture conditions at the end of culture (day 28).

Notably, despite GAG contents of co- and triculture lysates were reduced only at day 7, increased levels of soluble GAGs were detected in supernatants of BMSC, chondrocyte and mixed co- and tricultures with OA-subchondral bone compared to monocultures. Experiments with cell-free OA-subchondral bone explants showed a similar soluble GAG level than co- and tricultures and normal cell-free explants released even significantly higher amounts of soluble GAGs into their supernatant. Thus, enhanced GAG levels in co- and tricultures might stem from both - explants and cells in the fibrin gel. This means an additive effect is responsible for observed differences in GAG level and not a true increase in degradation of proteoglycans.

To understand which factors are in detail responsible for the observed inhibition of ECM components, further analysis of supernatants were performed. Degradation of ECM is typical for OA progression and is the origin of high concentration of ECM molecule fragments in the tissue microenvironment. These fragments on the other hand promote further cartilage loss.

No differences in total soluble collagen content with respect to hydroxyproline were found in supernatants of mono-, co- or tricultures with OA-subchondral bone. Additionally, also no alterations in soluble fibronectin fragments were detected.

One of the most important transcription factors during chondrogenesis is Sox9. Consequently, gene expression of this factor was investigated. Osteoblasts from OA-subchondral bone are reported to inhibit gene expression of *SOX9* in cocultured chondrocytes (Sanchez, Deberg et al. 2005). This appears to be in contrast to results from this study because no significant differences in Sox9 gene and protein expression or activity were observed between mono-, co- and tricultures with OA-subchondral bone. One possible reason for these discrepancies might be a different time line as Sanchez et al. observed Sox9 inhibition only after 4 days of coculture while the effect was abolished after 10 days (Sanchez, Deberg et al. 2005). Notably, in the present study *SOX9* gene expression of chondrocytes was downregulated during culture time along with *COL2A1* gene expression, indicating

an inhibitory effect of OA-subchondral bone on newly synthesized ECM molecules and proper matrix formation. Very important was the observation that *SOX9* gene expression in cocultures of ASC with normal subchondral bone was significantly induced during culture time and was drastically increased compared with mono- or OA-subchondral bone cocultures. This clearly hints to a positive, chondrogenesis promoting effect of normal subchondral bone on ASC.

A known suppressor of hypertrophy in BMSC and chondrocytes is PTHrP (Jiang, Leong et al. 2008; Aung, Gupta et al. 2011), which was not affected in the present study by co- or triculture with OA-subchondral bone but clearly increased over culture time. An increase of PTHrP indicates induction of chondrogenic differentiation in BMSC and promotes stabilization of a chondrogenic phenotype, partly by regulation of *COL10A1* (Fischer, Dickhut et al. 2010). With respect to downregulation of *COL10A1* gene expression and upregulation of PTHrP, the present study may indicate that coculture with subchondral bone stabilizes a chondrogenic phenotype and does not enhance hypertrophy. However, the collagen X inhibitory effect likely is unrelated to PTHrP, since no differences between monocultures and subchondral bone co- and tricultures were observed. Nevertheless, a decrease in *PTHrP/PTH*-receptor gene expression was reported to be induced in chondrocytes by cocultured osteoblasts (Sanchez, Deberg et al. 2005). Further tests would be necessary to confirm whether a regulation of the *PTHrP/PTH*-receptor is altered by co- or triculture with subchondral bone.

Another possible factor which might interfere with chondrogenic differentiation is bFGF, a negative regulator of chondrogenesis and chondrocyte differentiation known to suppress chondrocyte maturation and hypertrophy (Szuts, Mollers et al. 1998; Nagai and Aoki 2002). Cocultures of BMSC, ASC and chondrocytes with OA-subchondral bone and cocultures of ASC with normal bone induced bFGF release at the early phase of coculture. This is in line with a previous study, which showed that bFGF secretion by articular chondrocytes was restricted to the first seven days of culture (Fischer, Dickhut et al. 2010). During pathophysiological situations like injuries of tissue or during chronic inflammation, bFGF which is bound to heparin- and chondroitin sulfate molecules of ECM proteoglycans (Smith, West et al. 2007), might be released together with other growth factors (D'Amore 1990).

Cell-free OA- and normal subchondral bone explants also released bFGF into their supernatant thus in cocultures it might originate from both explants and fibrin gel embedded cells additionally. Moreover, OA-subchondral bone explants released more bFGF compared to normal subchondral bone explants indicating an induction of bFGF release either due to an altered disease status (OA vs. trauma) or because of different mean age of respective tissue donors. Analysis of ASC cocultures with OA- and normal subchondral bone confirmed induction of bFGF release specifically in the presence of OA subchondral bone cells. Since Weiss et al. revealed that bFGF stimulation inhibits the

TGF-ß responsive *COL2A1* and *COL10A1* gene expression of BMSC (Weiss, Hennig et al. 2010), approaches should be performed to figure out whether these effects could be induced in monocultures by bFGF supplementation.

Although many studies have examined effects of factors released from cartilage and chondrocytes on chondrogenesis of BMSC, only few have focused on the influence of pro-inflammatory cytokines from OA-subchondral bone on chondrogenic differentiation and matrix forming capacity of BMSC (Dozin, Quarto et al. 1990; Sanchez, Deberg et al. 2005). Pro-inflammatory cytokines are also produced by BMSC and OA-chondrocytes and are supposed to contribute to OA-pathogenesis (Fernandes, Martel-Pelletier et al. 2002). Hence, pro-inflammatory cytokines like IL-1ß, IL-6 and IL-8 were analyzed in the present study. Coculture with OA-subchondral bone strongly induced the release of these cytokines / chemokines into the culture supernatant in all culture conditions. This was most prevalent in the early phase of culture except for IL-1ß, which remained induced throughout the culture period. Notably, comparison of inflammatory factors released by cell-free OA- and normal subchondral bone explants also revealed differences. Firstly, normal subchondral bone released less IL-1ß than OA- subchondral bone and IL-1ß release was not induced in ASC by coculture with normal subchondral bone. Secondly, release of IL-6 and to some extent IL-8 was higher in cocultures of normal than of OA-subchondral bone.

One reason for these differences in cytokine expression between normal and OA-subchondral bone might be diversity of age and health status of donors for both tissues. Normal subchondral bone explants were obtained from younger trauma patients and reveal a differently composed subchondral bone ECM and an increased osteoblast number with a different metabolism, which also might lead to a higher bone-forming capacity, compared to elderly OA-subchondral bone donors (Luder 1998; Kouri and Lavalle 2006). Therefore, it is likely that cytokine expression of osteoblasts and importantly release of ECM bound cytokines is altered in normal osteoblasts and subchondral bone compared to old OA-osteoblasts and OA-subchondral bone.

It was demonstrated that OA-osteoblasts clearly secreted a high level of mediators as IL-6 which are involved in structural matrix changes, decrease aggrecan production in chondrocytes and play an important role in bone remodeling. This suggests that these cytokines are partly responsible for suppression of collagen and GAG synthesis in the present study, what would be in line with data from literature (Sanchez, Deberg et al. 2005; Ryu and Chun 2006)

Reduced synthesis of matrix components likely influences mechanical properties of newly generated matrix tissue (Poole, Kojima et al. 2001; Hollander, Dickinson et al. 2010). Therefore, biomechanical properties of newly formed ECM of mono-, co- and tricultures with OA-subchondral bone were

analyzed. BMSC and mixed cultures showed significantly reduced Young´s modulus in OA-subchondral bone co- and tricultures whereas aggregate modulus was reduced in all co- and tricultures compared to monocultures. No clear trend was observed for hydraulic permeability of mixed tricultures and chondrocyte cocultures with OA-subchondral bone. In line with these findings, a study from Erickson et al. showed that BMSC seeded hydrogel constructs possess significantly lower mechanical properties than chondrocyte seeded hydrogel constructs (Erickson, Huang et al. 2009). OA-subchondral bone coculture of BMSC leads to reduced loading capacity of matrix and increased permeability. In contrast, a trend to an increased Young´s modulus depending on applied strain was detected for OA-subchondral bone cocultured chondrocytes while hydraulic permeability and aggregate modulus remained unchanged.

A study of Li et al. revealed that OA-chondrocytes in a 3D culture demonstrate a significantly higher expression of inflammatory genes even after 3 weeks of culture. Consequently, their collagen network organization is perturbed resulting in a decreased stiffness and strength of matrix (Li, Davison et al. 2012). This alteration of the OA-chondrocyte phenotype would explain why no differences between mono- and cocultures of OA-chondrocytes with OA-subchondral bone were detectable in this study. Both culture conditions seemed to produce a similar matrix with respect to mechanical properties. Anyhow, this matrix is not comparable to an ECM synthesized by normal chondrocytes.

According to present data, coculture with OA-subchondral bone leads to weaker mechanical properties of BMSC and mixed cultures. In a previous study, our group indicated distinct modulating influences of OA-cartilage explants, which affect collagen composition of ECM in co- and tri-cultured cells leading to impaired biochemical and mechanical matrix properties caused by fibrillar network alterations. In detail, Young´s modulus and Aggregate modulus were decreased particularly in cocultures of BMSC and tricultures of mixed cells compared with respective controls, whereas hydraulic permeability was not influenced (Leyh, Seitz et al. 2014 a).

Differentiated chondrocytes in close cell-to-cell contact with undifferentiated BMSC could improve or enhance inhibitory effects of subchondral bone explants on BMSC. To answer this question mixed mono- and tricultured populations of OA-chondrocytes and BMSC with or without OA-subchondral bone were included. Overall, collagen gene expression was mainly not affected in OA-subchondral bone tricultures compared with mixed monocultures, while protein expression and biomechanical properties resembled rather the effects of BMSC cocultures than of chondrocyte cocultures. Presumably, BMSC metabolism in mixed cultures is not altered by chondrocytes in tricultures with subchondral bone explants.

All BMSC were obtained from OA-affected donors, thus key experiments were repeated with normal ASC as a reference. With respect to collagen gene expression together with cytokine, bFGF and GAG release, no striking differences between subchondral bone cocultures of both cell types were observed. As a result no effect of cell type (ASC opposed to BMSC), donor age or disease status (normal opposed to OA) is suggested on chondrogenic differentiation and matrix forming capacity.

In summary, collagen mRNA expression and production of collagens and GAG (only temporary) was inhibited in co- and tricultures with OA-subchondral bone compared with monocultures. Experiments with normal ASC confirmed inhibitory effects for OA-subchondral bone, and co- and triculture with normal cartilage or subchondral bone explants showed no or only reduced inhibitory effects on chondrogenic differentiation or collagen gene expression which might either hint to disease status induced effects (OA vs. trauma) or to a effect caused by different mean age of cell and tissue donors. Further, repetition of key experiments with subchondral bone from trauma patients revealed a relation of effects to OA age or trauma, because IL-1ß, bFGF and GAG release of cell-free OA-subchondral bone and normal bone were different. This was confirmed by results of gene expression and cytokine release of OA- versus normal subchondral bone cocultures. Therefore, at least some of the observed effects might be due to OA, trauma or differences in mean age of donors.

Additionally a profoundly upregulation of IL-1ß, IL-6 and IL-8 was detected in co-and tricultures and might partly mediate effects on newly formed extracellular matrix, leading to reduction of biochemical properties and diminished biomechanical properties.

5.4 Part III: Stimulation with IL-1β, IL-6 and IL-8 during chondrogenesis

Cells in the neighborhood of OA-cartilage and subchondral bone are exposed to a chondrogenesis inhibiting microenvironment. In part I and II inhibitory effects of OA-cartilage and OA-subchondral bone on collagen gene and protein expression and in case of OA-subchondral bone also temporary on GAG production were observed in addition to alterations in biomechanical properties. Presumably, secreted factors, primarily high levels of pro-inflammatory cytokines mainly IL-1ß, IL-6 and IL-8 in the first days of coculture were - beside other factors - responsible for inhibition of differentiation and ECM production. It is known that traumatic injury of joints causes an immediate rise in pro-inflammatory cytokines like IL-1ß or IL-6 in synovial fluid (Lu, Evans et al. 2011). These cytokine levels remained high for several days and decreased within 7 days to levels found in chronic OA (Irie, Uchiyama et al. 2003). To create artificial OA-conditions, stimulation setups of fibrin gels were performed with BMSC, mixed and chondrocyte monocultures. Monocultures were supplemented with IL-1ß, IL-6 or IL-8 in concentrations giving best results in a previous tested dose-response curve. Readout parameters were gene expression of *COL1A1, COL2A1, COL3A1*, *COL10A1, ACAN*, *MMP2, MMP3* and *MMP13* at days 7 and 28 as well as ECM production at day 28, with respect to deposited GAG, collagen I, II and III.

In detail, monocultures kept in chondrogenic medium containing TGF-ß3 and dexamethasone were supplemented for the first 7 days of culture with IL-1ß, IL-6 or IL-8. Stimulation with IL-6 and IL-8 was performed with similar concentrations previously found in co- and triculture supernatants, while stimulation with IL-1ß was performed with a higher IL-1ß concentration. It was adapted because a previous dose-response curve showed clearer defined changes in collagen gene expression patterns for higher IL-1ß concentrations. Nevertheless, this previous dose-response study revealed no differences in effects induced by stimulation with a higher IL-1ß concentration compared with a lower IL-1ß concentration. Stimulation of monocultures was performed for the first 7 days of culture, which was in contrast to the time dependent pattern of IL-1ß in the supernatants of co- and triculture explants. IL-1ß increased in supernatants of co- and triculture explants during culture time and was significant only at day 28. Notably, IL-1ß represents a factor of chronically inflammation, which probably is present in supernatants of co- and tricultures during the whole culture time (Maldonado and Nam 2013).

Stimulated monocultures revealed a similar phenotype like under OA-coculture conditions. In general, stimulation of monocultures with IL-1ß resulted in an inhibition of *COL1A1, COL2A1* and *COL10A1* gene expression in BMSC and mixed cultures whereas chondrocytes showed an induction particularly at the end of the culture period. This confirms inhibitory effects of IL-1ß on BMSC and mixed cell populations and could be interpreted as an anabolic effect of IL-1ß on OA-chondrocytes.

They are known to exhibit a phenotype switch to repair mode in order to replace destroyed cartilage accompanied with increased collagen synthesis, possibly in part induced by IL-1ß stimulation. Fan et al. demonstrated that OA chondrocytes are less catabolically stimulated by cytokines than normal cells – probably because of an initially high basal level of catabolic gene expression (Fan, Bau et al. 2005). In contrast, *COL3A1* gene expression in IL-1ß stimulated BMSC monocultures was significantly upregulated while it was significantly reduced in mixed and chondrocyte monocultures compared to unstimulated controls. *COL10A1* gene expression decreased in BMSC and mixed cultures induced by IL-1ß supplementation. Contrary, *COL10A1* gene expression was significantly upregulated in chondrocytes what suggests an IL-1ß initiated shift of chondrocytes from a stable articular to a hypertrophic phenotype. This discrepancy between BMSC and chondrocytes might be a result of the IL-1ß induced inhibition, which prevents not only chondrogenic but also terminal differentiation of BMSC.

In accordance with collagen gene expression, IL-1ß stimulation of BMSC and mixed monocultures induced in general a significant downregulation of *ACAN* gene expression, which is in consent with resent literature (Fernandes, Martel-Pelletier et al. 2002). However, *ACAN* gene expression in stimulated chondrocytes was upregulated at day 28. This is in line with results from Salter et al. suggesting involvement of IL-1ß in a chondroprotective signaling cascade, which is defined by increasing *ACAN* mRNA in chondrocytes (Salter, Millward-Sadler et al. 2002).

Additionally, gene expression of *MMP2, MMP3* and in case of BMSC and chondrocytes also *MMP13* was principally induced during IL-1ß stimulation. This might hint to migration and inhibition of differentiation.

Changes in gene expression induced by stimulation with IL-1ß showed most effects during stimulation at day 7, but not at day 28, indicating that they were reversed at the end of cultivation period. IL-1ß increased from the beginning of OA-coculture and kept a permanently high level. Therefore, it is likely, that IL-1ß induced effects are continuing during the whole culture time *in vitro* in coculture setups.

Overall, in this study stimulation with IL-6 resulted in a comparable outcome than stimulation with IL-1ß. Further, gene regulation of OA-cocultures and stimulation setups of monocultures showed similarities. In general, monocultures stimulated with IL-6 revealed a downregulation of *COL1A1, COL2A2* and *COL3A1* gene expression in comparison to respective unstimulated controls. Again, downregulation of *COL2A2* could be interpreted as a differentiation inhibiting effect, while downregulation of collagen gene expression in general might facilitate cell migration and could be a hint for enhanced proliferation. Gene expression of *COL10A1* was not affected in IL-6 stimulated

BMSC and mixed monocultures. In contrast, gene expression of *COL10A1* in chondrocytes was significantly reduced during IL-6 stimulation compared to respective unstimulated controls. This might hint to inhibition of hypertrophy in chondrocytes during IL-6 treatment (Goldring, Otero et al. 2008).

Stimulation of monocultures with IL-6 inhibited *ACAN* gene expression what is in concert with recent literature. IL-6 is able to amplify effects of IL-1ß stimulation (Fernandes, Martel-Pelletier et al. 2002) nevertheless, IL-6 initiated effects on aggrecan expression were less pronounced as after IL-1ß stimulation. IL-6 was found to be involved in bone and cartilage crosstalk and studies showed that IL-6 in combination with other cytokines could switch osteoblasts to a sclerotic phenotype (Sanchez, Deberg et al. 2005). However, IL-6 was thought to be beneficial in the early phase of experimental arthritis because it reduces cartilage proteoglycan loss (van de Loo, Kuiper et al. 1997).

MMP2 gene expression was not affected after IL-6 stimulation while *MMP3* and *MMP13* expression were mainly downregulated in all monocultures. Auxiliary, IL-6 is reported to induce TIMP production (Tsuchida, Beekhuizen et al. 2012) and thus is involved in feedback mechanisms by limiting proteolytic damage of cartilage matrix. Taken together, these IL-6 induced effects can be interpreted as a trial for tissue repair or at least for protection of ECM from degradation. Anyhow, the explicit role of IL-6 during OA and inflammation is critically discussed in literature, because it can promote both pro- and anti-inflammatory effects (Dinarello 2010).

Since IL-8 was detected in LC-MS analyses as one of the strongest differentially expressed factors in co- and triculture supernatants with OA-cartilage and OA-subchondral bone, stimulation of monocultures with IL-8 was performed. Taken together, IL-8 stimulation provoked only modest changes in collagen gene expression. Controversial effects of IL-8 on gene expression were demonstrated for BMSC and mixed cultures while chondrocytes were unaffected. In detail, IL-8 induced a decrease in *COL1A1, COL2A1* and *COL3A1* gene expression of BMSC, while *COL10A1* was only affected in mixed cultures. Additionally *ACAN* was significantly upregulated in BMSC monocultures during stimulation with IL-8 and significantly downregulated at the end of culture time. Mixed and chondrocyte monocultures did not respond on IL-8 stimulation. Possibly downregulation of ECM production favors BMSC migration and hints to BMSC proliferation instead of differentiation. Both would positively affect cartilage repair and suggests that IL-8 is required to initiate tissue repair *in vivo* (Hollander, Dickinson et al. 2010).

Moreover, *MMP2* and *MMP13* gene expression was downregulated in IL-8 stimulated BMSC, while they were elevated along with *MMP3* in mixed monocultures. Chemotaxis in cells can be induced by IL-8 stimulation and leads to cell migration associated with MMP upregulation. This could be seen as

an attempt for cartilage repair with local BMSC and suggests that initiation of tissue repair *in vivo* requires IL-8 (Mishima and Lotz 2008). Remarkably, chondrocytes were not affected by IL-8 stimulation perhaps, because OA-chondrocytes are less capable to cytokine stimulation than normal cells (Fan, Bau et al. 2005).

Response of mixed cultures to proinflammatory factors like IL-1ß, IL-6 or IL-8 in general rather resembled that of BMSC than that of chondrocyte monocultures, therefore chondrocytes in the mixed cultures might not alter BMSC metabolism.

In addition to gene expression, the collagens and GAGs were quantified in stimulated fibrin gels after 28 days of culture. In contrast to IL-1ß, IL-6 or IL-8 induced inhibition of gene expression, stimulation with these factors had only minimal effects on synthesis of ECM molecules. The newly generated matrix of monocultures stimulated with IL-1ß revealed significantly diminished amounts of ECM molecules (GAG and collagen II), what correlates with literature (Wehling, Palmer et al. 2009). No significant effects on protein expression of collagens I or III were determined for stimulations with IL-1ß. Contrary to gene expression, IL-6 or IL-8 stimulation setups had no significant effects on collagen and GAG concentration.

Taken together, stimulation of monocultures with IL-1ß and also partly IL-6 but not IL-8 induced similar gene expression patterns than observed in cocultures with OA-tissue and thus are suggested to partially mediate observed effects.

5.5 Part IV: Influence of coculture with normal ovine cartilage on BMSC differentiation

To gain insight in tissue intrinsic healing mechanisms cartilage defect models are the method of choice. Unfortunately, in the majority of cases only diseased human cartilage is on hand for *in vitro* studies. Normal human cartilage is hardly available, therefore cartilage from other species like sheep are an alternative tissue source. Even though species specificities like joint size, different load and weight distribution or cartilage thickness (McLure, Fisher et al. 2012), impedes comparison of human and animal cartilage, an ovine model provides the opportunity to study influences of normal cartilage on matrix production of chondrocytes and chondrogenic differentiated oBMSC *in vitro*. Soluble factors derived from normal ovine cartilage or chondrocytes, might enhance chondrogenic differentiation of oBMSC and influence ECM production of oCh and oBMSC *in vitro*.

Comparison of results gained from human OA-cartilage and subchondral bone co- and tricultures with normal ovine cartilage co- and tricultures, is interesting since pro-inflammatory factors induced by OA-tissue are partly responsible for inhibition of differentiation and ECM production what is in line with literature. For example, inhibitory effects of an inflammatory ovine joint environment were described for chondrogenic differentiation of oBMSC (Ando, Heard et al. 2012).

In this experimental setup, the microenvironment provided by normal ovine articular cartilage in general seemed to have no influence on ECM gene expression (with respect to *SOX9*, *ACAN*, *COL1A1*, *COL2A1* and *COL3A1*) of fibrin gel embedded oBMSC, mixed cultures or oCh. Gene expression of *COL2A1, COL3A1* and *ACAN* in both mixed culture conditions was significantly upregulated during culture time suggesting a positive synergistic effect of normal oBMSC with normal chondrocytes in mixed cultures.

Tang et al. demonstrated positive chondrogenic differentiation of oBMSC cultivated in a 3D scaffold supplemented with TGF-ß3 (Tang, Shakib et al. 2009). Therefore, normal ovine cartilage in this setup was suggested to have no additional effect on chondrogenic differentiation or functional status of cells than provided by a 3D environment and chondrogenic medium containing TGF-ß3.

In summary, no inhibitory or negative effects of normal ovine articular cartilage on differentiation or ECM production of oBMSC, ovine chondrocytes or mixed cultures were detectable.

6 Summary and conclusion

Positive chondrogenic differentiation was shown for fibrin gel embedded BMSC in monoculture and co- or triculture with cartilage explants but not with subchondral bone explants with respect to production of cartilage like ECM (collagen II and aggrecan). In contrast, chondrocytes, which served as a control, had almost no collagen I and X but were primarily rich in collagen type II, III and aggrecan. Furthermore, BMSC and chondrocyte monocultures exhibited an acceptable biomechanical stability. Coculture of fibrin gel embedded BMSC and chondrocytes with OA-cartilage resulted in an inhibition of collagen production and coculture with OA-subchondral bone resulted in an inhibition of chondrogenic differentiation and ECM assembly with respect to collagen and GAG (only temporary) production. Biomechanical properties of both OA-tissue cocultures were impaired mainly due to reduced collagen synthesis and disturbed formation of a stable and well-interconnected fibrillar collagen network.

Influences of known factors involved in chondrogenic differentiation (Sox9 and PTHrP) were excluded. However, bFGF seemed to be altered in OA-subchondral bone and cocultures stimulation attempts of monocultures with bFGF should be performed for verification.

Coculture with OA-cartilage and OA-subchondral bone induced release of proinflammatory cytokines IL-1ß, IL-6 and IL-8 into the supernatant. Analyses of cell-free OA- and normal cartilage or OA- and normal subchondral bone explant supernatants proved release of cytokines, GAGs and in case of subchondral bone bFGF. Consequently, cytokines in cocultures were released from explants and maybe additively from cocultured cells.

Verification of cytokines as origin for inhibitory effects was performed via stimulation of monocultures with IL-1ß, IL-6 and IL-8. IL-1ß and also partly IL-6 but not IL-8 induced similar gene expression patterns as observed in cocultures with OA-tissue and are suggested to partially mediate observed effects.

Normal ovine cartilage revealed no influences on chondrogenic differentiation or matrix generation of oBMSC or oCh with respect to collagen and *ACAN* gene expression. Additionally, repetition of key experiments with ASC confirmed inhibitory effects for OA-subchondral bone but not for normal subchondral bone cocultures. Normal cartilage or subchondral bone tissue in this experimental setup showed no or only reduced inhibitory effects on chondrogenic differentiation or collagen gene expression what might either hint to a disease status induced effect (OA vs. trauma) or to an effect caused by different mean age of donors.

To keep in mind, differentiation took place in a 3D surrounding in presence of TGF-ß3, which is a potent promoter of chondrogenesis (Tang, Shakib et al. 2009). Therefore, positive effects of coculture with normal ovine cartilage on oBMSC differentiation and matrix production might have been masked.

It is important to consider the microenvironment provided by surrounding cartilage and subchondral bone tissue especially before implanting cells into lesions and fissures of late stage OA-cartilage. The microenvironment of OA-cartilage seems to provide both: Inhibiting signals for chondrogenic differentiation of undifferentiated BMSC and promoting factors for phenotype stability of differentiated chondrocytes and BMSC with respect to hypertrophic markers. This suggests that balance of these factors determines the destiny of BMSC and chondrocytes and is of interest for future therapeutic strategies to stop or reverse OA progression. Identification of differentially expressed and released factors of OA- and normal cartilage- and subchondral bone explants should be analyzed using a proteomic approach and might provide important information which could essentially improve future treatment of OA pathology.

References

Abramson, S. B., M. Attur, et al. (2006). "Prospects for disease modification in osteoarthritis." Nat Clin Pract Rheumatol **2**(6): 304-312.

Aggarwal, S. and M. F. Pittenger (2005). "Human mesenchymal stem cells modulate allogeneic immune cell responses." Blood **105**(4): 1815-1822.

Ahmed, N., R. Dreier, et al. (2007). "Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells." Cell Physiol Biochem **20**(5): 665-678.

Aigner, T., M. Hemmel, et al. (2001). "Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage." Arthritis Rheum **44**(6): 1304-1312.

Aigner, T. and L. McKenna (2002). "Molecular pathology and pathobiology of osteoarthritic cartilage." Cell Mol Life Sci **59**(1): 5-18.

Akiyama, H., M. C. Chaboissier, et al. (2002). "The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6." Genes Dev **16**(21): 2813-2828.

Akizuki, S., V. C. Mow, et al. (1987). "Tensile properties of human knee joint cartilage. II. Correlations between weight bearing and tissue pathology and the kinetics of swelling." J Orthop Res **5**(2): 173-186.

Albro, M. B., R. J. Nims, et al. (2013). "Dynamic mechanical compression of devitalized articular cartilage does not activate latent TGF-beta." J Biomech **46**(8): 1433-1439.

Alsalameh, S., R. Amin, et al. (2004). "Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage." Arthritis Rheum **50**(5): 1522-1532.

Amin, A. K., J. S. Huntley, et al. (2009). "Chondrocyte survival in articular cartilage: the influence of subchondral bone in a bovine model." J Bone Joint Surg Br **91**(5): 691-699.

Anderson, H. C., P. T. Hodges, et al. (2000). "Bone morphogenetic protein (BMP) localization in developing human and rat growth plate, metaphysis, epiphysis, and articular cartilage." J Histochem Cytochem **48**(11): 1493-1502.

Ando, W., B. J. Heard, et al. (2012). "Ovine synovial membrane-derived mesenchymal progenitor cells retain the phenotype of the original tissue that was exposed to in-vivo inflammation: evidence for a suppressed chondrogenic differentiation potential of the cells." Inflamm Res **61**(6): 599-608.

Appleton, C. T., D. D. McErlain, et al. (2007). "Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis." Arthritis Res Ther **9**(1): R13.

Armstrong, S. J., R. A. Read, et al. (1995). "Topographical variation within the articular cartilage and subchondral bone of the normal ovine knee joint: a histological approach." Osteoarthritis Cartilage **3**(1): 25-33.

Ashraf, S. and D. A. Walsh (2008). "Angiogenesis in osteoarthritis." Curr Opin Rheumatol **20**(5): 573-580.

Aspenberg, P. and H. Van der Vis (1998). "Migration, particles, and fluid pressure. A discussion of causes of prosthetic loosening." Clin Orthop Relat Res(352): 75-80.

Aung, A., G. Gupta, et al. (2011). "Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells." Arthritis Rheum **63**(1): 148-158.

Barry, F., R. E. Boynton, et al. (2001). "Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components." Exp Cell Res **268**(2): 189-200.

Bedi, A., B. T. Feeley, et al. (2010). "Management of articular cartilage defects of the knee." J Bone Joint Surg Am **92**(4): 994-1009.

Benedek, T. G. (2006). "A history of the understanding of cartilage." Osteoarthritis Cartilage **14**(3): 203-209.

Berry, J. L., D. A. Thaeler-Oberdoerster, et al. (1986). "Subchondral pathways to the superior surface of the human talus." Foot Ankle **7**(1): 2-9.

Bian, L., D. Y. Zhai, et al. (2011). "Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage." Tissue Eng Part A **17**(7-8): 1137-1145.

Bianco, P., F. D. Cancedda, et al. (1998). "Bone formation via cartilage models: the "borderline" chondrocyte." Matrix Biol **17**(3): 185-192.

Birmingham, E., G. L. Niebur, et al. (2012). "Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche." Eur Cell Mater **23**: 13-27.

Bohme, K., M. Conscience-Egli, et al. (1992). "Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin, or thyroxine." J Cell Biol **116**(4): 1035-1042.

Bohme, K., K. H. Winterhalter, et al. (1995). "Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-beta 2 and basic fibroblast growth factor in synergy." Exp Cell Res **216**(1): 191-198.

Bonewald, L. F. (2002). "Osteocytes: a proposed multifunctional bone cell." J Musculoskelet Neuronal Interact **2**(3): 239-241.

Boyce, B. F., Z. Yao, et al. (2009). "Osteoclasts have multiple roles in bone in addition to bone resorption." Crit Rev Eukaryot Gene Expr **19**(3): 171-180.

Breedveld, F. C. (2004). "Osteoarthritis--the impact of a serious disease." Rheumatology (Oxford) **43 Suppl 1**: i4-8.

Brittberg, M., A. Lindahl, et al. (1994). "Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation." N Engl J Med **331**(14): 889-895.

Brittberg, M., L. Peterson, et al. (2003). "Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments." J Bone Joint Surg Am **85-A Suppl 3**: 109-115.

Brochhausen, C., N. Sanchez, et al. (2013). "Phenotypic redifferentiation and cell cluster formation of cultured human articular chondrocytes in a three-dimensional oriented gelatin scaffold in the presence of PGE2--first results of a pilot study." J Biomed Mater Res A **101**(8): 2374-2382.

Bruckner, P. and M. van der Rest (1994). "Structure and function of cartilage collagens." Microsc Res Tech **28**(5): 378-384.

Buckwalter, J. A., D. D. Anderson, et al. (2013). "The Roles of Mechanical Stresses in the Pathogenesis of Osteoarthritis: Implications for Treatment of Joint Injuries." Cartilage **4**(4): 286-294.

Buckwalter, J. A. and H. J. Mankin (1998). "Articular cartilage repair and transplantation." Arthritis Rheum **41**(8): 1331-1342.

Bullough, P. G., P. S. Yawitz, et al. (1985). "Topographical variations in the morphology and biochemistry of adult canine tibial plateau articular cartilage." J Orthop Res **3**(1): 1-16.

Burdon, T. J., A. Paul, et al. (2011). "Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential." Bone Marrow Res **2011**: 207326.

Burr, D. B. (2004). "Anatomy and physiology of the mineralized tissues: role in the pathogenesis of osteoarthrosis." Osteoarthritis Cartilage **12 Suppl A**: S20-30.

Burr, D. B. (2004). "Bone quality: understanding what matters." J Musculoskelet Neuronal Interact **4**(2): 184-186.

Burr, D. B. (2004). "The importance of subchondral bone in the progression of osteoarthritis." J Rheumatol Suppl **70**: 77-80.

Burr, D. B. and E. L. Radin (2003). "Microfractures and microcracks in subchondral bone: are they relevant to osteoarthrosis?" Rheum Dis Clin North Am **29**(4): 675-685.

Busby, G. A., M. H. Grant, et al. (2013). "Confined compression of collagen hydrogels." J Biomech **46**(4): 837-840.

Cameron, M., A. Buchgraber, et al. (1997). "The natural history of the anterior cruciate ligament-deficient knee. Changes in synovial fluid cytokine and keratan sulfate concentrations." Am J Sports Med **25**(6): 751-754.

Canalis, E., T. McCarthy, et al. (1988). "Isolation of growth factors from adult bovine bone." Calcif Tissue Int **43**(6): 346-351.

Caplan, A. I. and J. E. Dennis (2006). "Mesenchymal stem cells as trophic mediators." J Cell Biochem **98**(5): 1076-1084.

Caplan, A. I., M. Elyaderani, et al. (1997). "Principles of cartilage repair and regeneration." Clin Orthop Relat Res(342): 254-269.

Chambers, M. G., L. Cox, et al. (2001). "Matrix metalloproteinases and aggrecanases cleave aggrecan in different zones of normal cartilage but colocalize in the development of osteoarthritic lesions in STR/ort mice." Arthritis Rheum **44**(6): 1455-1465.

Cheng, A., T. E. Hardingham, et al. (2014). "Generating cartilage repair from pluripotent stem cells." Tissue Eng Part B Rev **20**(4): 257-266.

Cheng, T., N. C. Maddox, et al. (2012). "Comparison of gene expression patterns in articular cartilage and dedifferentiated articular chondrocytes." Journal of Orthopaedic Research **30**(2): 234-245.

Coimbra, I. B., S. A. Jimenez, et al. (2004). "Hypoxia inducible factor-1 alpha expression in human normal and osteoarthritic chondrocytes." Osteoarthritis Cartilage **12**(4): 336-345.

D'Amore, P. A. (1990). "Modes of FGF release in vivo and in vitro." Cancer Metastasis Rev **9**(3): 227-238.

Dahlberg, L., R. C. Billinghurst, et al. (2000). "Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1 (matrix metalloproteinase 1)." Arthritis Rheum **43**(3): 673-682.

Dallas, S. L., M. Prideaux, et al. (2013). "The osteocyte: an endocrine cell ... and more." Endocr Rev **34**(5): 658-690.

Day, J. S., M. Ding, et al. (2001). "A decreased subchondral trabecular bone tissue elastic modulus is associated with pre-arthritic cartilage damage." J Orthop Res **19**(5): 914-918.

de Crombrugghe, B., V. Lefebvre, et al. (2000). "Transcriptional mechanisms of chondrocyte differentiation." Matrix Biol **19**(5): 389-394.

de Seny, D., G. Cobraiville, et al. (2013). "Acute-phase serum amyloid a in osteoarthritis: regulatory mechanism and proinflammatory properties." PLoS One **8**(6): e66769.

Denker, A. E., S. B. Nicoll, et al. (1995). "Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1." Differentiation **59**(1): 25-34.

Dickhut, A., E. Gottwald, et al. (2008). "Chondrogenesis of mesenchymal stem cells in gel-like biomaterials in vitro and in vivo." Front Biosci **13**: 4517-4528.

Diederichs, S., K. Baral, et al. (2012). "Interplay between local versus soluble transforming growth factor-beta and fibrin scaffolds: role of cells and impact on human mesenchymal stem cell chondrogenesis." Tissue Eng Part A **18**(11-12): 1140-1150.

Dinarello, C. A. (2010). "Anti-inflammatory Agents: Present and Future." Cell **140**(6): 935-950.

Djouad, F., B. Delorme, et al. (2007). "Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes." Arthritis Res Ther **9**(2): R33.

Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.

Donnelly, E., D. X. Chen, et al. (2010). "Contribution of mineral to bone structural behavior and tissue mechanical properties." Calcif Tissue Int **87**(5): 450-460.

Dore, D., S. Quinn, et al. (2009). "Correlates of subchondral BMD: a cross-sectional study." J Bone Miner Res **24**(12): 2007-2015.

Dozin, B., R. Quarto, et al. (1990). "Stabilization of the mRNA follows transcriptional activation of type II collagen gene in differentiating chicken chondrocyte." J Biol Chem **265**(13): 7216-7220.

Dreier, R. (2010). "Hypertrophic differentiation of chondrocytes in osteoarthritis: the developmental aspect of degenerative joint disorders." Arthritis Res Ther **12**(5): 216.

Dreier, R., S. Grassel, et al. (2004). "Pro-MMP-9 is a specific macrophage product and is activated by osteoarthritic chondrocytes via MMP-3 or a MT1-MMP/MMP-13 cascade." Exp Cell Res **297**(2): 303-312.

Endres, M., K. Neumann, et al. (2012). "An ovine in vitro model for chondrocyte-based scaffold-assisted cartilage grafts." J Orthop Surg Res **7**: 37.

Erickson, I. E., A. H. Huang, et al. (2009). "Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels." Tissue Eng Part A **15**(5): 1041-1052.

Fan, Z., B. Bau, et al. (2005). "Freshly isolated osteoarthritic chondrocytes are catabolically more active than normal chondrocytes, but less responsive to catabolic stimulation with interleukin-1beta." Arthritis Rheum **52**(1): 136-143.

Farndale, R. W., D. J. Buttle, et al. (1986). "Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue." Biochim Biophys Acta **883**(2): 173-177.

Felson, D. T. (2004). "An update on the pathogenesis and epidemiology of osteoarthritis." Radiol Clin North Am **42**(1): 1-9, v.

Felson, D. T. and T. Neogi (2004). "Osteoarthritis: is it a disease of cartilage or of bone?" Arthritis Rheum **50**(2): 341-344.

Fernandes, J. C., J. Martel-Pelletier, et al. (2002). "The role of cytokines in osteoarthritis pathophysiology." Biorheology **39**(1-2): 237-246.

Findlay, D. M. (2010). "If good things come from above, do bad things come from below?" Arthritis Res Ther **12**(3): 119.

Fischer, J., A. Aulmann, et al. (2014). "Intermittent PTHrP(1-34) exposure augments chondrogenesis and reduces hypertrophy of mesenchymal stromal cells." Stem Cells Dev **23**(20): 2513-2523.

Fischer, J., A. Dickhut, et al. (2010). "Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis." Arthritis Rheum **62**(9): 2696-2706.

Francioli, S. E., C. Candrian, et al. (2010). "Effect of three-dimensional expansion and cell seeding density on the cartilage-forming capacity of human articular chondrocytes in type II collagen sponges." J Biomed Mater Res A **95**(3): 924-931.

Frank, R. S. and R. M. Hochmuth (1987). "An investigation of particle flow through capillary models with the resistive pulse technique." J Biomech Eng **109**(2): 103-109.

Fussenegger, M., J. Meinhart, et al. (2003). "Stabilized autologous fibrin-chondrocyte constructs for cartilage repair in vivo." Ann Plast Surg **51**(5): 493-498.

Gannon, A. R., T. Nagel, et al. (2012). "The role of the superficial region in determining the dynamic properties of articular cartilage." Osteoarthritis Cartilage **20**(11): 1417-1425.

Garner, B. C., A. M. Stoker, et al. (2011). "Using animal models in osteoarthritis biomarker research." J Knee Surg **24**(4): 251-264.

Gehmert, S., M. Hidayat, et al. (2011). "Angiogenesis: the role of PDGF-BB on adipose-tissue derived stem cells (ASCs)." Clin Hemorheol Microcirc **48**(1): 5-13.

George, J., Y. Kuboki, et al. (2006). "Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds." Biotechnol Bioeng **95**(3): 404-411.

Geyer, M., S. Grassel, et al. (2009). "Differential transcriptome analysis of intraarticular lesional vs intact cartilage reveals new candidate genes in osteoarthritis pathophysiology." Osteoarthritis Cartilage **17**(3): 328-335.

Goldring, M. B. (2000). "Osteoarthritis and cartilage: the role of cytokines." Curr Rheumatol Rep **2**(6): 459-465.

Goldring, M. B. (2012). "Articular cartilage degradation in osteoarthritis." HSS J **8**(1): 7-9.
Goldring, M. B. and S. R. Goldring (2007). "Osteoarthritis." J Cell Physiol **213**(3): 626-634.
Goldring, M. B., M. Otero, et al. (2008). "Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism." Ann Rheum Dis **67 Suppl 3**: iii75-82.
Goldring, M. B., K. Tsuchimochi, et al. (2006). "The control of chondrogenesis." J Cell Biochem **97**(1): 33-44.
Grabowski, P. (2009). "Physiology of bone." Endocr Dev **16**: 32-48.
Grassel, S. (2014). "The role of peripheral nerve fibers and their neurotransmitters in cartilage and bone physiology and pathophysiology." Arthritis Res Ther **16**(6): 485.
Grassel, S. and N. Ahmed (2007). "Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation." Front Biosci **12**: 4946-4956.
Grassel, S. and J. Lorenz (2014). "Tissue-engineering strategies to repair chondral and osteochondral tissue in osteoarthritis: use of mesenchymal stem cells." Curr Rheumatol Rep **16**(10): 452.
Grassel, S., M. Rickert, et al. (2010). "Coculture between periosteal explants and articular chondrocytes induces expression of TGF-beta1 and collagen I." Rheumatology (Oxford) **49**(2): 218-230.
Greek, R. and A. Menache (2013). "Systematic reviews of animal models: methodology versus epistemology." Int J Med Sci **10**(3): 206-221.
Green, W. T., Jr., G. N. Martin, et al. (1970). "Microradiographic study of the calcified layer of articular cartilage." Arch Pathol **90**(2): 151-158.
Guevremont, M., J. Martel-Pelletier, et al. (2003). "Human adult chondrocytes express hepatocyte growth factor (HGF) isoforms but not HgF: potential implication of osteoblasts on the presence of HGF in cartilage." J Bone Miner Res **18**(6): 1073-1081.
Guilak, F. (2011). "Biomechanical factors in osteoarthritis." Best Pract Res Clin Rheumatol **25**(6): 815-823.
Hagg, R., P. Bruckner, et al. (1998). "Cartilage fibrils of mammals are biochemically heterogeneous: differential distribution of decorin and collagen IX." J Cell Biol **142**(1): 285-294.
Hamperl, S., C. R. Brown, et al. (2014). "Compositional and structural analysis of selected chromosomal domains from Saccharomyces cerevisiae." Nucleic Acids Res **42**(1): e2.
Hardingham, T. E., A. J. Fosang, et al. (1994). "The structure, function and turnover of aggrecan, the large aggregating proteoglycan from cartilage." Eur J Clin Chem Clin Biochem **32**(4): 249-257.
Harris, J. D., R. A. Siston, et al. (2010). "Autologous chondrocyte implantation: a systematic review." J Bone Joint Surg Am **92**(12): 2220-2233.
Hatami-Marbini, H. and E. Etebu (2013). "A new method to determine rate-dependent material parameters of corneal extracellular matrix." Ann Biomed Eng **41**(11): 2399-2408.
Hattori, T., C. Muller, et al. (2010). "SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification." Development **137**(6): 901-911.
Hennig, T., H. Lorenz, et al. (2007). "Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6." J Cell Physiol **211**(3): 682-691.
Hiligsmann, M., C. Cooper, et al. (2013). "Health economics in the field of osteoarthritis: an expert's consensus paper from the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO)." Semin Arthritis Rheum **43**(3): 303-313.
Hill, C. L., D. G. Gale, et al. (2001). "Knee effusions, popliteal cysts, and synovial thickening: association with knee pain in osteoarthritis." J Rheumatol **28**(6): 1330-1337.
Ho, S. T., S. M. Cool, et al. (2010). "The influence of fibrin based hydrogels on the chondrogenic differentiation of human bone marrow stromal cells." Biomaterials **31**(1): 38-47.
Hollander, A. P., S. C. Dickinson, et al. (2010). "Stem cells and cartilage development: complexities of a simple tissue." Stem Cells **28**(11): 1992-1996.
Holtzman, D. J., A. A. Theologis, et al. (2010). "T(1rho) and T(2) quantitative magnetic resonance imaging analysis of cartilage regeneration following microfracture and mosaicplasty cartilage resurfacing procedures." Journal of Magnetic Resonance Imaging **32**(4): 914-923.

Homminga, G. N., P. Buma, et al. (1993). "Chondrocyte behavior in fibrin glue in vitro." Acta Orthop Scand **64**(4): 441-445.

Huang, K. and L. D. Wu (2008). "Aggrecanase and aggrecan degradation in osteoarthritis: a review." J Int Med Res **36**(6): 1149-1160.

Huebner, K. D., N. G. Shrive, et al. (2014). "Dexamethasone inhibits inflammation and cartilage damage in a new model of post-traumatic osteoarthritis." J Orthop Res **32**(4): 566-572.

Hunter, D. J. and D. T. Felson (2006). "Osteoarthritis." BMJ **332**(7542): 639-642.

Hwang, N. S., S. Varghese, et al. (2008). "Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration." PLoS One **3**(6): e2498.

Ikeda, D., H. Ageta, et al. (2013). "iTRAQ-based proteomics reveals novel biomarkers of osteoarthritis." Biomarkers **18**(7): 565-572.

Inoue, H. (1981). "Alterations in the collagen framework of osteoarthritic cartilage and subchondral bone." Int Orthop **5**(1): 47-52.

Irie, K., E. Uchiyama, et al. (2003). "Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee." Knee **10**(1): 93-96.

Jazrawi, L. M., M. J. Alaia, et al. (2011). "Advances in magnetic resonance imaging of articular cartilage." J Am Acad Orthop Surg **19**(7): 420-429.

Jiang, J., N. L. Leong, et al. (2008). "Interaction between zonal populations of articular chondrocytes suppresses chondrocyte mineralization and this process is mediated by PTHrP." Osteoarthritis Cartilage **16**(1): 70-82.

Jiang, J., S. B. Nicoll, et al. (2005). "Co-culture of osteoblasts and chondrocytes modulates cellular differentiation in vitro." Biochem Biophys Res Commun **338**(2): 762-770.

Jikko, A., Y. Kato, et al. (1999). "Inhibition of chondrocyte terminal differentiation and matrix calcification by soluble factors released by articular chondrocytes." Calcif Tissue Int **65**(4): 276-279.

Juncosa-Melvin, N., K. S. Matlin, et al. (2007). "Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair." Tissue Eng **13**(6): 1219-1226.

Kang, N., X. Liu, et al. (2012). "Effects of co-culturing BMSCs and auricular chondrocytes on the elastic modulus and hypertrophy of tissue engineered cartilage." Biomaterials **33**(18): 4535-4544.

Karsdal, M. A., D. J. Leeming, et al. (2008). "Should subchondral bone turnover be targeted when treating osteoarthritis?" Osteoarthritis Cartilage **16**(6): 638-646.

Kim, M. K., S. W. Choi, et al. (2010). "Autologous chondrocyte implantation in the knee using fibrin." Knee Surg Sports Traumatol Arthrosc **18**(4): 528-534.

Kobayashi, M., G. R. Squires, et al. (2005). "Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage." Arthritis Rheum **52**(1): 128-135.

Kolf, C. M., E. Cho, et al. (2007). "Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation." Arthritis Res Ther **9**(1): 204.

Kouri, J. B., J. M. Aguilera, et al. (2000). "Apoptotic chondrocytes from osteoarthrotic human articular cartilage and abnormal calcification of subchondral bone." J Rheumatol **27**(4): 1005-1019.

Kouri, J. B. and C. Lavalle (2006). "Do chondrocytes undergo "activation" and "transdifferentiation" during the pathogenesis of osteoarthritis? A review of the ultrastructural and immunohistochemical evidence." Histology and Histopathology **21**(7): 793-802.

Ku, C. H., P. H. Johnson, et al. (2006). "Collagen synthesis by mesenchymal stem cells and aortic valve interstitial cells in response to mechanical stretch." Cardiovasc Res **71**(3): 548-556.

Lai, W. M., J. S. Hou, et al. (1991). "A triphasic theory for the swelling and deformation behaviors of articular cartilage." J Biomech Eng **113**(3): 245-258.

Lane, L. B., A. Villacin, et al. (1977). "The vascularity and remodelling of subchondrial bone and calcified cartilage in adult human femoral and humeral heads. An age- and stress-related phenomenon." J Bone Joint Surg Br **59**(3): 272-278.

Lee, H. J., C. Yu, et al. (2008). "Enhanced chondrogenesis of mesenchymal stem cells in collagen mimetic peptide-mediated microenvironment." Tissue Eng Part A **14**(11): 1843-1851.

Lefebvre, V., R. R. Behringer, et al. (2001). "L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway." Osteoarthritis Cartilage **9 Suppl A**: S69-75.

Lefebvre, V. and B. de Crombrugghe (1998). "Toward understanding SOX9 function in chondrocyte differentiation." Matrix Biol **16**(9): 529-540.

Lettry, V., K. Hosoya, et al. (2010). "Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells." Jpn J Vet Res **58**(1): 5-15.

Leyh, M., A. Seitz, et al. (2014 b). "Subchondral bone influences chondrogenic differentiation and collagen production of human bone marrow-derived mesenchymal stem cells and articular chondrocytes." Arthritis Res Ther **16**(5): 453.

Leyh, M., A. Seitz, et al. (2014 a). "Osteoarthritic cartilage explants affect extracellular matrix production and composition in cocultured bone marrow-derived mesenchymal stem cells and articular chondrocytes." Stem Cell Res Ther **5**(3): 77.

Li, F., N. Song, et al. (2014). "Pigment epithelium derived factor suppresses expression of Sost/sclerostin by osteocytes: Implication for its role in bone matrix mineralization." J Cell Physiol.

Li, H., N. Davison, et al. (2012). "Evaluating Osteoarthritic Chondrocytes through a Novel 3-Dimensional *In Vitro* System for Cartilage Tissue Engineering and Regeneration." Cartilage **3**(2): 128-140.

Li, W. J., H. Chiang, et al. (2009). "Evaluation of articular cartilage repair using biodegradable nanofibrous scaffolds in a swine model: a pilot study." J Tissue Eng Regen Med **3**(1): 1-10.

Lin, Y. Y., N. Tanaka, et al. (2010). "Applying an excessive mechanical stress alters the effect of subchondral osteoblasts on chondrocytes in a co-culture system." Eur J Oral Sci **118**(2): 151-158.

Little, C. B., C. R. Flannery, et al. (1999). "Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan in vitro." Biochem J **344 Pt 1**: 61-68.

Lu, X. L. and V. C. Mow (2008). "Biomechanics of articular cartilage and determination of material properties." Med Sci Sports Exerc **40**(2): 193-199.

Lu, Y. H. C. S., C. H. Evans, et al. (2011). "Effects of short-term glucocorticoid treatment on changes in cartilage matrix degradation and chondrocyte gene expression induced by mechanical injury and inflammatory cytokines." Arthritis Res Ther **13**(5).

Luder, H. U. (1998). "Age changes in the articular tissue of human mandibular condyles from adolescence to old age: a semiquantitative light microscopic study." Anat Rec **251**(4): 439-447.

Lutolf, M. P. and J. A. Hubbell (2005). "Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering." Nat Biotechnol **23**(1): 47-55.

Madry, H., C. N. van Dijk, et al. (2010). "The basic science of the subchondral bone." Knee Surg Sports Traumatol Arthrosc **18**(4): 419-433.

Majumdar, M. K., E. Wang, et al. (2001). "BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1." J Cell Physiol **189**(3): 275-284.

Malda, J., J. C. de Grauw, et al. (2013). "Of mice, men and elephants: the relation between articular cartilage thickness and body mass." PLoS One **8**(2): e57683.

Maldonado, M. and J. Nam (2013). "The role of changes in extracellular matrix of cartilage in the presence of inflammation on the pathology of osteoarthritis." Biomed Res Int **2013**: 284873.

Mandelbaum, B., J. E. Browne, et al. (2007). "Treatment outcomes of autologous chondrocyte implantation for full-thickness articular cartilage defects of the trochlea." Am J Sports Med **35**(6): 915-921.

Mankin, H. J. (1982). "Alterations in the structure, chemistry, and metabolism of the articular cartilage in osteoarthritis of the human hip." Hip: 126-145.

Mansell, J. P., J. F. Tarlton, et al. (1997). "Biochemical evidence for altered subchondral bone collagen metabolism in osteoarthritis of the hip." Br J Rheumatol **36**(1): 16-19.

Marlovits, S., M. Hombauer, et al. (2004). "Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes." J Bone Joint Surg Br **86**(2): 286-295.

Maroudas, A., M. T. Bayliss, et al. (1980). "Further studies on the composition of human femoral head cartilage." Ann Rheum Dis **39**(5): 514-523.

Maroudas, A. I. (1976). "Balance between swelling pressure and collagen tension in normal and degenerate cartilage." Nature **260**(5554): 808-809.

Massicotte, F., D. Lajeunesse, et al. (2002). "Can altered production of interleukin-1beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients." Osteoarthritis Cartilage **10**(6): 491-500.

Mastrogiacomo, M., R. Cancedda, et al. (2001). "Effect of different growth factors on the chondrogenic potential of human bone marrow stromal cells." Osteoarthritis Cartilage **9 Suppl A**: S36-40.

Mauck, R. L., X. Yuan, et al. (2006). "Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture." Osteoarthritis Cartilage **14**(2): 179-189.

McCarty, R. C., S. Gronthos, et al. (2009). "Characterisation and developmental potential of ovine bone marrow derived mesenchymal stem cells." J Cell Physiol **219**(2): 324-333.

McLure, S. W., J. Fisher, et al. (2012). "Regional cartilage properties of three quadruped tibiofemoral joints used in musculoskeletal research studies." Proc Inst Mech Eng H **226**(8): 652-656.

Meachim, G. and R. Allibone (1984). "Topographical variation in the calcified zone of upper femoral articular cartilage." J Anat **139 (Pt 2)**: 341-352.

Mendler, M., S. G. Eich-Bender, et al. (1989). "Cartilage contains mixed fibrils of collagen types II, IX, and XI." J Cell Biol **108**(1): 191-197.

Mesa, J. M., V. Zaporojan, et al. (2006). "Tissue engineering cartilage with aged articular chondrocytes in vivo." Plast Reconstr Surg **118**(1): 41-49; discussion 50-43.

Meunier, P. J. and G. Boivin (1997). "Bone mineral density reflects bone mass but also the degree of mineralization of bone: therapeutic implications." Bone **21**(5): 373-377.

Millward-Sadler, S. J., M. O. Wright, et al. (2000). "Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes." Arthritis Rheum **43**(9): 2091-2099.

Milz, S. and R. Putz (1994). "Quantitative morphology of the subchondral plate of the tibial plateau." J Anat **185 (Pt 1)**: 103-110.

Mishima, Y. and M. Lotz (2008). "Chemotaxis of human articular chondrocytes and mesenchymal stem cells." J Orthop Res **26**(10): 1407-1412.

Mo, X. T., S. C. Guo, et al. (2009). "Variations in the ratios of co-cultured mesenchymal stem cells and chondrocytes regulate the expression of cartilaginous and osseous phenotype in alginate constructs." Bone **45**(1): 42-51.

Moldovan, F., J. P. Pelletier, et al. (2000). "Diacerhein and rhein reduce the ICE-induced IL-1beta and IL-18 activation in human osteoarthritic cartilage." Osteoarthritis Cartilage **8**(3): 186-196.

Mow, V. C. and X. E. Guo (2002). "Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies." Annu Rev Biomed Eng **4**: 175-209.

Mow, V. C., M. H. Holmes, et al. (1984). "Fluid transport and mechanical properties of articular cartilage: a review." J Biomech **17**(5): 377-394.

Mow, V. C., S. C. Kuei, et al. (1980). "Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments." J Biomech Eng **102**(1): 73-84.

Mrugala, D., C. Bony, et al. (2008). "Phenotypic and functional characterisation of ovine mesenchymal stem cells: application to a cartilage defect model." Ann Rheum Dis **67**(3): 288-295.

Mundlos, S. and B. R. Olsen (1997). "Heritable diseases of the skeleton. Part II: Molecular insights into skeletal development-matrix components and their homeostasis." FASEB J **11**(4): 227-233.

Munirah, S., O. C. Samsudin, et al. (2007). "Articular cartilage restoration in load-bearing osteochondral defects by implantation of autologous chondrocyte-fibrin constructs: an experimental study in sheep." J Bone Joint Surg Br **89**(8): 1099-1109.

Murab, S., S. Chameettachal, et al. (2013). "Matrix-embedded cytokines to simulate osteoarthritis-like cartilage microenvironments." Tissue Eng Part A **19**(15-16): 1733-1753.

Nagai, H. and M. Aoki (2002). "Inhibition of growth plate angiogenesis and endochondral ossification with diminished expression of MMP-13 in hypertrophic chondrocytes in FGF-2-treated rats." J Bone Miner Metab **20**(3): 142-147.

Nakaoka, R., S. X. Hsiong, et al. (2006). "Regulation of chondrocyte differentiation level via co-culture with osteoblasts." Tissue Eng **12**(9): 2425-2433.

Neilson, M., A. White, et al. (2004). "Changes in bone architecture in the femoral head and neck in osteoarthritis." Clin Anat **17**(5): 378-391.

Newberry, W. N., J. J. Garcia, et al. (1998). "Analysis of acute mechanical insult in an animal model of post-traumatic osteoarthrosis." J Biomech Eng **120**(6): 704-709.

Newberry, W. N., C. D. Mackenzie, et al. (1998). "Blunt impact causes changes in bone and cartilage in a regularly exercised animal model." J Orthop Res **16**(3): 348-354.

Osyczka, A. M., D. L. Diefenderfer, et al. (2004). "Different effects of BMP-2 on marrow stromal cells from human and rat bone." Cells Tissues Organs **176**(1-3): 109-119.

Otsuki, S., S. P. Grogan, et al. (2010). "Tissue neogenesis and STRO-1 expression in immature and mature articular cartilage." J Orthop Res **28**(1): 96-102.

Pan, J., X. Zhou, et al. (2009). "In situ measurement of transport between subchondral bone and articular cartilage." J Orthop Res **27**(10): 1347-1352.

Park, J. S., H. N. Yang, et al. (2009). "In vitro and in vivo chondrogenesis of rabbit bone marrow-derived stromal cells in fibrin matrix mixed with growth factor loaded in nanoparticles." Tissue Eng Part A **15**(8): 2163-2175.

Pearle, A. D., R. F. Warren, et al. (2005). "Basic science of articular cartilage and osteoarthritis." Clinics in Sports Medicine **24**(1): 1-+.

Pereira, D., B. Peleteiro, et al. (2011). "The effect of osteoarthritis definition on prevalence and incidence estimates: a systematic review." Osteoarthritis Cartilage **19**(11): 1270-1285.

Pfander, D., B. Swoboda, et al. (2001). "Expression of early and late differentiation markers (proliferating cell nuclear antigen, syndecan-3, annexin VI, and alkaline phosphatase) by human osteoarthritic chondrocytes." Am J Pathol **159**(5): 1777-1783.

Pitsillides, A. A. and F. Beier (2011). "Cartilage biology in osteoarthritis--lessons from developmental biology." Nat Rev Rheumatol **7**(11): 654-663.

Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-147.

Poole, A. R. (2003). "What type of cartilage repair are we attempting to attain?" J Bone Joint Surg Am **85-A Suppl 2**: 40-44.

Poole, A. R., T. Kojima, et al. (2001). "Composition and structure of articular cartilage: a template for tissue repair." Clin Orthop Relat Res(391 Suppl): S26-33.

Poole, C. A. (1997). "Articular cartilage chondrons: form, function and failure." J Anat **191 (Pt 1)**: 1-13.

Pricola, K. L., N. Z. Kuhn, et al. (2009). "Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism." J Cell Biochem **108**(3): 577-588.

Prockop, D. J. (1997). "Marrow stromal cells as stem cells for nonhematopoietic tissues." Science **276**(5309): 71-74.

Regan, E., J. Flannelly, et al. (2005). "Extracellular superoxide dismutase and oxidant damage in osteoarthritis." Arthritis Rheum **52**(11): 3479-3491.

Reverte-Vinaixa, M. M., N. Joshi, et al. (2013). "Medium-term outcome of mosaicplasty for grade III-IV cartilage defects of the knee." J Orthop Surg (Hong Kong) **21**(1): 4-9.

Roddy, E. and M. Doherty (2006). "Changing life-styles and osteoarthritis: what is the evidence?" Best Pract Res Clin Rheumatol **20**(1): 81-97.

Rosenthal, A. K., C. M. Gohr, et al. (2011). "Proteomic analysis of articular cartilage vesicles from normal and osteoarthritic cartilage." Arthritis Rheum **63**(2): 401-411.

Ryu, J. H. and J. S. Chun (2006). "Opposing roles of WNT-5A and WNT-11 in interleukin-1beta regulation of type II collagen expression in articular chondrocytes." J Biol Chem **281**(31): 22039-22047.

Salter, D. M., D. E. Hughes, et al. (1992). "Integrin expression by human articular chondrocytes." Br J Rheumatol **31**(4): 231-234.

Salter, D. M., S. J. Millward-Sadler, et al. (2002). "Differential responses of chondrocytes from normal and osteoarthritic human articular cartilage to mechanical stimulation." Biorheology **39**(1-2): 97-108.

Sanchez, C., M. A. Deberg, et al. (2008). "Phenotypic characterization of osteoblasts from the sclerotic zones of osteoarthritic subchondral bone." Arthritis Rheum **58**(2): 442-455.

Sanchez, C., M. A. Deberg, et al. (2005). "Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1beta and oncostatin M pre-treated non-sclerotic osteoblasts." Osteoarthritis Cartilage **13**(11): 979-987.

Sanchez, C., M. A. Deberg, et al. (2005). "Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes." Osteoarthritis Cartilage **13**(11): 988-997.

Schagemann, J. C., E. H. Mrosek, et al. (2006). "Morphology and function of ovine articular cartilage chondrocytes in 3-d hydrogel culture." Cells Tissues Organs **182**(2): 89-97.

Schmid, T. M. and T. F. Linsenmayer (1985). "Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues." J Cell Biol **100**(2): 598-605.

Schreml, S., P. Babilas, et al. (2009). "Harvesting human adipose tissue-derived adult stem cells: resection versus liposuction." Cytotherapy **11**(7): 947-957.

Schulze-Tanzil, G., R. D. Muller, et al. (2009). "Differing in vitro biology of equine, ovine, porcine and human articular chondrocytes derived from the knee joint: an immunomorphological study." Histochem Cell Biol **131**(2): 219-229.

Schutze, N., U. Noth, et al. (2005). "Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation." Cell Commun Signal **3**(1): 5.

Sekiya, I., B. L. Larson, et al. (2005). "Comparison of effect of BMP-2, -4, and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma." Cell Tissue Res **320**(2): 269-276.

Sekiya, I., K. Tsuji, et al. (2000). "SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6." J Biol Chem **275**(15): 10738-10744.

Setton, L. A., V. C. Mow, et al. (1994). "Mechanical properties of canine articular cartilage are significantly altered following transection of the anterior cruciate ligament." J Orthop Res **12**(4): 451-463.

Sims, C. D., P. E. Butler, et al. (1998). "Tissue engineered neocartilage using plasma derived polymer substrates and chondrocytes." Plast Reconstr Surg **101**(6): 1580-1585.

Singh, I. (1978). "The architecture of cancellous bone." J Anat **127**(Pt 2): 305-310.

Smith, S. M., L. A. West, et al. (2007). "Heparan and chondroitin sulfate on growth plate perlecan mediate binding and delivery of FGF-2 to FGF receptors." Matrix Biol **26**(3): 175-184.

Snelling, S., R. Rout, et al. (2014). "A gene expression study of normal and damaged cartilage in anteromedial gonarthrosis, a phenotype of osteoarthritis." Osteoarthritis Cartilage **22**(2): 334-343.

Sofat, N. (2009). "Analysing the role of endogenous matrix molecules in the development of osteoarthritis." Int J Exp Pathol **90**(5): 463-479.

Soltz, M. A. and G. A. Ateshian (1998). "Experimental verification and theoretical prediction of cartilage interstitial fluid pressurization at an impermeable contact interface in confined compression." J Biomech **31**(10): 927-934.

Steinert, A. F., S. C. Ghivizzani, et al. (2007). "Major biological obstacles for persistent cell-based regeneration of articular cartilage." Arthritis Res Ther **9**(3): 213.

Stockwell, R. A. (1971). "The interrelationship of cell density and cartilage thickness in mammalian articular cartilage." J Anat **109**(Pt 3): 411-421.

Suri, S., S. E. Gill, et al. (2007). "Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis." Ann Rheum Dis **66**(11): 1423-1428.

Syftestad, G. T. and A. I. Caplan (1984). "A fraction from extracts of demineralized adult bone stimulates the conversion of mesenchymal cells into chondrocytes." Dev Biol **104**(2): 348-356.

Sylvester, K. G. and M. T. Longaker (2004). "Stem cells: review and update." Arch Surg **139**(1): 93-99.

Szuts, V., U. Mollers, et al. (1998). "Terminal differentiation of chondrocytes is arrested at distinct stages identified by their expression repertoire of marker genes." Matrix Biol **17**(6): 435-448.

Tang, Q. O., K. Shakib, et al. (2009). "TGF-beta3: A potential biological therapy for enhancing chondrogenesis." Expert Opin Biol Ther **9**(6): 689-701.

Taylor, D. W., N. Ahmed, et al. (2010). "Proteoglycan and collagen accumulation by passaged chondrocytes can be enhanced through side-by-side culture with primary chondrocytes." Tissue Eng Part A **16**(2): 643-651.

Tchetina, E. V. (2011). "Developmental mechanisms in articular cartilage degradation in osteoarthritis." Arthritis **2011**: 683970.

Tchetina, E. V., M. Kobayashi, et al. (2007). "Chondrocyte hypertrophy can be induced by a cryptic sequence of type II collagen and is accompanied by the induction of MMP-13 and collagenase activity: implications for development and arthritis." Matrix Biol **26**(4): 247-258.

Tchetina, E. V., G. Squires, et al. (2005). "Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions." J Rheumatol **32**(5): 876-886.

Tetlow, L. C., D. J. Adlam, et al. (2001). "Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes." Arthritis Rheum **44**(3): 585-594.

Ting, V., C. D. Sims, et al. (1998). "In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes." Ann Plast Surg **40**(4): 413-420; discussion 420-411.

Torzilli, P. A., R. Grigiene, et al. (1999). "Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content." J Biomech Eng **121**(5): 433-441.

Tsuchida, A. I., M. Beekhuizen, et al. (2012). "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): R262.

van de Loo, F. A., S. Kuiper, et al. (1997). "Interleukin-6 reduces cartilage destruction during experimental arthritis. A study in interleukin-6-deficient mice." Am J Pathol **151**(1): 177-191.

van den Berg, W. B. (2011). "Osteoarthritis year 2010 in review: pathomechanisms." Osteoarthritis Cartilage **19**(4): 338-341.

van den Berg, W. B., L. A. Joosten, et al. (1999). "Role of tumour necrosis factor alpha in experimental arthritis: separate activity of interleukin 1beta in chronicity and cartilage destruction." Ann Rheum Dis **58 Suppl 1**: I40-48.

van der Harst, M. R., P. A. Brama, et al. (2004). "An integral biochemical analysis of the main constituents of articular cartilage, subchondral and trabecular bone." Osteoarthritis Cartilage **12**(9): 752-761.

van der Kraan, P. M. and W. B. van den Berg (2012). "Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration?" Osteoarthritis Cartilage **20**(3): 223-232.

Van der Vis, H. M., P. Aspenberg, et al. (1998). "Fluid pressure causes bone resorption in a rabbit model of prosthetic loosening." Clin Orthop Relat Res(350): 201-208.

van Susante, J. L., P. Buma, et al. (1999). "Resurfacing potential of heterologous chondrocytes suspended in fibrin glue in large full-thickness defects of femoral articular cartilage: an experimental study in the goat." Biomaterials **20**(13): 1167-1175.

Vats, A., R. C. Bielby, et al. (2006). "Chondrogenic differentiation of human embryonic stem cells: the effect of the micro-environment." Tissue Eng **12**(6): 1687-1697.

Verzijl, N., J. DeGroot, et al. (2000). "Effect of collagen turnover on the accumulation of advanced glycation end products." J Biol Chem **275**(50): 39027-39031.

Walsh, D. A., C. S. Bonnet, et al. (2007). "Angiogenesis in the synovium and at the osteochondral junction in osteoarthritis." Osteoarthritis Cartilage **15**(7): 743-751.

Wehling, N., G. D. Palmer, et al. (2009). "Interleukin-1beta and tumor necrosis factor alpha inhibit chondrogenesis by human mesenchymal stem cells through NF-kappaB-dependent pathways." Arthritis Rheum **60**(3): 801-812.

Weiss, S., T. Hennig, et al. (2010). "Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells." J Cell Physiol **223**(1): 84-93.

Westacott, C. I., G. R. Webb, et al. (1997). "Alteration of cartilage metabolism by cells from osteoarthritic bone." Arthritis Rheum **40**(7): 1282-1291.

Wiegant, K., P. M. van Roermund, et al. (2013). "Sustained clinical and structural benefit after joint distraction in the treatment of severe knee osteoarthritis." Osteoarthritis Cartilage **21**(11): 1660-1667.

Williams, C. G., T. K. Kim, et al. (2003). "In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel." Tissue Eng **9**(4): 679-688.

Yamane, S., E. Cheng, et al. (2007). "Gene expression profiling of mouse articular and growth plate cartilage." Tissue Eng **13**(9): 2163-2173.

Yan, D., G. Zhou, et al. (2009). "The impact of low levels of collagen IX and pyridinoline on the mechanical properties of in vitro engineered cartilage." Biomaterials **30**(5): 814-821.

Yu, C. L., K. H. Sun, et al. (1994). "Interleukin 8 modulates interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha release from normal human mononuclear cells." Immunopharmacology **27**(3): 207-214.

Zscharnack, M., P. Hepp, et al. (2010). "Repair of chronic osteochondral defects using predifferentiated mesenchymal stem cells in an ovine model." Am J Sports Med **38**(9): 1857-1869.

Publications, Awards, Posters and Presentations

Publications

Leyh, M., Seitz, A., Dürselen, L., Schaumburger, J., Ignatius, A., et al. Subchondral bone influences chondrogenic differentiation and collagen production of human bone marrow-derived mesenchymal stem cells and articular chondrocytes. Arthritis Research & Therapy. 10/2014; 16(5):453

Leyh, M., Seitz, A., Dürselen, L., Springorum, HR., Angele, P., et al. Osteoarthritic cartilage explants affect extracellular matrix production and composition in cocultured bone marrow-derived mesenchymal stem cells and articular chondrocytes. Stem Cell Res Ther (2014); 5: 77.

Haubner, F., Leyh, M., Ohmann, E., Pohl, F., Prantl, L., Gassner, HG. Effects of external radiation in a co-culture model of endothelial cells and adipose-derived stem cells. Radiat Oncol (2013); 8(1):66.

Haubner, F., Leyh, M., Ohmann, E., Sadick, H., Gassner, HG. Effects of botulinum toxin A on patient specific keloid fibroblasts in vitro. Laryngoscope (2013)

Awards

Highest Broicher Award of the "Deutsche Gesellschaft für Hals-Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie, e.V. " (May 2014) for: Michaela Leyh, Regensburg, with research group H. G. Gassner, F. Pohl, F. Haubner, Regensburg; Poster: "Modulation radiogener Effekte durch plättchenreiches Plasma auf Zellen der kutanen Wundheilung in vitro"

Posters and Presentations:

17 April 2009	Forschungssymposium (Bad Abbach)	presentation
3 - 5 June 2009	Joint Meeting of the French and German Societies for Connestive Tissue (DGBF, Reims),	poster
	grant: Reisekostenbeihilfe der FAS Frauenförderung	
4 - 6 October 2009	Ernst Klenk Symposium (Köln),	poster
	grant: Reisestipendium der Freunde der Uni Regensburg	
4 - 5 December 2009	German Cartilage Club Meeting der ICRS (Bad Abbach)	
10 - 13 March 2010	33rd Annual Meeting of the German Society for Cell Biology (GSCB, Regensburg),	poster
18 - 20 March 2010	Joint Meeting of the British and German Societies for	

	Matrix Biology (DGBF, Frankfurt),	poster
16 April 2010	Forschungssymposium (Bad Abbach)	presentation
26 - 27 November 2010	Translation in Regenerative Medicine (TIRM, Regensburg)	
23 - 26 September 2010	OARSI World Congress on Osteoarthritis (Brüssel),	poster
	grant: Reisekostenbeihilfe der FAS Frauenförderung	
3 - 7 July 2010	FECTS Meeting (Davos),	poster
	grant: Reisekostenbeihilfe der GlaxoSmithKline Stiftung	
25 March 2011	Forschungssymposium (Bad Abbach),	presentation
31 March -2 April 2011	Jahrestagung der Deutschen Gesellschaft für Bindegewebs-forschung (DGBF, Köln),	poster
2 - 3 September 2011	Symposium of AO Exploratory Research (Davos),	presentation
	grant: Reisekostenbeihilfe der FAS Frauenförderung	
6 - 8 October 2011	Münster DFG Abschluss- Symposium	
2 - 3 March 2012	Münchner Symposium für exp. Orthopädie, Unfallchirurgie und muskuloskelettale Forschung (München),	presentation
23 - 25 May 2012	Würzburger Initiative Tissue Engineering (WITE, Würzburg),	presentation
28 - 31 May 2014	85. Jahrestagung der Deutschen Gesellschaft für Hals- Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie e.V. (Dortmund)	poster &
	grant: Deutsche Gesellschaft für HNO e.V.	presentation