The effect of mechanical stimulation and biological factors on human mesenchymal stem cell and human articular cartilage progenitor cell chondrogenesis and hypertrophy

By

Alexander Neumann

Supervised by

Dr. M.J. Stoddart

&

Prof. M. Alini

AO Research Institute Davos

Davos, Switzerland

&

Prof. C.W. Archer

School of Medicine

Swansea University

Wales, UK

&

Dr. C.A. Boulter

School of Biosciences

Cardiff University

Wales, UK

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AO Research Institute Davos
Clavadelerstrasse 8

7270 Davos Platz
Switzerland

School of Biosciences
Cardiff University

Cardiff, Wales, UK
CF10 3AX

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Thesis summary

Adult articular cartilage has a limited repair capacity. This leads to an increasing demand for optimised repair techniques. Furthermore, current procedures to regenerate articular cartilage fail to achieve sufficient results. Previous work within our group suggested that combination of functional tissue engineering and gene transfer represents a promising alternative approach.

In this thesis, different viral gene transfer methods were investigated and optimised. A clinically relevant three dimensional transduction model was developed. These results were directly implemented in further work aiming to investigate the combined effect of multiaxial mechanical stimulation and adenoviral-mediated over-expression of bone morphogenetic protein 2 on human chondroprogenitor cell chondrogenesis and progression towards hypertrophy. Two cell sources were investigated, namely human mesenchymal stem cells and human articular cartilage progenitor cells. The combined approached enhanced human mesenchymal stem cell chondrogenesis. Yet, it was not possible to completely prevent progression towards hypertrophy. For human articular cartilage progenitor cells, over-expression of bone morphogenetic protein 2 did enhance their chondrogenic differentiation potential. However, mechanical stimulation alone, in the absence of exogenous growth factors, led to stable chondrogenic induction without signs of hypertrophic differentiation. This suggests these cells should be further investigated.

Additionally, the potential of Dorsomorphin, as possible agent to block hypertrophic differentiation by inhibition of bone morphogenetic protein signalling, was investigated in a fibrin polyurethane composite system, using human mesenchymal stem cells. As opposed to the pellet culture model, application of Dorsomorphin led to a cytotoxic effect which decreased the general differentiation potential.

Finally, the chondrogenic potential of the two cell types was directly compared, using the pellet culture model. Under serum-free conditions, human articular cartilage progenitor cells were not able to undergo chondrogenesis. The reasons for this remain to be elucidated. The combined results of the thesis can help to develop a novel one-step procedure to treat articular cartilage defects.

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Abbreviations

2D	Two dimensional
3D	Three dimensional
18s rRNA	Eighteen s ribosomal RNA
α-MEM	Alpha Minimal Essential Medium
AA	Ascorbic acid 2-phosphate
AAV	Adeno-associated virus
AB	Avidin-biotinylated enzyme
ACPS(s)	Articular cartilage progenitor cell(s)
ACT	Autologous chondrocyte transplantation
Ad.BMP-2	First generation, E1-, E5-deleted, serotype 5 adenoviral vector carrying the cDNA for human BMP-2
ALK	Activin receptor-like kinase
ALP	Alkaline phosphatase
bFGF	Basic fibroblast growth factor
BMP(s)	Bone morphogenetic protein(s)
BMP-2	Bone morphogenetic protein 2
BSA	Bovine serum albumin
CAR	coxsackie-adenovirus receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
Col I	Collagen type I
Col II	Collagen type II
Col X	Collagen type X
DAB	(3, 3’-diaminobenzidine)

DEPC	Diethyl pyrocarbonate-treated
DMMB	1,9-Dimethyl-methylene blue
DMEM	Dulbecco's Modified Eagle Medium high glucose (4.5g/L)
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPBS	Phosphate buffered saline containing 2 M sodium chloride
DTT	DL-Dithiothreitol
EACA	ε-aminocaproic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESC(s)	Embryonic stem cell(s)
FBS	Foetal bovine serum
g	Times gravity
GAG	Glycosaminoglycan
GAG/DNA	GAG content normalised to DNA content
GDF	Growth and differentiation factor
hACPC(s)	Human articular cartilage progenitor cell(s)
HGF	Hepatocyte growth factor
hMSC FBS	human MSC tested FBS
hMSC(s)	Human bone-marrow derived mesenchymal stem cell(s)
HRP	Horseradish peroxidase
IGF-1	Insulin like growth factor 1
Ihh	Indian hedgehog
IPS(s)	Induced pluripotent stem cell(s)
ITS+1	Insulin-transferrin-sodium selenite, linoleic BSA

kb	Kilo-bases
LTR	Long terminal repeat
mRNA	Messenger ribonucleic acid
MIF	Macrophage migration inhibitory factor
MilliQ water	Purified and de-ionised water
MMLV	Moloney murine leukaemia virus
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MSC(s)	Mesenchymal stem cell(s)
MSC FBS	Human MSC tested FBS
ND	Not detectable
NEAA	MEM non-essential amino acids
OA	Osteoarthritis
P	Passage
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline for histology supplemented with 0.1% Tween 20
PCR	Polymerase chain reaction
PD(s)	Population doubling(s)
PEEK	Polyetheretherketone
P/S	Penicillin/Streptomycin
PTHrP	Parathyroid hormone-related protein
PU	Polyurethane
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
RPM	Rounds per minute
RT	Reverse transcription

RT-PCR	Real-time PCR
Runx2	Run-related transcription factor 2
Rv.BMP-2	First generation VSG.G pseudo-typed retroviral vector expressing human BMP-2
Rv.eGFP	First generation VSG.G pseudo-typed retroviral vector expressing enhanced green fluorescent protein
SMAD	Small Mothers Against Decapentaplegic
SOX	SRY-related HMG-box
TE	Tissue engineering
T/E	Trypsin-EDTA
TE buffer	Tris-EDTA buffer
TGF-β	Transforming growth factor beta
TMB	Tetramethylbenzidine
VSV.G	Vesicular stomatitis virus G protein
Wnt	Wingless/integrated

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Chapter 1: General Introduction.

1.1 Abstract.

Hyaline, articular cartilage covers the osseous ends in diarthrodial joints. Its function is to absorb and distribute forces generated through loads. Thereby, it allows for smooth, almost frictionless motion between the articulating surfaces (Buckwalter and Mankin, 1998a). Articular cartilage is subjected to loading forces on a daily basis and its integrity is crucial for the normal function of the joint during everyday life. Despite having the capability to endure loads for decades, its intrinsic healing capacity is very low. Once damaged, articular cartilage has a very limited capability for self-repair. Today, there exists a fast growing need for optimised replacement tissue. Nevertheless, currently available procedures within clinical practice, aiming to generate a complete regeneration of articular cartilage, fail to demonstrate reproducible success. Hence, new approaches for the treatment of articular cartilage defects are desperately needed. Tissue engineering (TE) is believed to be one of the most promising alternative approaches when trying to regenerate articular cartilage.

1.2 Articular cartilage.

1.2.1 Articular cartilage development.

During embryonic development, bone and cartilage formation are closely linked. Bones can form through two different mechanisms; intra-membranous ossification and endochondral ossification. During intra-membranous ossification, mesenchymal stem cells (MSCs) directly develop into osteoblasts which form the bones of the skull. During endochondral ossification, which occurs in any other type of bone within the body, a cartilage template is built first. This template is then replaced by bone, sparing only the articular cartilage of the joints (figure 1-1).

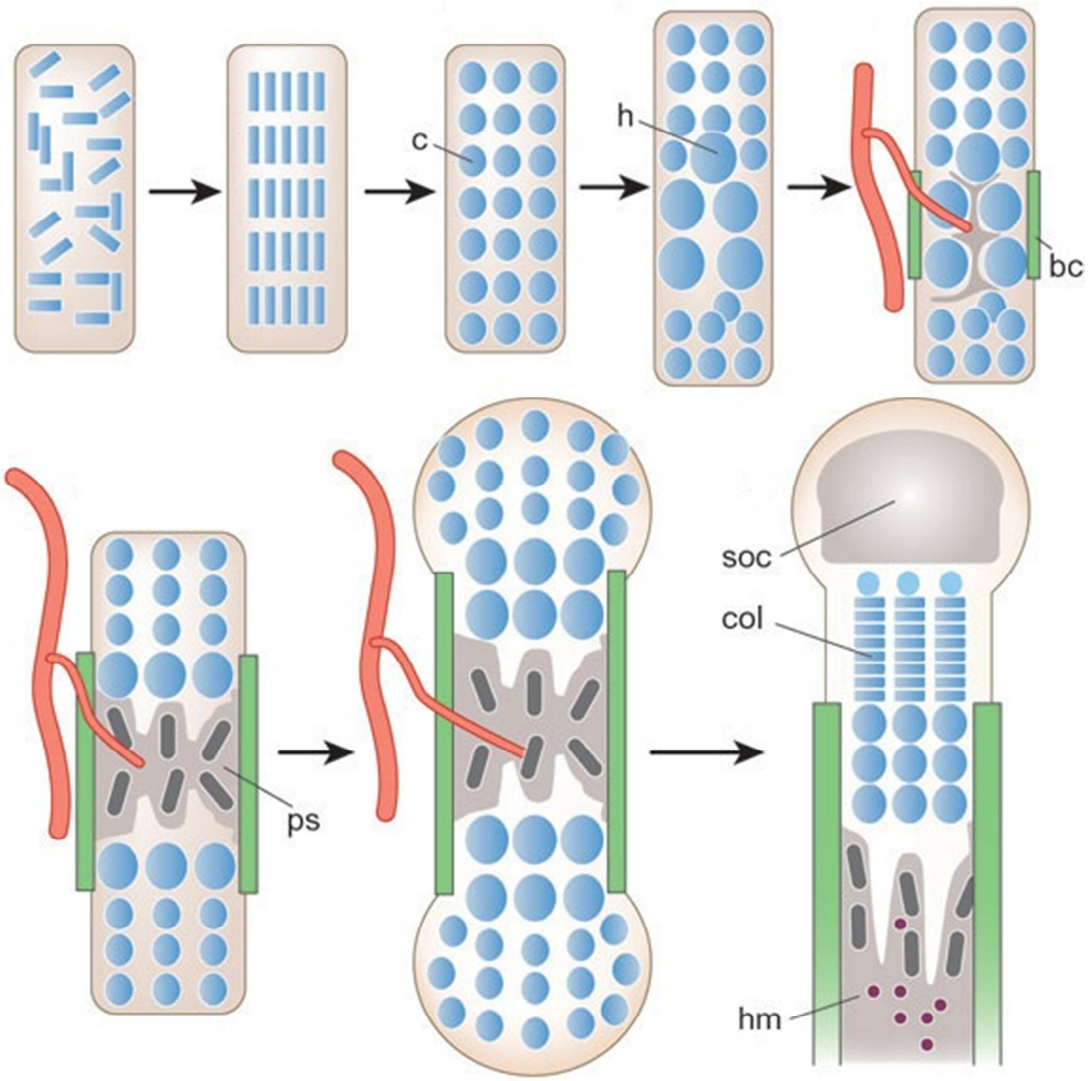

Figure 1-1: Schematic display of the process of endochondral ossification. First, mesenchymal cells condense and form a cartilaginous template. Chondrocytes (c) in the middle of the template start to undergo hypertrophy (h). Next, a bone collar (bc) is formed and hypertrophic chondrocytes attract vascular invasion. Then, the primary spongosia (ps), the primary centre of ossification, is formed. The bone elongates through chondrocyte proliferation, matrix synthesis and chondrocyte hypertrophy. At the end of the long bones, the second centre of ossification is formed (soc). Below the soc, a growth plate with columnar chondrocytes (col) is formed and haematopoietic marrow (hm) is starting to expand into the marrow space. Figure adapted from (Kronenberg, 2003).

The whole process starts with the condensation of mesenchymal cells. These cells then grow and start to differentiate into chondrocytes which form a cartilaginous template of the developing bone. Next, chondrocytes in the middle of the template start to enter hypertrophy. They stop growing and start to enlarge and secrete collagen type X (Col X) protein. The hypertrophic chondrocytes start to secrete vascular endothelial growth factor and begin to mineralise their matrix. Afterwards, the so-called bone collar is beginning to form. Perichondral cells differentiate into osteoblast and start to produce matrix. This process is guided by the hypertrophic chondrocytes, which then undergo apoptotic cell death. Within their matrix, the primary spongosia (primary centre of ossification) is beginning to form. Osteoblasts and blood vessels invade the matrix that has been produced by the hypertrophic chondrocytes and replace it with bone. The long bones elongate through three different processes; proliferation of columnar chondrocytes, chondrocyte matrix production and chondrocyte hypertrophy. Finally, secondary centres of ossification are starting to form at the end of the long bones. Chondrocytes stop proliferating, begin to undergo hypertrophy and attract vascular and osteoblast invasion. In the long bones of the limbs, which are connected by articulating joints, growth plates are additionally formed. These growth plates are located between the primary and the secondary centre of ossification at the end of the bones. They consists of cartilage which can be separated into four zones; resting cartilage, proliferating cartilage, maturing (hypertrophic) cartilage and calcified cartilage (figure 1-2). During skeletal maturation, bones continue to grow by proliferation of columnar chondrocytes, chondrocyte matrix production and chondrocyte hypertrophy within the growth plate. After skeletal maturity, the growth plate is replaced by bone and the only cartilage that is left is the articular cartilage of the joint.

The process how synovial joints and articular cartilage form during embryonic development and the plethora of molecules (e.g. signalling molecules and patterning molecules), which are involved during this process, have not been completely unravelled yet. Nevertheless, the general mechanisms has already been established (reviewed e.g. in: (Khan et al., 2007)). The process starts with condensation of mesenchymal cell. Next, a so called interzone is formed. This zone is located at the site where the future joint is going to form and comprises a thin layer of mesenchymal cells. These interzone cells together with adjacent mesenchymal cells are involved in the cavitation process, during which the two opposing skeletal elements separate.

The three-dimensional (3D) configuration of the future joint is established in a process called morphogenesis. Finally, the mature joint, including the articular cartilage and the synovial capsule, is formed. The interzone cells are critically involved in these processes. Fate tracking experiments in chicken embryos revealed that peri-joint mesenchymal cell became part of the interzone and that these cells were involved in the formation of the articular cartilage and the joint capsule (Pacifici et al., 2006). In the same study, these interzone cells were isolated and shown to express growth and differentiation factor (GDF)-5, wingless-integrated (Wnt)-14 and cluster of differentiation (CD)-44 and that they differentiate into chondrocytes. The important role of interzone cells, expressing GDF-5, during joint formation was further established in the mouse model (Koyama et al., 2008). Fate mapping (using GDF-5-cre transgenic mice), revealed that interzone cells displayed a gradient-like distribution along the ventral-to-dorsal axis during joint formation and that these cells gave rise to joint tissues, such as articular cartilage and the synovial lining. Isolated interzone cells displayed a propensity to undergo chondrogenesis and this process could be enhanced by over-expression of GDF-5 and blocked by over-expression of Wnt9a (Koyama et al., 2008).

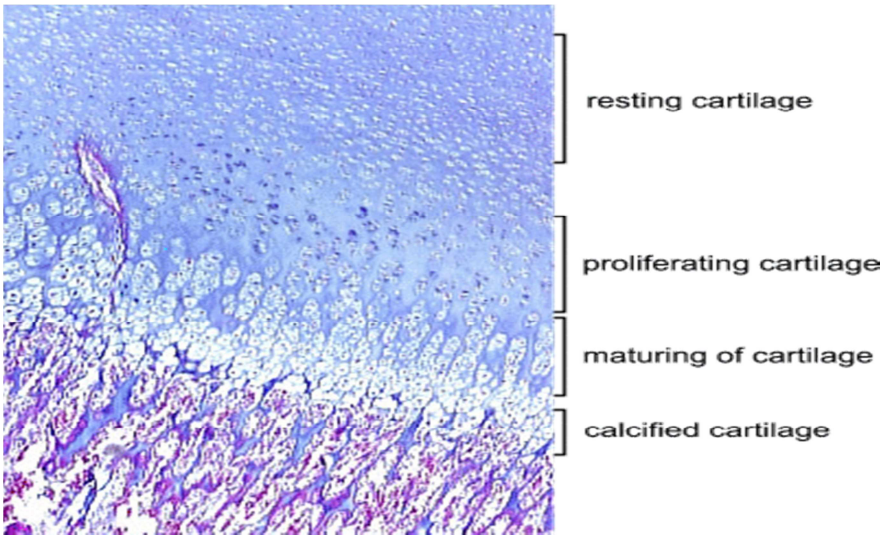

Figure 1-2: Histological staining of an epiphyseal growth plate. The resting zone comprises small scattered chondrocytes. They probably form a reserve for the proliferating columnar chondrocytes. These cells form stacks which are aligned in the direction of growth. In the third zone, chondrocytes start to mature and become hypertrophic. In the zone of calcified cartilage, chondrocytes start to calcify their matrix and undergo apoptotic cell death. Finally, this template is invaded by blood vessels and osteoblasts. Figure adapted from http://www.gla.ac.uk.

1.2.2 Articular cartilage composition.

Articular cartilage is a relatively simple tissue. It lacks vasculature, a lymphatic system and it is not innervated. Previously, it was believed to consist only of one type of cell, the chondrocyte (for a detailed overview about chondrocytes see: (Archer and Francis-West, 2003)), and the extracellular matrix (ECM) these cells produce. However, recently there exists growing evidence that articular cartilage also contains a stem/progenitor cell population (Dowthwaite et al., 2004; Hayes et al., 2008; Khan et al., 2009). These progenitors were originally isolated from the surface zone of bovine articular cartilage through differential adhesion to fibronectin (Dowthwaite et al., 2004). In 2010, it was demonstrated that these cells can also be obtained from human articular cartilage (Williams et al., 2010).

Articular cartilage is only very sparsely populated with cells. In humans, approximately 2% of its total volume is occupied by chondrocytes (Hunziker et al., 2002). The chondrocytes main functions are to secrete and to maintain the ECM. Also, chondrocytes are known to sense loading forces and modify their matrix accordingly (Buckwalter and Mankin, 1998b). The ECM of articular cartilage consists of three main components; water (up to 80% wet weight), collagen (50-80% dry weight), mainly type II with smaller amounts of type VI, IX, X and XI and negatively charged proteoglycans (5-10% dry weight) (Buckwalter and Mankin, 1998b; Riesle et al., 1998). The collagens type II, IX and XI form a mesh with provides tensile strength and is able to entrap macromolecules. The negatively charged proteoglycans consist of a protein core and one or more glycosaminoglycan (GAG) chain(s). GAGs that are typically presented within articular cartilage are, for example, hyaluronic acid, chondroitin sulphate, keratan sulphate and heparan sulphate (Buckwalter and Mankin, 1998b; Temenoff and Mikos, 2000). The two main classes of GAG in articular cartilage are large aggregating proteoglycans (aggrecans) and smaller proteoglycans, such as decorin and bigylcan. Aggrecans are able to attract water, due to their high sulphate content, leading to a negative charge. This feature is very important for the stress distribution within articular cartilage and also for the resilience of this tissue. The small proteoglycans do not contribute to the tissues mechanical properties. Instead, they are believed to be important for the organisation of the ECM structure (Buckwalter and Mankin, 1998b).

Articular cartilage possesses a highly ordered structure. Due to morphological changes it can be divided into four different zones; the superficial zone, the middle zone, the deep zone and the zone of calcified cartilage (figure 1-3).

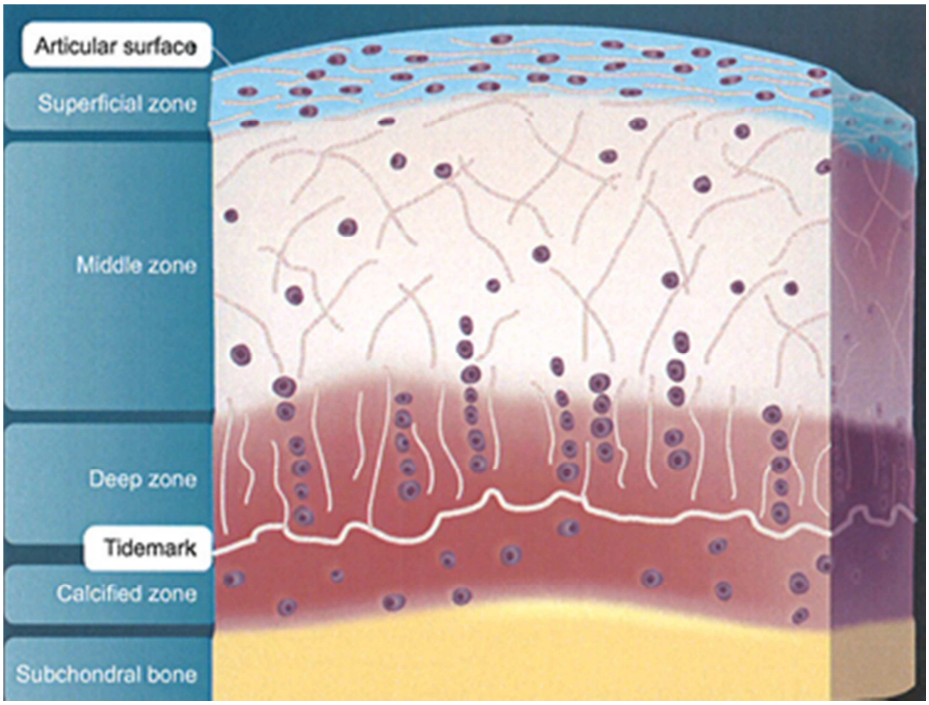

Figure 1-3: Schematic illustration of the zonal structure of articular cartilage. Articular cartilage can be divided into four zones; 1) the superficial zone which contains flattened chondrocytes and collagen fibres which are aligned parallel to the articular surface, 2) the middle zone where spherical chondrocytes are embedded in a matrix of randomly orientated collagen fibres, 3) the deep zone where rounded chondrocytes and collagen fibres are aligned in stacks perpendicular to the surface 4) the zone of calcified cartilage which is separated from the other zones through the tidemark. Chondrocytes in the zone of calcified cartilage are small and their matrix starts to calcify. Figure adapted from (Stoddart et al., 2009) with permission of Future Medicine Ltd.

These zones differ in matrix components, the alignment of collagen fibres, relative amount of collagen, relative amount of proteoglycans and chondrocyte phenotype. These differences are of great functional importance. The proportion of the four zones differs between species but also between different joints within the same species. Generally, collagen content decreases and aggrecan content increases from the superficial zone to the zone of calcified cartilage. The water content is highest in the superficial zone and lowest in the deep zone. The superficial zone is closest to the joint space and the thinnest zone within articular cartilage. It consists of two layers. The first layer, the acellular lamina splendens, covers the joint. Within the second layer, flattened chondrocytes and collagen fibres are aligned parallel to the surface.

Within the joint, only chondrocytes in the superficial zone and synovial cells are able to synthesise superficial zone protein/lubricin (Schumacher et al., 1999). This unique protein plays a major role for lubrication properties within the articulating joint. The middle zone separates the superficial zone from the deep zone. Chondrocytes within the middle zone have a spherical morphology. Compared to the superficial zone, collagen fibres are larger and randomly aligned. In the deep zone, chondrocytes have a rounded morphology and are aligned in columns perpendicular to the surface. Collagen fibres are larger than in the first two zones and they are aligned perpendicular to the surface. The zone of calcified cartilage is separated from the other three zones through a structure called the tidemark. This zone lies adjacent to the sub-chondral bone and represents a transition between articular cartilage and bone. The chondrocytes within the zone of calcified cartilage are small and their matrix is starting to calcify (Buckwalter and Mankin, 1998b).

Not only articular cartilage as a tissue, but also its ECM, can be sub-divided into different regions/zones (Buckwalter and Mankin, 1998b). Namely, these are a pericellular region, a territorial region and an interterritorial region. The pericellular region of the ECM is directly attached to the chondrocyte membrane. Interestingly, it does not contain any (or very few) fibrillar collagens. The territorial matrix surrounds the pericellular matrix, mainly of single chondrocytes yet also of pairs, clusters or even columns (within the middle zone) of chondrocytes. Pericellular and intercellular matrix share a uniform function. They allow the binding of the chondrocytes cell membrane to molecules of the ECM. Furthermore, they protect the chondrocyte from forces generated through load and from deformation. In contrast, the main function of the interterritorial matrix is to provide the mechanical properties of the cartilaginous tissue itself. It contains large collagen fibrils with changing geometry. These fibres are aligned parallel to the articulating surface in the superficial zone, random in the transitional zone and perpendicular to the articulating surface in the deep zone.

1.2.3 Articular cartilage homeostasis.

Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) form a negative feedback loop which is responsible for the balance between proliferating and hypertrophic chondrocytes. This mechanism controls when chondrocytes leave the proliferating pool and when they begin to undergo hypertrophy. Figure 1-4 gives a short overview about the Ihh/PTHrP feedback loop. More detailed information can be found in (Kronenberg, 2003; Vortkamp et al., 1996).

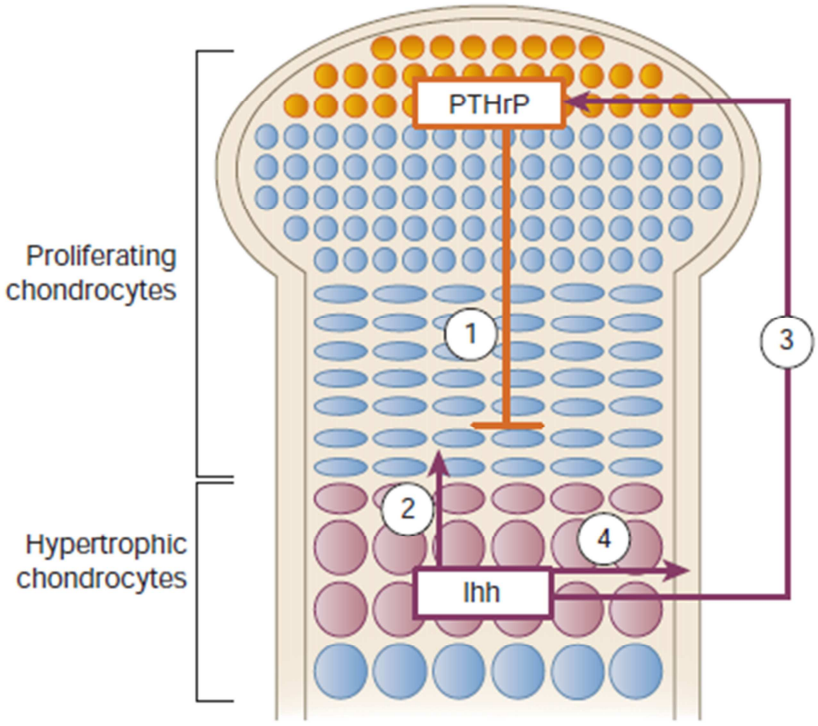

Figure 1-4: Schematic overview of the Ihh/PTHrP feedback loop. PTHrP is produced in the perichondrium and from chondrocytes at the end of long bones. It acts on pre-hypertrophic chondrocytes and prevents them from undergoing hypertrophy (1). Hypertrophic chondrocytes are too far away from the source of PTHrP production. The stop proliferating and produce Ihh. Ihh induces proliferation of chondrocytes in the proliferating zone (2), stimulates the production of PTHrP at the end of long bones (3) and potentially induces the conversion of perichondral cells into osteoblasts (4). Figure adapted from (Kronenberg, 2003).

PTHrP is produced in the perichondrium and from chondrocytes at the end of long bones. It acts on pre-hypertrophic chondrocytes which possess a high level of PTHrP receptor and prevents them from undergoing hypertrophy (Lee et al., 1996). Thereby, these cells stay within the proliferating pool. Binding of PTHrP to its receptor also leads to a reduced expression of runt-related transcription factor 2 (Runx2) and phosphorylation of the master chondrogenic transcription factor SRY-related HMG-box (SOX) 9.

Chondrocytes which are located too far away from the PTHrP producing cells are no longer exposed to sufficient concentrations of PTHrP. These cells begin to produce Ihh and stop proliferating. Ihh has three main modes of action. First, it acts on chondrocytes in the proliferating zone and induces proliferation. Second, it acts on chondrocytes at the end of long-bones and simulates them to produce PTHrP. The specific mechanism of this action is still unknown. Finally, exposure of perichondral cells to Ihh leads to their conversion into osteoblasts.

1.2.4 Clinical significance of articular cartilage damage.

When articular cartilage is damaged or degenerates, patients will suffer from pain, joint deformation and limited joint movement capacity. This causes great discomfort to the patient and is one of the major causes for disability in middle-aged and older people, especially in western countries. Damage to articular cartilage is mainly caused by either traumatic injury or pathological conditions, such as rheumatoid arthritis (RA) or osteoarthritis (OA). RA is an autoimmune disease characterised by two main symptoms; joint inflammation and tissue destruction. On the other hand, OA is a more complex, progressive disease. It represents the most common musculoskeletal disease in the elderly population and leads to a high socio-economic burden (Reginster, 2002). OA can affect every joint in the body yet some joints, such as knee or hip, are more prone to OA. Further, OA can either be restricted to a single joint or it can be more generic. Even though OA influences the whole joint, its main hallmark is articular cartilage degradation (including loss of proteoglycans from the ECM, disruption of the collagen network and, finally, cell loss). Other symptoms are, for example, intra-articular cartilage inflammation or changes in peri-articular and sub-chondral bone (such as osteophytes or sub-chondral bone cysts) (Goldring and Goldring, 2007). Major factors that influence the onset and progression of OA are joint instability, obesity, muscle weakness, peripheral neuropathy and increasing age (Goldring and Goldring, 2007).

Generally, cartilage defects can be divided into two major classes; partial-thickness/full-thickness defects and osteochondral defects. In partial-thickness/full-thickness defects, the damage is restricted entirely to the cartilaginous tissue and does not penetrate the sub-chondral bone. Partial-thickness defects (which do not span the whole thickness of the articular cartilage) resemble the clefts and fissures seen during the early stages of human OA (Hunziker, 1999). Full-thickness defects span the whole thickness of the articular cartilage but do not penetrate the sub-chondral bone. These defects will not heal spontaneously (figure 1-5 a). Partially, because the cell number in cartilage is very low (Poole et al., 2001). In addition to that, mature chondrocytes have a limited proliferative and biosynthetic activity. In healthy, adult cartilage, they are able to provide matrix turnover, yet there ability to build new matrix is very limited. Moreover, articular cartilage possesses a rare intrinsic biology. As it is an avascular tissue, neither blood cells nor MSCs from outside the tissue can reach the defect and contribute to a healing response (Hunziker and Rosenberg, 1996).

In osteochondral defects, the damage penetrates the sub-chondral bone. In this case, a healing response is induced (figure 1-5 b). Blood first enters the lesion from damaged vasculature or marrow and a fibrin clot is formed (Buckwalter and Mankin, 1998a; Steinert et al., 2007). Therein, platelets are trapped which start to secrete bioactive factors, such as platelet derived growth factor or different isoforms of transforming growth factor beta (TGF-β). These factors then attract vascular invasion and migration of mesenchymal progenitor cells into the defect (Steinert et al., 2007). Eventually, this repair response leads to the generation of a fibrocartilage-like repair tissue. In the human, the normal cartilage of the joint is hyaline. The fibrocartilage-like repair tissue is not adapted to the loading forces within the joint and, thereby, mechanically inferior to articular cartilage. This will ultimately lead to its disintegration under the load forces it has to bear (Hunziker, 2002). As opposed to humans, some joints in different vertebrate species can comprise fibrocartilage (Barnett, 1954).

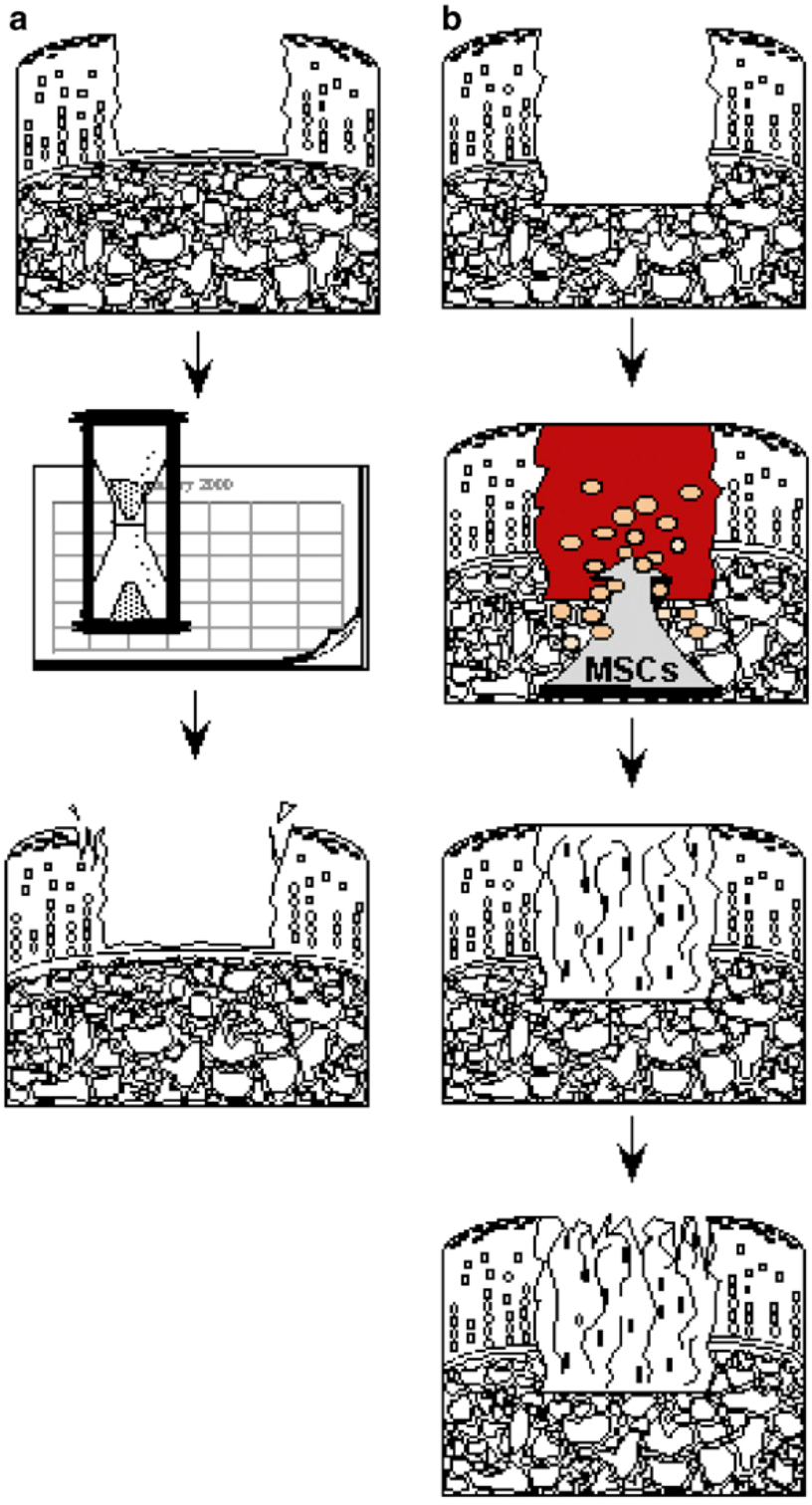

Figure 1-5: Schematic presentation of a full-thickness (a) and an osteochondral (b) defect. Full-thickness defects are restricted to the cartilage tissue and will not heal spontaneously. In osteochondral defects, the damage does penetrate the sub-chondral bone. A fibrin clot is formed which induces migration of MSCs and a repair response is induced. This leads to the generation of a fibrocartilage tissue which is mechanically inferior and degenerates with time. Figure adapted from (Trippel et al., 2004).

1.2.5 Tissue engineering as an alternate approach for articular cartilage repair.

Currently, there exists a great variety of approaches, within clinical practice, which aim to heal articular cartilage defects. Conservative treatment options include, for example, weight loss, physiotherapy and pain-relieving medication. Surgical interventions are the next option (reviewed for example in: (Ahmed and Hincke, 2010)). They are indicated if conservative treatment methods failed or if the defect is already too advanced for such treatment options. Usually, for a first line surgical treatment, different marrow-stimulating techniques, such as pridie drilling (Muller and Kohn, 1999) or microfracture (Steadman et al., 2002), are conducted. These techniques are relatively straightforward and cost effective. Principally, they are based on the natural healing response of osteochondral defects. Small holes are created within the sub-chondral bone. Thus, an access to the bone-marrow space is created which leads to the formation of a fibrin clot, containing cells, within the defect. Finally, these techniques result in the formation of a fibrocartilagenous repair tissue. Other options are, for example, replacement of lost articular cartilage with soft tissue grafts, such as periosteum or perichondrium (Homminga et al., 1990), mosaicplasty (Matsusue et al., 1993) or autologous chondrocyte transplantation (ACT) (Brittberg et al., 1994). ACT is a two-step cell-based therapy. First, cartilage is harvested from a non-weight-bearing area of the joint. Next, chondrocytes are isolated and propagated in cell culture, in order to increase their number. Finally, in a second operation, they are transplanted into the defect which is then covered with a periosteal flap. ACT results in the formation of a hyaline-like cartilaginous repair tissue. Nevertheless, ACT possesses some disadvantages, such as donor-site morbidity, chondrocyte leakage from the defect site, the requirement for two surgical interventions, chondrocyte de-differentiation during monolayer culture and periosteal hypertrophy. Some of these were overcome using later generation ACT which included the use of Collagen I/III scaffolds or sponges to contain the cells.

The mentioned treatment options can improve the clinical situation of the single patient, such as pain relief or an increased ability to move. Yet, the clinical outcome of these procedures seems to be dependent on many different parameters, such as the age of the patient, the size of the defect and the pre-operative duration of symptoms. In general, the younger the patient, the smaller the defect and the less time prolonged between the occurrence of the symptoms and the beginning of the treatment the better the clinical outcome will be (Homminga et al., 1990; Marlovits et al., 2006; Mithoefer et al., 2006). Unfortunately, none of the mentioned techniques could yet demonstrate an ability to reproducibly regenerate articular cartilage within every patient and, thus, provide a long-term solution (Getgood et al., 2009).

In summary, although William Hunter first described damage of articular cartilage more than two and a half centuries ago (the original publication from 1743 was re-printed in: (Hunter, 1995)), there is no procedure available that has the ability to fully reconstitute the biological function and composition of articular cartilage. Even nowadays, it remains a very challenging task for surgeons and basic scientists. TE, a field that bridges basic and applied research, is believed to be a promising alternate approach to the currently available applications. It was first introduced by Langer and Vacanti in 1993 (Langer and Vacanti, 1993). Hunziker describes TE as “the art of reconstituting mammalian tissues, both structurally and functionally” (Hunziker, 2002). Its ultimate goal is to create a functional tissue *ex vivo* and to integrate this tissue into the (human) body. Generally, TE consists of three major building blocks; cells, a scaffold and stimulating or bioactive factors.

1.3 Cells.

1.3.1 Introduction.

This section will mainly focus on human bone-marrow-derived mesenchymal stem cells (hMSCs), sometimes also termed mesenchymal stromal cells. Other possible cell sources for TE of articular cartilage will be discussed. Mainly, these are mature chondrocytes and embryonic stem cells (ESCs). Recently, induced pluripotent stem cells (iPSCs) have been discovered and they might represent a valid cell source for cartilage TE and many other diseases in the long term. Further, the isolation of a progenitor cell population from articular cartilage was recently described. These cells might harbour great potential for articular cartilage TE. Each cell type has its own set of advantages and disadvantages which will be briefly discussed thereafter.

1.3.2 Chondrocytes.

Mature chondrocytes are widely used in TE and clinical practice. Usually, the number of chondrocytes that can be harvested is quite low and not sufficient for many applications, although recent studies using minced cartilage are beginning to question that opinion (Lu et al., 2006; Marmotti et al., 2012). For most applications, chondrocytes have to be expanded in monolayer culture in order to generate sufficient cell numbers. Afterwards, they can be either injected into the defect site (ACT) or used as a cell source in TE applications. However, there exists one major obstacle. During monolayer proliferation, chondrocytes tend to de-differentiate and lose their phenotype (von der Mark et al., 1977). Successful re-differentiation is possible in 3D culture and was demonstrated in several studies from different groups (Benya and Shaffer, 1982; Bonaventure et al., 1994; Stoddart et al., 2006). Sailor and co-workers were also able to demonstrate that a population of bovine chondrocytes, in monolayer culture, was able to retain its phenotype if 100ng/ml human recombinant bone morphogenetic protein 2 (BMP-2) was added to the culture medium (Sailor et al., 1996).

The application of autologous chondrocytes faces another drawback which has to be considered. The harvesting step requires an invasive operation which has to be conducted prior to the actual implantation step or any cell culture experiment. This surgical procedure, as any surgical intervention, represents a potential risk and a burden for the patient. On top of that, the harvesting of chondrocytes from healthy cartilage is often coupled with donor site morbidity. This will contribute to the damaging of a formerly healthy tissue.

1.3.3 Mesenchymal stem cells.

MSCs belong to the class of multipotent adult stem cells. Friedenstein and his co-workers were the first to discover their existence and demonstrated that they can be isolated from bone-marrow (Friedenstein et al., 1976). In the human body, they can be derived from several other tissues, such as adipose tissue (Zuk et al., 2002), muscle (Kuroda et al., 2006), trabecular bone (Tuli et al., 2003), periosteum (Arnold et al., 2002) and synovium (De Bari et al., 2001). In the year 2006, the International Society for Cellular Therapy proposed minimal criteria to describe MSCs (Dominici et al., 2006). Namely these were; their ability to adhere to cell culture plastic, their capacity to differentiate along the chondrogenic, osteogenic and adipogenic lineage and their surface marker expression profile (negative for CD14 or CD11b, CD19 or CD79α, Cd34, Cd45 and HLA-D-; positive for CD73, CD90 and CD105).

Yet, it has to be considered that these criteria are not perfect. The cells, isolated through adherence to cell culture plastic, are not a pure population. Further, selection with the mentioned surface markers will only separate mesenchymal cells from hematopoietic cells. Additionally, unless working with clonal populations, it cannot be ruled out that different cell types within the MSC population differentiate towards chondrocytes, osteocytes and adipocytes. Therefore, these criteria will likely result in an enrichment and not in a defined isolation of a single cell type. That’s why so-called MSCs are probably not consisting of only a single cell type. More likely, they are highly heterogeneous and consist of many different cell types.

Furthermore, some people hesitate to call these cells MSCs because they lack true stem cell characteristics. MSCs are only multipotent not totipotent or pluripotent. Thus, they do not have the ability to differentiate into any kind of cell derived from all three germ layers (e.g. heart muscle cells, neurons, kidney cells etc.) or cells from the germ lineage. Instead, they can only differentiate into a restricted number of cell types which belong to the mesenchymal lineage (figure 1-6). This has been shown in different studies (Pittenger et al., 1999; Tuli et al., 2003). Additionally, they do not have an unlimited capacity for self-renewal. Instead, they are known to become senescent around 30 population doublings (PDs) (Bonab et al., 2006). This is why they are sometimes referred to as mesenchymal progenitor cells or mesenchymal stromal cells.

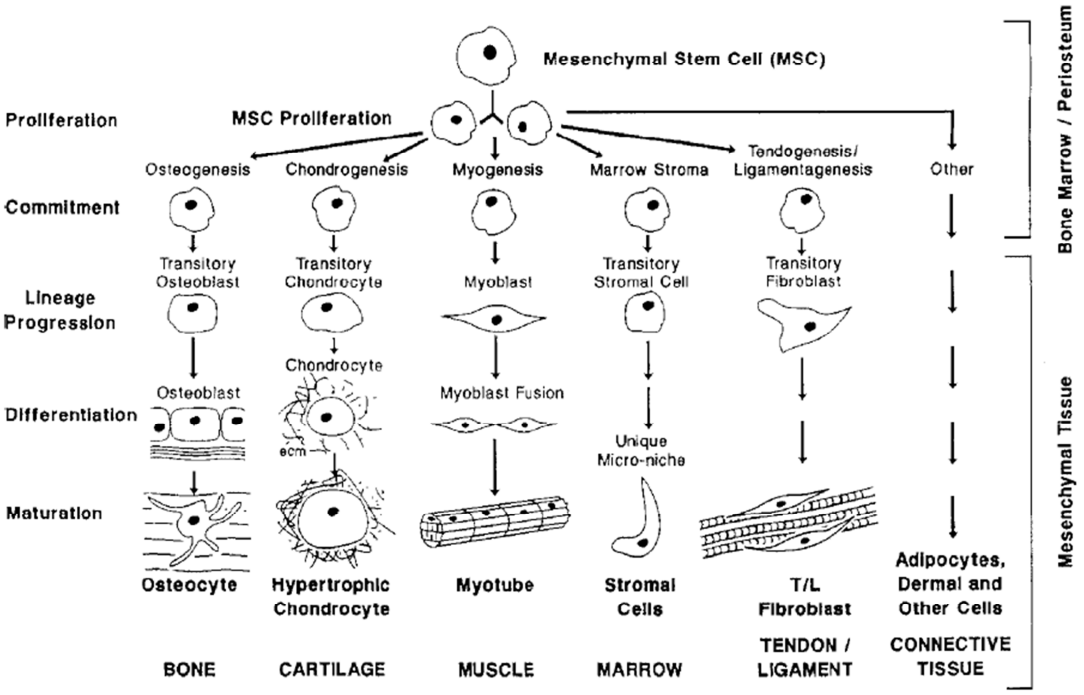

Figure 1-6: Schematic summary of the process of mesenchymal stem cell differentiation. Mesenchymal stem cells can differentiate into several cell types which belong to the mesenchymal lineage. Examples are osteocytes, chondrocytes and adipocytes. Figure adapted from (Caplan, 2009).

MSCs can be differentiated towards the chondrogenic lineage and numerous different approaches for *in vitro* chondrogenic differentiation exist (reviewed for example in: (Heng et al., 2004)). The molecular pathway MSCs undergo during chondrogenesis is very complicated and a vast number of different molecules, such as transcription factors and growth hormones, are involved (for detailed information read for example: (Goldring et al., 2006; Jorgensen et al., 2008)). The first study that showed the successful chondrogenic differentiation of MSCs was done by Ashton and co-workers in 1980 (Ashton et al., 1980). The next milestone was set by Johnstone et al. in 1998. They were the first to describe a defined medium for the *in vitro* chondrogenesis of MSCs in 3D micromass culture (Johnstone et al., 1998). This chondrogenic medium lacked foetal bovine serum (FBS) and contained dexamethasone and TGF-β1 as essential agents for the induction of chondrogenesis. The fact that FBS is omitted from the culture medium is very desirable for any potential application within clinical practice. Thus, potential risks, such as immunological rejection or disease transmission (Will et al., 1996), can be avoided. Moreover, serum contains a vast number of biologically active substances, such as hormones or growth factors. Its exact composition differs with the source from which it is obtained but also between different batches from the same source. Therefore, media that lack serum provide a milieu which is chemically more defined and is favourable for any kind of biological study.

Although much work is carried out in the field at the moment, *in vitro* chondrogenic induction of MSCs still faces several shortcomings. First, the proliferative capacity of MSCs, and consequently their cell number, in bone-marrow declines with age (Caplan, 2007). The reason for that phenomenon is currently still under debate. Second, the cells that are isolated from bone-marrow are, in fact, a highly heterogeneous population. Only few cells within this population are able to undergo chondrogenic differentiation. Hence, new and better isolation and purification protocols are urgently needed. Further, when chondrogenesis of MSCs is induced *in vitro*, chondrocytes cannot be stably arrested in culture and advance to their terminal phenotype the hypertrophic chondrocyte (described for example in: (Bosnakovski et al., 2006; Pelttari et al., 2006; Yoo et al., 1998)). This progression towards hypertrophy is accompanied by changes in the cell morphology, a decline in collagen type II (Col II) expression, an elevated expression of Col X and a marked increase in the activity of the enzyme alkaline phosphatase (ALP) that changes all lead to terminal differentiation.

If TE of articular cartilage using MSCs as cell source should become reality, currently existing protocols will have to be improved. It is an absolute requirement that the progression towards the hypertrophic state is prevented. Possible approaches to tackle this problem are, for instance, the addition of inhibitors of hypertrophy, the use of a special tailored scaffold or the application of mechanical load. Besides their use as a cell source in TE, MSCs may also be applied in regenerative medicine. It is becoming increasingly clear that they can home to injured tissues and act as trophic mediators and/or modulate the immune response (Augello et al., 2010; Caplan, 2007; Caplan and Dennis, 2006; Caplan, 2009; Chamberlain et al., 2007). Their function *in vivo* might be more similar to a conductor not to an orchestra. This proposes that they do not form the regenerated tissue themselves but rather contribute to the healing response by providing a wide spectrum of different biological active macromolecules which then act on cells within the defect.

1.3.4 Articular cartilage progenitor cells.

The isolation and identification of articular cartilage progenitor cells (ACPCs), from bovine cartilage samples, was described by Dowthwaite et al,. in 2004 (Dowthwaite et al., 2004). It was hypothesised that growth of articular cartilage is achieved from the appositional surface. Thereby, a population of progenitor or stem cells must reside within this zone. The group was able to isolate a chondrocyte sub-population with progenitor-like characteristics from the surface zone of articular cartilage by differential adhesion to fibronectin. These cells were shown to possess a high colony forming efficiency, to express Notch 1 and to exhibit phenotypic plasticity. In 2008, Hayes et al., demonstrated that these cells possess novel chondroitin sulphate sulphation motifs and that monoclonal antibodies against these epitopes could be used to select this cell population by flow cytometry (Hayes et al., 2008). In 2009, Khan et al., further characterised ACPCs from bovine origin (Khan et al., 2009). The focus of the study was on the growth kinetics and if prolonged culture influences these cells. ACPCs were isolated though differential adhesion to fibronectin and clonal cells were proliferated up to 50 PDs. Cells were assessed by measuring growth kinetics, telomere length, telomerase activity, β-galactosidase activity and gene expression analyses of SOX9 and Notch1.

It was demonstrated that ACPCs grow exponentially for approximately 20 PDs. Afterwards, growth slowed down and became more linear. The mean telomere length was significantly higher, when compared to de-differentiated chondrocytes that underwent a similar number of PDs. Further, telomerase activity was approximately 2.6-fold higher in ACPCs. The SOX9 activity was similar in ACPCs, when compared to de-differentiated chondrocytes. Finally, if ACPCs were induced to undergo chondrogenesis in pellet culture, co-ordinated growth and differentiation was monitored. Recently, it was demonstrated that ACPCs can be derived from human articular cartilage through differential adhesion to fibronectin (Williams et al., 2010). Williams and co-workers showed that clonal cell lines can be derived and proliferated up to high PDs. Also, it was demonstrated that these cells possess a restricted differentiation potential and that they can be chondrogenically induced within the pellet culture system. Further, the group found evidence for high telomerase activity and for the maintenance of telomere length within these cells, both features of a MSC population. Consequently, these cells represent an interesting alternative cell source in cell-based applications for cartilage repair. In a recent study of McCarthy et al.,, ACPCs and MSCs were directly compared as potential cell sources for cartilage repair in the horse (McCarthy et al., 2011). Both cells types were induced to undergo chondrogenesis within the pellet culture model. Immunohistochemistry revealed positive labelling for the Col II protein and the aggrecan protein in both cell populations. However, labelling for the Col X protein was only evident in pellets derived from MSCs. Further, MSCs labelled positive for the proteins Matrillin 1 and Runx2, whereas labelling was diminished or absent in ACPCs. Both cell sources were shown to be able to undergo osteogenesis and adipogenesis under standard conditions. In summary, this study showed that both cell types possess multilineage potential. However, ACPCs seemed to differentiate into stable articular chondrocytes, whereas MSCs appeared to undergo hypertrophy. As this progression towards hypertrophy is the main drawback for MSC based TE of articular cartilage, ACPCs might represent a superior cell source.

1.3.5 Embryonic stem cells.

ESCs have several major advantages. They possess the unlimited capacity to self-renew and they are pluripotent. Further, they are able to maintain their pluripotency in culture. It was demonstrated that ESCs can be differentiated into mesenchymal precursor cells under the right culture conditions (Barberi et al., 2005). Other studies demonstrated that they can be differentiated along the chondrogenic, osteogenic or adipogenic lineage (zur Nieden et al., 2005) or that they selectively undergo chondrogenesis when cultured under specific conditions (Hwang et al., 2006; Toh et al., 2007). Disadvantages of ESCs are, for instance, immunological rejection or the risk of teratoma formation after transplantation (Trounson, 2001). Last, but not least, people will have to consider the major ethical aspect if they use human ESCs as a cell source for cartilage TE or basic research.

1.3.6 Induced pluripotent stem cells.

iPSCs are pluripotent cells derived from somatic cells and can be differentiated into cells from all three germ layers. Their production from mouse fibroblasts was first described in the pioneering work of Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka, 2006). One year later, the same group was able to generate iPSCs from human fibroblasts (Takahashi et al., 2007). In both cases, re-programming into a pluripotent state was achieved through the retroviral-mediated expression of four factors; Oct3/4, SOX2, c-Myc and Klf4. iPSCs harbour the great potential to create patient-specific pluripotent stem cells. These cells could be used to treat several diseases and also for other applications, such as drug screening. In recent work, Wei et al., showed that iPSCs can be generated from osteoarthritic chondrocytes and that these disease-specific iPSCs can be successfully differentiated towards the chondrogenic lineage (Wei et al., 2012). Although, research using iPSCs is still at its infancy, potential problems have already been associated with their use. Differences in gene expression between iPSCs cells from different sources have been reported (Saric and Hescheler, 2008). These differences suggest that re-programming is incomplete. Furthermore, the creation of chimeras is more problematic in iPSCs, when compared to ESCs. This feature has been associated with a higher risk of tumour formation (Geoghegan and Byrnes, 2008).

1.3.7 Cell number.

A key factor in TE studies that is very often not addressed is the initial cell seeding density. In general, articular cartilage contains very few cells and has one of the lowest cellular densities among all tissues within the human body. Hence, the initial number of cells, seeded into different matrices or scaffolds, has to be carefully considered for every experiment. For example, Li and co-workers showed that the initial cell seeding density of hMSCs, in a composite fibrin gel-polyurethane (PU) scaffold system, influenced their ability to undergo chondrogenesis (Li et al., 2009a). It was demonstrated that 5 x 10^6 cells per scaffold (which equals to app. 33.25 x 10^6 cells per ml) are optimal within this system. Proteoglycan and Col II protein deposition was more evenly distributed throughout the scaffolds, when compared to higher (10 x 10^6) or lower (2 x 10^6) cell seeding densities. In addition, higher aggrecan and Col II gene expression was detected, when compared with the pellet culture system, the current "gold standard" for *in vitro* chondrogenesis. The gene expression of collagen type I (Col I) and Col X and the GAG to DNA ratio was comparable between both culture systems.

1.4 Scaffolds.

1.4.1 Introduction.

There exist a plethora of different scaffolds which can be used in TE of articular cartilage. This section will only provide a short general overview. Further, it will focus on a composite system consisting of a fibrin gel and a PU sponge-like scaffold. This composite system has been used for most experimental work within the thesis. More detailed general information about scaffolds for articular cartilage repair can be found, for example, in: (Balakrishnan and Banerjee, 2011; Frenkel and Di Cesare, 2004; Stoddart et al., 2009; Tortelli and Cancedda, 2009). A scaffold should mimic the 3D environment in which the cell normally reside. In order to accomplish this task, it has to fulfil several criteria:

- A scaffold, like any other device that should be implanted into the body, must be biocompatible. That means that neither the intact scaffold nor its degradation products will cause any inflammatory, immunogenic or otherwise harmful reaction. This point is especially important in articular cartilage which is an avascular tissue. As a result, degradation products can only slowly be evacuated by passive diffusion.
- Biodegradability is another important feature. The implanted scaffold should degrade and allow for a physiological remodelling. Ideally, the scaffold material should degrade with the same speed that the new tissue needs to form. This would allow for a gradually replacement of the scaffold with the freshly built tissue. At the same time, it is important to keep in mind that a scaffold will change its mechanical and structural properties while degrading.
- Scaffolds that should be utilised in TE applications must have a sufficient porosity. Cells loaded into the scaffold should be able to migrate through its intermediate space and/or native cells from the surrounding tissue should be able to infiltrate it.
- Further, a scaffold must allow for sufficient cell attachment. In order to accomplish this task, its surface might have to be adjusted depending on the cell type that is used. Factors, such as starting material, hydrophobicity or the possibility to attach cell adhesion promoting peptides have to be considered carefully.
- The scaffold should provide sufficient mechanical integrity. If necessary, it has to be implanted by a surgical procedure. In this case, it must be able to withstand the loading forces that might prevail *in vivo*, for example within an articulating joint. Mechanical integrity is also crucial if the scaffold is subjected to mechanical stimulation in a bioreactor system.
- Finally, if implanted, the scaffold has to be retained at the site of implantation.

Scaffolds used for cartilage repair can be manufactured from a wide range of different starting materials. If combined systems and chemical modifications of the starting material(s) are considered, the options are virtually unlimited. Broadly, these materials can be divided into two main classes, according to their origin. Scaffolds can be derived from natural materials or they can be synthetic. These two main groups can be further sub-divided. Scaffolds made of natural materials can be based on proteins or carbohydrates. Some examples, for scaffolds derived from natural materials, are fibrin, collagen, hyaluronan, chitosan, alginate and agarose. Synthetic materials that are commonly applied are PU and poly(α-hydroxyacids), such as polylactic acid and poly(ε-caprolactone), to name but a few.

1.4.2 A composite fibrin gel – polyurethane sponge scaffold system for tissue engineering of articular cartilage.

Fibrin is the polymerised form of fibrinogen. It is formed through a reaction between fibrinogen and thrombin. Its natural function in the body is to build a 3D matrix. This matrix prevents blood loss for example after a vascular lesion has occurred. Fibrin has been applied as scaffold system in many different studies. It can be either used as scaffold material or as a carrier substance for different cell types and different growth factors (Breen et al., 2009b; Eyrich et al., 2007; Ho et al., 2010; Li et al., 2009a; Pelaez et al., 2009; Salzmann et al., 2009; Xu et al., 2005)

PUs are a highly miscellaneous family of elastomers which are commonly used in clinical practice (explicitly reviewed in: (Pinchuk, 1994)). One prime requirement for several clinical applications is their stability *in vivo*. In contrast, for TE applications, there is an increasing interest in the design and the synthesis of biodegradable PUs. For cartilage TE, this system is intensively studied since 2003 (Eyrich et al., 2007; Grad et al., 2003; Lee et al., 2005; Li et al., 2009b; Li et al., 2009a; Li et al., 2009c; Salzmann et al., 2009; Schatti et al., 2011).

In 2003, Grad and co-workers investigated the potential and limitations of biodegradable PU scaffolds for cartilage TE (Grad et al., 2003). Their idea was that PU scaffolds have favourable mechanical properties and, therefore, mechanical stimulation can be applied to cells seeded within this type of scaffold. The mechanical load should help to stimulate the formation of a more cartilage-like ECM. The scaffolds potential to support the attachment, the growth and the maintenance of differentiated chondrocytes was investigated (in a time-frame up to 42 days). The group demonstrated that the DNA content of the constructs remained constant. Furthermore, they monitored an increase in collagen and GAG content. Shortcomings of this system were a significant release of matrix molecules into the culture medium and de-differentiation of mature chondrocytes. As possible improvement, it was suggested to either decrease the pore size of the scaffold or to suspend the cells in a hydrogel (such as fibrin, alginate or agarose) and afterwards seed them into the PU scaffold. Both measures can potentially improve the retention of matrix molecules. Further, the application of a hydrogel would be superior for the preservation of the chondrocytes differentiated phenotype.

In 2005, Lee and colleagues conducted a follow up study where a hybrid system, consisting of a fibrin hydrogel and a PU scaffold, was used (Lee et al., 2005). PU scaffolds, both alone or in combination with a fibrin gel, were seeded with chondrocytes and cultured for up to 28 days. Short-term mechanical stimulation was applied to some constructs. The composite system was superior, when compared to the application of PU scaffolds alone. In the composite system, chondrocytes were more homogenously distributed within the scaffold and higher cell viability during seeding was obtained. Furthermore, an increase in GAG retention and higher levels of aggrecan and Col II gene expression were measured. In conclusion, this study demonstrated that the additional use of a fibrin hydrogel is a significant improvement of the PU scaffold system for TE of articular cartilage. The group proposed several possibilities for further improvement. For example, an optimised loading protocol, the control of oxygen levels or the administration of growth or differentiation factors.

More recent studies revealed that mechanical stimulation induces hMSC chondrogenesis through the TGF-β pathway (Li et al., 2009b), that chondrogenesis of hMSCs can be enhanced by modifying frequency and amplitude of complex mechanical stimulation (Li et al., 2009c) and that shear plays an important role during hMSC chondrogenesis (Schatti et al., 2011). Furthermore, it was demonstrated that hMSCs are able to undergo chondrogenesis without application of exogenous growth factors and dexamethasone through the combination of adenoviral-mediated over-expression of SOX9 and mechanical stimulation (Kupcsik et al., 2010). Finally, work with bovine chondrocytes revealed that retroviral-mediated over-expression of BMP-2 in combination with mechanical stimulation synergistically enhances *in vitro* chondrogenesis of these cells (Salzmann et al., 2009).

1.5 Stimulating factors.

1.5.1 Introduction.

In TE of articular cartilage, different stimulating factors are used with the goal to either improve chondrogenesis of stem/progenitor cells or to better retain the chondrocytes phenotype. This section will mainly focus on two commonly applied stimuli; application of bioactive factors and stimulation through mechanical load.

1.5.2 Bioactive factors.

Bioactive factors, such as growth hormones, transcription factors or differentiation factors, play a pivotal role in embryogenesis and they influence a plurality of cellular functions within the human body. Chondrogenic cells (chondrocytes, MSCs and periosteal/perichondral cells) are influenced by and respond to them. For cellular based TE applications, it is widely accepted that exposure of cells to specific factors can induce chondrogenic differentiation and/or help to maintain the chondrocyte phenotype. The following section will focus on members of the TGF superfamily. Other factors that can be utilised include insulin like growth factor 1 (IGF-1) (Gelse et al., 2003; Madry et al., 2013; Palmer et al., 2005; Steinert et al., 2009a), fibroblast growth factors (Kato and Iwamoto, 1990; Weisser et al., 2001) and members of the SOX family (Kupcsik et al., 2010).

The bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the TGF superfamily. They play a role in various biological processes including embryogenesis, cell proliferation, postnatal bone formation and cartilage development. For TE of articular cartilage, their roles in skeletal development and chondrogenesis are of major interest (reviewed for example in: (Hoffmann and Gross, 2001; Kawabata et al., 1998; Wu et al., 2007; Yoon and Lyons, 2004)). BMPs are synthesised as inactive precursors. These precursors must be cleaved before they can form dimers, which is their biological active conformation. Historically, they were first recorded in the 1960s (Urist, 1965) and human BMPs were first cloned and purified in the late 1980s (Wozney et al., 1988). Currently, around twenty different BMPs have been identified and described.

BMPs bind to and signal through serine/threonine kinase receptors, which are composed of different type I and type II sub-types (figure 1-7). Ligand binding to the type I receptor leads to its phosphorylation by the type II receptor. The type II receptor is already phosphorylated in its basal state. Downstream signalling is mediated through "Small Mothers Against Decapentaplegic" (SMAD) proteins 1, 5 and 8. They are activated, through phosphorylation by their type I receptor, bind to and form a complex with SMAD 4, translocate into the nucleus and activate the transcription of target genes. SMADs 6 and 7 act as inhibitors of BMP signalling, on the receptor level, as they can bind to and form stable complexes with the receptors.

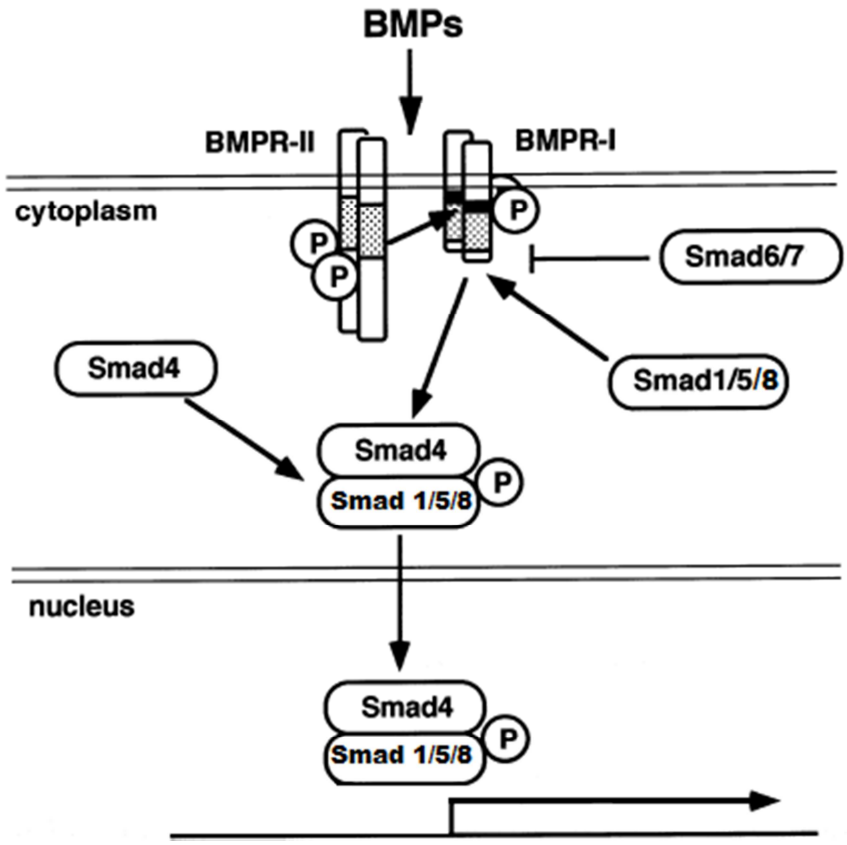

Figure 1-7: Schematic display of the BMP signalling pathway. BMPs bind to serine/threonine receptor kinases. This leads to phosphorylation of SMAD 1, 5 and 8. Phosphorylated SMADs 1, 5 and 8 form a complex with SMAD 4 and translocate to the nucleus where they can activate transcription of target genes. BMP signalling can be inhibited at the receptor level by SMAD 6 and 7. Figure adapted from (Kawabata et al., 1998).

Different members of the bone morphogenetic protein (BMP) family, such as BMP-2, -4, -6, -9,-12, and -13, are routinely applied for articular cartilage TE. Some studies compare the effect of different BMPs on the chondrogenesis of hMSCs (Majumdar et al., 2001; Sekiya et al., 2005) or their ability to promote cartilage formation when chondrocytes are used as cell source (Gooch et al., 2002). Other studies apply only one single BMP, often BMP-2, and study its effect on cartilage repair or on the chondrogenesis of progenitor cells (MSCs, periosteal cells) in different species, such as human or rabbit (Schmitt et al., 2003; Sellers et al., 2000; Uusitalo et al., 2001; Weisser et al., 2001).

The three isoforms of TGF-β are also part of the transforming growth factor superfamily. Namely, these are TGF-β1, TGF-β2 and TGF-β3. They influence various cellular activities, such as differentiation, proliferation and the production of ECM. All isoforms are secreted as inactive precursors, which bind to an associated peptide. In order to become active, this peptide has to dissociate, which will permit the binding of the TGF-β protein to its receptor complex. As in BMP-2 signalling, this receptor complex is composed of type I and type II TGF-β receptor sub-types. Initially, the ligand binds to its type II receptor which is phosphorylated in its basal state. This process lead to the recruitment of the type I receptor, the formation of a receptor complex and phosphorylation of the type I receptor. Downstream signalling is similar to downstream signalling within the BMP-2 signalling pathway. However, it is mediated by SMADs 2 and 3 instead of SMADs 1, 5 and 8. A detailed overview about TGF-β signalling can be found in: (Massague, 1998).

A vast number of studies, describing possible applications of TGF-β family members in cartilage TE and regeneration, can be found in the literature. Commonly, they are used for chondrogenic induction in different cells types from different species, such as human ESCs, rabbit bone-marrow-derived MSCs, hMSCs and equine bone-marrow-derived or adipose-derived MSCs (Barberi et al., 2005; Fan et al., 2008; Hwang et al., 2006; Kisiday et al., 2008; Miyanishi et al., 2006).

1.5.3 Gene transfer.

Routinely, growth and differentiation factors, deployed in articular cartilage TE, are applied exogenously. Due to their short half-lives, this approach is problematic for an application in clinical practice. The concept of gene therapy/gene transfer is an alternative conception that may help to overcome this shortcoming. Gene transfer is based on the delivery of a complementary deoxyribonucleic acid (cDNA), specific for the desired factor, to a target cell. Afterwards, the cell itself is able to produce the desired transgene. There exist two approaches for gene transfer applications, a direct ("*in vivo"*) and an indirect ("*ex vivo"*) one. The decision which approach is used is based on several criteria, such as which gene should be delivered, which vector is used and which cell type is targeted. When using the direct approach, the vector is injected straight into the final destination, for instance the knee joint (Gouze et al., 2002; Nita et al., 1996; Roessler et al., 1993). On the other hand, when the indirect approach is used, cells are first transduced *ex vivo.* Afterwards, the genetically modified cells are transplanted or injected into the targeted site within the body (Gelse et al., 2003; Kang et al., 1997; Mason et al., 1998). A wide range of different vectors can be used for gene transfer applications. They can be broadly divided into two main classes, non-viral vector (for example naked DNA or liposomes) and viral vectors (for example adenovirus, adeno-associated virus (AAV) and different kinds of retroviruses, such as lentivirus, foamyvirus, and moloney murine leukaemia virus). Each vector type possesses its own set of advantages and disadvantages. In comparison to viral vectors, non-viral vectors are relatively safe. Nevertheless, they are considered to be less efficient, when compared to viral vectors (Dinser et al., 2001; Steinert et al., 2008; Thomas et al., 2003). On the other hand, viral vectors are much more effective but their application raises safety concerns (Steinert et al., 2008; Thomas et al., 2003).

Gene therapy, in cartilage repair, features many different candidate genes and different cell types that can be targeted. The following section will only provide a very brief overview. In order to gain a more detailed, general view, readers are referred to: (Cucchiarini and Madry, 2005; Steinert et al., 2008; Trippel et al., 2004). The selection of the candidate gene product depends on the desired therapeutic approach, for example stimulation of cell proliferation or cartilage matrix synthesis, inhibition of senescence or apoptosis, stimulation of chondrogenic differentiation or inhibition of hypertrophy. For example, if chondrogenic differentiation and/or cartilage matrix synthesis should be stimulated, anabolic growth factors, such as BMP-2, IGF-1 and different TGF-βs, transcription factors, such as SOX9, or signal transduction molecules, such as different SMAD proteins, are valid options. Growth factors are particularly attractive candidates because they can act in both autocrine (stimulation of the transduced cell itself) and paracrine (stimulation of naïve surrounding cells) fashion. Three major groups of cells are routinely targeted in TE of articular cartilage; articular chondrocytes (Kang et al., 1997), progenitor/stem cells (Kupcsik et al., 2010) and synoviocytes (Gouze et al., 2002). Chondrocytes represent a prime target because they are the cell type that should reconstitute the cartilage defect. The other two cell types are able to differentiate into mature chondrocytes and, thereby, can help to fill and repair the defect. Furthermore, transduced cells can enhance the endogenous repair by providing a plethora of bioactive factors (dependent on the transgene which has been introduced into the cell). The first gene therapy trial in the cartilage field was executed by Evans and co-workers in 1996 (Evans et al., 1996). Ever since, gene therapy has become a field which is extensively studied regarding an application in cartilage repair and TE of articular cartilage.

1.5.4 Mechanical factors.

Healthy articular cartilage is subjected to mechanical forces during everyday life and it is widely accepted that they play a pivotal role for the maintenance and the development of this tissue. In order to mimic the *in vivo* situation more precisely, the concept of functional TE was developed. It implements mechanical stimulation with the goal to generate a tissue construct that is able to withstand the mechanical demands which prevail *in vivo.* Mechanical stimulation can be either applied solely or in combination with other stimuli, usually growth or differentiation factors. The implication of mechanical stimulation might be a crucial step towards generating a tissue that is functionally more adequate and that closer resembles native articular cartilage. The following section will provide a short general overview of different stimuli utilised in cartilage TE. Afterwards, complex mechanical loading will be described in more detail (more information about mechanical stimulation in cartilage TE can be found for example in: (Freed et al., 2006; Grad et al., 2011; Grodzinsky et al., 2000)).

Mechanical compression (Buschmann et al., 1995; Campbell et al., 2006), fluid flow (Freed et al., 1998), tissue shear (Jin et al., 2001) and hydrostatic pressure (Miyanishi et al., 2006) are the four types of physical stimuli that are most commonly investigated and utilised in functional TE. Mechanical compression can be static or dynamic. It can be directly applied to cartilage explants or to cells seeded in different types of scaffolds. Dynamic compression protocols utilise a great variety of different amplitudes and frequencies. It is well known that compression will induce a metabolic response, such as changes in chondrocyte gene expression and/or synthesis of ECM molecules, in the cartilage tissue. This response can differ dramatically, depending on which type of mechanical compression (static or dynamic) is applied. Implementation of static compression down-regulates the biosynthesis of proteoglycans and proteins whereas dynamic compression has the exact opposite effect. This tendency has been demonstrated for example in the study of Buschmann et al., in 1995. The group investigated the effects of short-term and long-term administration of static or dynamic compression on the biosynthesis of bovine chondrocytes (Buschmann et al., 1995).

The stimulatory effect of fluid flow originates from two different factors. First, fluid flow induces shear forces within the tissue. Furthermore, it can increase the rate of transport of growth factors and nutrients. Fluid flow can be generated with different types of bioreactors, such as spinner-flasks, rotating-wall bioreactors and perfusion culture system (summarised in: (Temenoff and Mikos, 2000)).

In contrast to dynamic loading, tissue shear deformation hardly provokes any volumetric changes, fluid flow or pressure gradients within the cartilage tissue. Hence, tissue shear decouples the effect of fluid flow from the effects induced by cell or matrix deformation. The impact of shear stress on cartilage explants was, for example, evaluated by Frank et al., and Jin et al., (Frank et al., 2000; Jin et al., 2001). Both studies focussed on how dynamic matrix and cell deformation will affect the cell metabolism. Interestingly, in this setup, shear strain led by preference to an up-regulation of collagen gene expression over proteoglycan gene expression.

Hydrostatic pressure is one of the most important mechanical stimuli within articular cartilage. It is generated once cartilage is subjected to mechanical forces. Loading forces lead to fluid flow within the tissue (cartilage is a highly hydrated tissue with a water content of 70-80%). However, there exists an initial resistance to this fluid flow which results in the generation of hydrostatic pressure within the cartilage tissue. In TE of articular cartilage, hydrostatic pressure has different possible scopes of application. It can be used to enhance the metabolic activity of chondrocytes, to differentiate progenitor cells towards the chondrogenic phenotype or to mediate a chondroprotective effect. One advantage of hydrostatic pressure is that it can be applied to cells in monolayer culture, 3D constructs and cartilage explants. Yet, different studies exhibit conflicting results. For examples, similar levels of hydrostatic pressure have been shown to either up- or down-regulate proteoglycan biosynthesis in bovine chondrocytes (summarised in: (Elder and Athanasiou, 2009)).

These physical stimuli can help and already have helped to improve the outcome of studies with the aim to generate cartilaginous tissue. Nevertheless, they are everything but perfect. The normal physiological movement of the joint is kinetically very complex. Hence, application of a single, simple stimulus, such as tissue shear, compression or pressurisation, will not adequately reflect the situation that prevails *in vivo*. In order to better meet the necessary requirements, a bioreactor was developed by the group of Prof. Alini (Wimmer et al., 2004). This bioreactor system is able to generate several different stimuli, namely compression, shear and flexion. These stimuli can be either applied individually or simultaneously. Thus, this bioreactor system is able to simulate the complex kinematics of the joint more precisely. The bioreactor was designed based on tribological considerations. A tribological system consists of a body, a counterbody, an interfacial medium and a surrounding environment. Within an articulating joint, the body and the counterbody are the two opposing cartilage surfaces. The interfacial medium is represented by the synovial fluid and the surrounding environment is defined by the human body. In a human articular joint, sliding wear is the prevailing type of surface interaction. The crossing angles of the trajectories are different for different types of joints and the wear paths of the forward and backward fraction do not lie in identical geometrical lines. All these factors were taken into account during the process of development and a so-called “ball-on-pin” concept was finally evolved. A scaffold, in which cells can be embedded, or a cartilaginous tissue (pin) and a commercially available alumina ball (later replaced by a ceramic hip ball) equal the two bodies of the tribological system. The interstitial fluid is the culture medium and the surrounding environment is a CO_2 incubator. With this sophisticated bioreactor system, compression, shear and flexion can be applied alone or simultaneously in varying combinations. Thereby, a complex shear force pattern can be generated. The new bioreactor system has already been used in several studies with different types of cells, such as bovine chondrocytes and hMSCs, and promising results were generated (Kupcsik et al., 2010; Li et al., 2009b; Li et al., 2009c; Salzmann et al., 2009; Schatti et al., 2011).

For example, in 2009, Li et al., demonstrated that mechanical stimulation modulates chondrogenesis of hMSCs through the TGF-β pathway (Li et al., 2009b). Interestingly, the effect of mechanical stimulation was dependent on the concentration of TGF-β1 in the culture medium. A lower growth factor concentration (1µg/ml instead of 10µg/ml) or the absence of exogenous TGF-β1, within the culture medium, led to a more pronounced chondrogenic effect of the loading regime. This was shown by large increases in chondrogenic marker gene expression (Col II and aggrecan). Further, it was demonstrated that mechanical stimulation resulted in endogenous production of TGF-β1 and TGF-β3. When TGF-β signalling was blocked at the receptor level, mechanical stimulation was no longer able to induce chondrogenesis.

1.6 Thesis aim.

The current thesis had two main aims: the first aim was to investigate and optimise viral gene transfer methods for basic research and a possible clinical application (chapter 3). In the first study of chapter 3, the effect of transduction with a first generation VSG.G pseudo-typed retroviral vector expressing human bone morphogenetic protein 2 (Rv.BMP-2) on the gene expression profile of hMSCs, during monolayer proliferation, was investigated. In the second study of chapter 3, a new and optimised 3D transduction protocol for a first generation, E1-, E5-deleted, serotype 5 adenoviral vector carrying the cDNA for human BMP-2 (Ad.BMP-2) was proposed and compared to routine two-dimensional (2D) adenoviral transduction. This comparison was conducted, using hMSCs, in alginate and agarose, two hydrogels routinely applied in cartilage TE applications, and, further, in a clinically relevant fibrin hydrogel-PU composite scaffold. In the last study of chapter 3, the feasibility of a novel protocol for a timed transduction of hMSCs, with Ad.BMP-2, in the fibrin hydrogel-PU composite scaffold system was demonstrated. The second aim was to investigate the potential of two possible human cell sources for cartilage TE (chapter 4, chapter 5, chapter 6 and chapter 7). These cells sources were hMSCs and human articular cartilage progenitor cells (hACPCs). In chapter 4, the effect of mechanical stimulation and adenoviral-mediated over-expression of human BMP-2, alone and in combination, on the chondrogenic differentiation and the progression towards hypertrophy of monolayer-expanded hMSCs was investigated. Chapter 5 investigated the effect of dorsomorphin, a potential candidate substance to suppress hypertrophy by blocking of BMP signalling, on the chondrogenic differentiation and the progression towards hypertrophy of monolayer-expanded hMSCs. The cells were cultured in fibrin hydrogel- PU composite scaffolds in medium containing exogenous TGF-β1. In chapter 6, the chondrogenic potential of hACPCs and hMSCs in the established pellet culture model (with the application of exogenous TGF-β1) was compared. Further, the effect of co-culturing these cells, in varying ratios, on their chondrogenic and hypertrophic potential was examined. Finally, chapter 7 had the same aim as chapter 4 but used hACPCs as cell source.

Chapter 2: Materials and Methods.

2.1 Materials.

- 1-Bromo-3-chloro-propane (Sigma, St.Louis, MO)
- 1, 9-dimethyl-methylene-blue (Sigma, St.Louis, MO)
- 10-fold Polymerase chain reaction buffer II and $MgCL_2$ set (500 mM KCl, 100 mM Tris/HCl, pH 8.3, 25 mM $MgCl_2$; Applied Biosystems, Carlsbad, CA)
- 6-Aminocaproic acid (Sigma, St.Louis, MO)
- Ad293 cells (Stratagene, Santa Clara, CA)
- Agarose (Low melting-point, preparative grade for small fragments, RNAse/DNAse free, melting-point ≤65°C; Promega, Madison, WI)
- Albumin from bovine serum (Fraction V, essentially protease free; Sigma, St.Louis, MO)
- Agarose (SeaPlaque, low melting-point; Lonza, Basel, Switzerland)
- Alginic acid sodium (Fluka, St.Louis, MO)
- Alpha Minimum Essential Medium (Deoxy-/Ribonucleoside and $NaHCO_3$ free; Gibco, Carlsbad, CA)
- Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma, St.Louis, MO)
- Basic fibroblast growth factor (recombinant human, E.Coli-derived; Peprotech, Rocky Hill, NY)
- Benzonase (384units per µl; Sigma, St.Louis, MO)
- Bone morphogenetic protein 2 (recombinant human, CHO cell-derived; R&D Systems, Minneapolis, MN)
- Bovine serum albumin (fraction V, fatty acid free; Roche, Basel, Switzerland)
- Caesium chloride (Thermo Fischer Scientific, Waltham, MA)
- Calcium chloride (Fluka, St.Louis, MO)
- Calf thymus DNA (LuBioScience, Luzern, Switzerland)
- Chondroitin 4-sulfate sodium salt from bovine trachea (Fluka, St.Louis, MO)
- Chondroitinase AC (from *Flavobacterium heparinum*, reconstituted to 2.5units/ml in 20mM phosphate buffer with pH 7.0; Sigma, St.Louis, MO)
- Coverslips (24x40 mm; Menzel Gläser, Braunschweig, Germany)
- Dako pen (Dako, Dako, Denmark)

- Deoxynuclesoside triphosphate mixture (2.5 mM each dNTP; Applied Biosystems, Carlsbad, CA)
- Dexamethasone (Sigma, St.Louis, MO)
- Diethyl pyrocarbonate (Fluka, St.Louis, MO)
- Dilution buffer F and dilution buffer T (both Baxter, Vienna, Austria)
- Dimethylsulfoxide (Fluka, St.Louis, MO)
- Disodium Ethylenediaminetetraacetic acid (EDTA; Sigma, St.Louis, MO)
- DL-Dithiothreitol (≥98% (TLC), ≥99.0% (titration); Sigma, St.Louis, MO)
- Dorsomorphin dihydrochloride (MW=472.41; Tocris Biosciences, Bristol, UK)
- Dulbecco's Modified Eagle Medium (high glucose, 4500mg/L glucose, - Pyruvate, - $NaHCO_3$; Gibco, Carlsbad, CA)
- Dulbecco's Modified Eagle Medium Glutamax (high glucose, 4500mg/L glucose; Gibco, Carlsbad, CA)
- Duo Set enzyme-linked immunosorbent assay (ELISA) Development System for human BMP-2 (R&D Systems, Minneapolis, MN)
- Duo Set ELISA Development System for human TGF-β1 (R&D Systems, Minneapolis, MN)
- Ethanol (≥99.8%, for molecular biology; Fluka, St.Louis, MO)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (Fluka, St.Louis, MO)
- Ethylenediaminetetraacetic acid solution (0.5 M in H_20; Fluka, St.Louis, MO)
- Eukitt® quick hardening mounting medium (Fluka, St.Louis, MO)
- Fast Green FCF (Fluka, St.Louis, MO)
- Fibrinogen (diluted 1:3 in dilution buffer F; Baxter, Vienna, Austria)
- Ficoll (Histopaque-1077; Sigma, St.Louis, MO)
- Filter paper for embedding cassettes (Medite Medizintechnik GmbH; Burgdorf, Germany)
- First generation, E1-, E5-deleted, serotype 5 adenoviral vector carrying the cDNA for human BMP-2 (Vector Biolabs, Philadelphia, PA)
- Foetal bovine serum (heat inactivated; Gibco, Carlsbad, CA)
- Folded filter paper (Ø 270 mm; Whatman, Maidstone, Kent, UK)
- Glacial acetic acid (Fluka, St.Louis, MO)
- Glucose (D (+); Sigma, St.Louis, MO)

- Glycerol (≥99,5%; Sigma, St.Louis, MO)
- Glycine (≥99%; Fluka, St.Louis, MO)
- Haematoxyline solution according to Mayer (Fluka, St.Louis, MO)
- HEPES buffer (1 M, 238.2 g/L; Gibco, Carlsbad, CA)
- High salt precipitation solution (0.8 M sodium citrate and 1.2 M sodium chloride; Molecular Research Center Inc., Cincinnati, OH)
- Hoechst 33258 dye (bisbenzimide; Polysiences Inc., Warrington, PA)
- Hyaluronidase (type I-S, reconstituted to 1 unit/µl in phosphate buffered saline for histology containing 0.1% Tween 20; Sigma, St.Louis, MO)
- Hydrochloric acid (37%; Fluka, St.Louis, MO)
- Hydrogen peroxide (30%; Fluka, St.Louis, MO)
- ImmPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA)
- Insulin-transferrin-sodium selenite, linoleic BSA (100 x, contains 0.625mg/ml insulin from bovine pancreas, 0.625mg/ml human transferrin, 0.625µg/ml sodium selenite, 125mg/ml BSA and 535µg/ml linoleic acid; Cyagen, Guangzhou, China)
- Iodoacetamide (≥99%, cristalline; Sigma, St.Louis, MO)
- Isopentane (Sigma, St.Louis, MO)
- Isopropanol (≥99.5%, for molecular biology; Sigma, St.Louis, MO)
- MEM non-essential amino acids (100 x, liquid; Gibco, Carlsbad, CA)
- Mesenchymal stem cell qualified foetal bovine serum (Gibco, Carlsbad, CA)
- Methanol (99.5%; Brenntag, Mülheim, Germany)
- Microtome blades (N35HR; Feather, Ozaka, Japan)
- Monoclonal IgG mouse anti-chicken collagen 2 antibody (CIICI, cross-reacts with human; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA)
- Monoclonal IgG2a mouse anti-rat aggrecan antibody (1-C-6, cross-reacts with human; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA)
- Monoclonal IgM mouse anti-porcine collagen type X antibody (cross-reacts with human; Sigma, St.Louis, MO)
- MultiScribe reverse transcriptase (50 U/µl; Applied Biosystems, Carlsbad, CA)

- O.C.T. compound (R.Jung GmbH, Nussloch, Germany)
- Parafilm (Pechiney plastic packaging, Chicago, IL)
- Penicillin / Streptomycin (10, 000units penicillin, 10, 000µg streptomycin; Gibco, Carlsbad, CA)
- Phosphate buffered saline tablets (Sigma, St.Louis, MO)
- PKH26 red fluorescent cell linker kits for general cell membrane labelling (Sigma, St.Louis, MO)
- PKH67 fluorescent cell linker kits for general cell membrane labelling (Sigma, St.Louis, MO)
- ProLong® antifade reagent with DAPI (Molecular Probes, Carlsbad, CA)
- Polyacryl carrier (Molecular Research Center Inc., Cincinnati, OH)
- "Polybrene" (10mg/ml; Millipore, Billerica, MA)
- Polyoxyethylen-Sorbitax-Monolaurat (Tween 20; Sigma St.Louis, MO)
- Potassium dihydrogen phosphate (Fluka, St.Louis, MO)
- Pre-developed TaqMan assay reagents human 18s ribosomal RNA (20 x; Applied Biosystems, Carlsbad, CA)
- Primocin (Invivogen, San Diego, CA)
- Proteinase K (recombinant PCR Grade; Roche, Basel, Switzerland)
- Quantakine BMP-2 Immunoassay (R&D Systems, Minneapolis, MN)
- Random hexamers (50µM; Applied Biosystems, Carlsbad, CA)
- Reagent diluent concentrate 1 (R&D Systems, Minneapolis, MN)
- RNase Inhibitor (20U/µl; Applied Biosystems, Carlsbad, CA)
- Safe lock Eppendorf tubes (Eppendorf, Hamburg, Germany)
- Safranin-O (water soluble; Chroma, Münster, Germany)
- Self-designed forward primers, reverse primers and probes (Microsynth, Balgach, Switzerland)
- Sodium chloride (puriss ≥99.5%; Fluka, St.Louis, MO)
- Sodium citrate (Sigma, St.Louis, MO)
- Sodium hydrogen carbonate (Sigma, St.Louis, MO)
- Sodium hydroxide (K ≤0.02%, ≥98.0%; Sigma, St.Louis, MO)
- Sodium phosphate (Fluka, St.Louis, MO)
- Sodium phosphate dibasic dihydrate (puriss ≥99.5%; Fluka, St.Louis, MO)

- Sodium phosphate monobasic monohydrate (puriss ≥99.0%; Fluka, St.Louis, MO)
- Sodium pyruvate (≥99%, cell culture tested; Sigma, St.Louis, MO)
- Sodium tetraborate (anhydrous; Fluka St.Louis, MO)
- Sucrose (Fluka, St.Louis, MO)
- Sulphuric acid (18.76 M, 95-98%; Fluka, St.Louis, MO)
- Superfrost plus microscope slides (Menzel Gläser, Braunschweig, Germany)
- TaqMan gene expression master mix (Applied Biosystems, Carlsbad, CA)
- TGF-β1 (recombinant human, CHO cell-derived; Fitzgerald, Acton, MA)
- TGF-β2 (recombinant human, HEK 293 cell-derived; Peprotech, Rocky Hill, NY)
- Thrombin (Baxter, Vienna, Austria)
- Tissue culture plastic (Techno plastic products, Trasadingen, Switzerland)
- TRI Reagent (Molecular Research Center Inc., Cincinnati, OH)
- Trypan Blue (Fluka, St.Louis, MO)
- Trypsin-Ethylenediaminetetraacetic acid (10 x, 5 g/L Trypsin, 2 g/L EDTA, 8.5 g/L NaCl; Gibco, Carlsbad, CA)
- TRIS-EDTA buffer solution (100 x, for molecular biology; Sigma, St.Louis, MO)
- Trizma base (Tris, minimum 99.9%; Sigma, St.Louis, MO)
- Vectastain elite ABC kit mouse IgG (includes normal horse serum, concentrated biotinylated horse anti-mouse IgG secondary antibody and reagents A (avidin) and B (paired biotinylated enzyme); Vector Laboratories, Burlingame, CA)
- VSV.G pseudotyped retroviral vector expressing human BMP-2 (kindly provided by Dr. Martina Anton, TU München, München, Germany)
- VSV.G pseudotyped retroviral vector expressing enhanced green fluorescent protein (kindly provided by Dr. Martina Anton, TU München, München Germany)
- Weigert´s iron haematoxyline kit (for nuclear staining in histology; Merck, Whitehouse Station, NY)
- Xylol (Brenntag, Mülheim, Germany)

2.2 Methods.

2.2.1. Preparation of culture media, solutions and growth factor stocks.

Alpha Minimal Essential Medium (α-MEM): α-MEM is a culture medium suited for the monolayer proliferation of hMSCs. For 1L of medium, 10.08g powdered medium and 2.2g sodium hydrogen carbonate were dissolved in 990ml of purified and de-ionised water (MilliQ water). Sodium hydrogen carbonate was added to provide essential carbonate ions. Next, 10ml of 100 x Penicillin/Streptomycin (P/S) stock solution was added in order to prevent bacterial contamination. Final concentrations were 100units of penicillin and 100µg of streptomycin per ml. The solution was sterilised in a laminar-flow hood into a sterile glass bottle using a 0.22µm filter. After filtration, α-MEM can be stored at 2-8°C for up to 1 month.

Dulbecco's Modified Eagle Medium high glucose (4.5g/L) (DMEM): DMEM is a culture medium which is well suited for supporting the growth of a broad spectrum of mammalian cells. For 1L of medium, 13.38g powdered medium, 3.7g sodium hydrogen carbonate and 110mg sodium pyruvate were completely dissolved in 990ml of MilliQ water. Sodium hydrogen carbonate was added to provide essential carbonate ions. Sodium pyruvate was added as it is not included in the powdered medium. Next, 10ml of 100 x P/S stock solution was added in order to prevent bacterial contamination. Final concentrations were 100units of penicillin and 100µg of streptomycin per ml. The solution was sterilised in a laminar-flow hood into a sterile glass bottle using a 0.22µm filter. After filtration, DMEM can be stored at 2-8°C for up to 1 month.

For the preparation of serum-containing media, components were dissolved in 890ml of MilliQ water. 10ml of P/S and 100ml of FBS were added. Serum-containing media was sterilised as described above. Afterwards, it can be stored at 2-8°C for up to 1 week.

MSC growth medium: α-MEM was supplemented with 10% human MSC tested FBS (hMSC FBS) and 5ng/ml of basic fibroblast growth factor (bFGF). The hMSC FBS is carefully pretested to ensure optimal properties for enhancing hMSC proliferation. The bFGF improves hMSC proliferation and prevents hMSC differentiation.

ACPC growth medium: DMEM Glutamax® was supplemented with 10% FBS, 1% P/S, 10mM HEPES buffer, 0.1mM ascorbic acid 2-phosphate (AA), 1mM sodium pyruvate and an additional 1g/L of glucose (final glucose concentration was 5.5g/L). HEPES acts as zwitterionic organic chemical buffering agent. AA acts as anti-oxidant and sodium pyruvate was added as it is not included in the powdered medium. Afterwards, the solution was sterilised in a laminar-flow hood into a sterile glass bottle using a 0.22µm filter. After filtration, medium was supplemented with 5ng/ml bFGF and 1ng/ml TGF-β2. The bFGF improves hACPC proliferation and prevents hACPC differentiation. The TGF-β, at this low concentration, improves chondrocyte (Barbero et al., 2004) and hACPC proliferation and survival (personal correspondence). ACPC growth medium can be stored at 2-8°C for up to 1 week.

Chondrogenic medium: Serum-free DMEM was supplemented with 1% insulin-transferrin-sodium selenite, linoleic bovine serum albumin (BSA) pre-mix (ITS+1), 50µg/ml AA, 1% MEM non-essential amino acids (NEAA), 10^{-7}M dexamethasone and 10ng/ml TGF-β1. Chondrogenic medium was prepared and sterilised, using a 0.22µm filter, freshly before every medium change. ITS+1 pre-mix (contains 0.625mg/ml insulin from bovine pancreas, 0.625mg/ml human transferrin, 0.625µg/ml sodium selenite, 125mg/ml BSA and 535µg/ml linoleic acid) provides cells with insulin, which enables the glucose-uptake of the cells, the micronutrient selenium and transferrin (iron-carrier). NEAA provides the amino acids glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline and L-serine. AA acts as anti-oxidant and is essential for collagen synthesis. Dexamethasone and TGF-β1 were added for the *in vitro* chondrogenic induction of MSCs (Johnstone et al., 1998). If chondrogenic medium was used for culture of fibrin-PU composite scaffolds, an additional 5µM of ε-aminocaproic acid (EACA) was added in order to prevent fibrin degradation (Kupcsik et al., 2009).

Chondro-permissive medium: Chondro-permissive medium was used for studies involving mechanical stimulation with the bioreactor system. It has the same composition as the chondrogenic medium with the exception that it lacks exogenous TGF-β1. Instead, endogenous production of TGF-β1 was enhanced through mechanical stimulation (Li et al., 2009b). As the bioreactor system represents a non-sterile environment, P/S was substituted with 100µg/ml of Primocin. Primocin is a new antibiotic formulation that prevents contamination with gram-positive and gram-negative bacteria, mycoplasma and fungi.

Phosphate Buffered Saline (PBS): PBS tablets were dissolved in MilliQ water. One tablet, in 200ml of MilliQ water, yields 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4 at 25°C. PBS was sterilised in a laminar-flow hood into a sterile glass bottle using a 0.22µm filter. After filtration, PBS can be stored at 2-8°C for up to 1 month.

Phosphate Buffered Saline containing 2M sodium chloride (DPBS): For 1L of DPBS, 3.4g potassium dihydrogen phosphate (25mM), 3.55g sodium phosphate (25mM) and 116.88g sodium chloride (2M) were dissolved in 950ml of MilliQ water. pH was adjusted to 7.4 with 5M sodium hydroxide solution. Afterwards, volume was adjusted to 1L with MilliQ water. DPBS can be stored at room temperature for up to one year.

Working solution Trypsin-ethylenediaminetetraacetic acid (EDTA) (T/E): Ten-fold T/E solution was diluted 1 to 10 with PBS and sterilised using a 0.22µm filter prior to use.

Diethyl pyrocarbonate-treated (DEPC) water: MilliQ water was supplemented with 0.1% diethyl pyrocarbonate and afterwards steam-sterilised at 121°C for 20 minutes.

Tris-EDTA buffer (TE buffer): 100-fold stock solution was dissolved in DEPC water.

10mM Tris-HCl, pH 7.9: For 1L, 1.2114g of Trizma base was completely dissolved in 990ml of MilliQ water. Next, pH was adjusted to 7.9 with hydrochloric acid. Finally, total volume was adjusted to 1L with MilliQ water.

Caesium chloride solution (1.2g/ml): 26.8g of caesium chloride were completely dissolved in 92ml of 10mM Tris-HCl pH 7.9. After the salt was completely dissolved, this yields 100ml of a caesium chloride solution with a total density of 1.2g/ml. Finally, solution was sterile-filtered using a 0.22µm filter.

Caesium chloride solution (1.4g/ml): 53g of caesium chloride were completely dissolved in 87ml of 10mM Tris-HCl pH 7.9. After the salt was completely dissolved, this yields 100ml of a caesium chloride solution with a total density of 1.4g/ml. Finally, solution was sterile-filtered using a 0.22µm filter.

10mM Tris-HCl, 0.1mM EDTA, pH 8: For 1L, 1.2114g of Trizma base was completely dissolved in 990ml of MilliQ water. Next, 200µl of 0.5M EDTA solution was added. The pH was adjusted to 8 using hydrochloric acid. Finally, total volume was adjusted to 1l with MilliQ water.

10mM Tris-HCl, 200mM sodium chloride, 1mM EDTA, 4% sucrose (w/v), pH 8: For 1L, 1.2114g of Trizma base, 11.668g of sodium chloride and 40g of sucrose were completely dissolved in 925ml of MilliQ water. Next, pH was adjusted to 8 with hydrochloric acid. Finally, total volume was adjusted to 1L with MilliQ water.

0.9% (w/v) sodium chloride: For 250ml, 2.25g of sodium chloride was completely dissolved in 250ml of MilliQ water. Afterwards, it was sterile-filtered using a 0.22µm filter. The solution can be stored at room temperature for up to 1 year.

102mM calcium chloride: For 100ml, 1.55g of calcium chloride was completely dissolved in 100ml of MilliQ water. Afterwards, it was sterile-filtered using a 0.22µm filter. The solution can be stored at room temperature for up to 1 year.

1.2% (w/v) alginate: For 100ml, 1.2g of alginic acid sodium salt was dissolved in 100ml of 0.9% sodium chloride solution. The solution was put into a beaker, covered with Parafilm® and stirred overnight using a magnet stirrer. On the next day, the solution was sterile-filtered using a 0.22µm filter. 1.2% alginate can be stored at room temperature for up to 1 month.

2% (w/v) low melting-point agarose: For 10ml, 200mg of low melting-point agarose were added to 10ml serum-free DMEM. The agarose was dissolved through heating in a microwave. Afterwards, the solution was steam-sterilised at 121°C for 20 minutes. 2% low melting-point agarose can be stored at room temperature for up to 1 month.

5% (w/v) low melting-point agarose: For 20ml, 1g of low melting-point agarose was added to 20ml of sterile PBS. The agarose was dissolved through heating in a microwave. Afterwards, the solution was steam-sterilised at 121°C for 20 minutes. Aliqouts of 10ml were prepared in 50ml Falcon tubes and stored at 4°C until use.

1.25% (w/v) low melting-point agarose in DMEM + 10% FBS + 1% P/S: For 40ml, 10ml of 5% low melting-point agarose were dissolved in a 60°C water-bath. DMEM + 10% FBS + 1% P/S was adjusted to 37°C in a water-bath. Finally, 10ml of dissolved 5% low melting-point agarose were mixed with 30ml of DMEM + 10% FBS + 1% P/S and immediately used.

150mM sodium chloride, 55mM sodium citrate, pH 6.8: For 250ml, 2.192g sodium chloride and 4.043g sodium citrate were completely dissolved in 245ml of MilliQ water. Next, pH was adjusted to 6.8 using hydrochloric acid. Then, the volume was adjusted to 250ml with MilliQ water and the solution was sterile-filtered using a 0.22µm filter. The solution can be stored at either room temperature or 2-8°C for up to 1 year.

Ten-fold primer/probe mix: For 100µl primer/probe mix, 40µl of TE buffer were mixed with 20µl of forward primer, 20µl of reverse primer and 20µl of probe. The 10-fold mix contains 9µM forward and reverse primers and 2.5µM of probe. It can be stored at - 20°C for up to 2 years.

Duo Set BMP-2 ELISA and Duo Set TGF-β1 ELISA wash buffer: For 1L of wash buffer, 1L of sterile PBS was supplemented with 500µl of Tween 20. Final concentration was 0.05% Tween 20 in sterile PBS. The wash buffer can be stored at 2-8°C for up to 1 week.

Duo Set BMP-2 ELISA reagent diluent: For 100ml of reagent diluent, 1g of protease-free BSA was dissolved in 100ml of sterile PBS. The final concentration was 1% protease-free BSA in sterile PBS. The reagent diluent can be stored at 2-8°C for up to 1 week.

Duo Set BMP-2 ELISA, Duo Set TGF-β1 ELISA and Quantakine BMP-2 Immunoassay substrate solution: For 10ml of substrate solution, 5ml of colour reagent A (hydrogen peroxide) were mixed with 5ml of colour reagent B (tetramethylbenzidine). The substrate solution has to be prepared freshly and is intended for immediate use.

Duo Set BMP-2 ELISA capture antibody (mouse anti-human BMP-2): The capture antibody was reconstituted in 1ml of PBS in order to create a 180µg/ml stock solution. The capture antibody was aliquoted and frozen at -20°C. The capture antibody is stable at -20°C for up to 6 months.

Duo Set BMP-2 ELISA detection antibody (biotinylated mouse anti-human BMP-2): The detection antibody was reconstituted in 1ml of reagent diluent in order to create a 180µg/ml stock solution. The detection antibody was aliquoted and frozen at -20°C. The detection antibody is stable at -20°C for up to 6 months.

Duo Set BMP-2 ELISA standard (recombinant human BMP-2): The standard was reconstituted in 0.5ml of reagent diluent in order to create a 190ng/ml stock solution. Aliquots were prepared and frozen at -20°C. The standard is stable at -20°C for up to 2 months.

Duo Set BMP-2 ELISA and Duo Set TGF-β1 ELISA streptavidin-conjugated HRP: 1ml of concentrated streptavidin-conjugated horseradish-peroxidase (HRP) was included in each kit. The concentrated streptavidin-conjugated HRP was diluted 1:200 in reagent dilution before use.

Duo Set TGF-β1 ELISA block buffer: For 100ml of block buffer, 100ml of sterile PBS was supplemented with 5ml of Tween 20. The final concentration was 5% Tween 20 in sterile PBS. The block buffer has to be used within 1-2 days. For long-term storage (1-2 weeks), block buffer has to be supplemented with an additional 0.05% of NaN_3.

Duo Set TGF-β1 ELISA reagent diluent: For 100ml of reagent diluent, 1.4ml of reagent diluent concentrate 1 were mixed with 98.6ml of wash buffer. The diluted reagent diluent concentrate has to be used within 8 hours.

Duo Set TGF-β1 ELISA capture antibody (mouse anti-human TGF-β1): Capture antibody was reconstituted in 1ml of PBS in order to create a 360µg/ml stock solution. The capture antibody was aliquoted and frozen at -20°C. The capture antibody is stable at -20°C for up to 6 months.

Duo Set TGF-β1 ELISA detection antibody (biotinylated chicken anti-human TGF-β1): The detection antibody was reconstituted in 1ml of reagent diluent in order to create a 54µg/ml stock solution. The detection antibody was aliquoted and frozen at -20°C. The detection antibody is stable at -20°C for up to 6 months.

Duo Set TGF-β1 ELISA standard (recombinant human TGF-β1): The standard was reconstituted in 0.5ml of reagent diluent in order to create a 220ng/ml stock solution. Aliquots were prepared and frozen at -20°C. The standard is stable at -20°C for up to 2 months.

1M sulphuric acid: For 200ml, 189.3ml of MilliQ water was mixed with 10.7ml of concentrated sulphuric acid. The solution can be stored at room temperature for up to 1 year.

1M HCl: For 100ml, 91.67ml of MilliQ water were mixed with 8.33ml of 37% HCl (12M). The solution can be stored at room temperature for up to 1 year.

1.2M NaOH/0.5M HEPES: For 100ml, 50ml of 1M HEPES buffer were mixed with 38ml of MilliQ water and 12ml of 10M NaOH. The solution can be stored at room temperature for up to 1 month.

Quantakine BMP-2 Immunoassay wash buffer: For 500ml of wash buffer, 20ml of wash buffer concentrate were mixed with 480ml of MilliQ water.

Quantakine BMP-2 Immunoassay calibrator diluent RD5P: For 200ml of calibrator diluent RD5P, 20ml of concentrate were mixed with 180ml of MilliQ water and stirred for at least 15 minutes.

Quantakine BMP-2 Immunoassay BMP-2 standard stock solution (recombinant human BMP-2): The standard was reconstituted in 1ml of MilliQ water in order to create a 20 ng/ml stock solution and mixed for at least 15 minute before further use.

1,9-Dimethyl-methylene blue (DMMB): For 1L of reagent, 16mg of DMMB, 3.04g of glycine and 2.37g of sodium chloride were dissolved in 1L of MilliQ water. The DMMB was stirred overnight in a beaker wrapped in alumina foil. On the next day, pH was adjusted to 3 with 1M hydrochloric acid. The DMMB is stable, in a brown bottle at room temperature, for up to 3 months.

Hoechst 32258 stock solution (1mg/ml): For 10ml of stock dye solution, 10mg of Hoechst 32258 were completely dissolved in MilliQ water. The solution has to be protected from light. It can be either stored in a brown bottle or in a Falcon tube wrapped in alumina foil. The stock dye solution is stable at 2-8°C for up to 6 months

DNA standard: Calf thymus DNA was dissolved in DPBS to a final concentration of 100µg/ml. Aliqouts were prepared and stored at -20°C for long-term storage.

75% ethanol in DEPC water: For 20ml, 15ml of ≤99.8% ethanol were mixed with 5ml of DEPC water. The solution can be stored at room temperature for up to 1 year.

bFGF stock (20, 000x, 100 µg/ml): Recombinant human bFGF was reconstituted in sterile MilliQ water to a final concentration of100 µg/ml. Aliqouts were prepared and frozen at -20°C for long-term storage.

5M sodium chloride: For 100ml, 29.22g of sodium chloride was completely dissolved in 100ml of MilliQ water. The solution can be stored at room temperature for up to 2 years.

1M Tris pH 7.5: For 100ml, 12.114g of Trizma base was completely dissolved in 95ml of MilliQ water. Afterwards, pH was adjusted to 7.5 with hydrochloric acid. Finally, the total volume was adjusted to 100ml with MilliQ water. The solution can be stored at room temperature for up to 1 year.

Dorsomorphin dihydrochloride stock solution (10, 000x, 100mM): Dorsomorphin dihydrochloride was dissolved in MilliQ water to a final concentration of 100mM. Aliqouts were frozen and can be stored at -20°C for up to 1 month.

5mM TRIS pH 7.5, 150mM sodium chloride, 0.1% BSA: For 1ml of sterile 5mM TRIS pH 7.5, 150mM sodium chloride, 0.1% BSA, 965µl of MilliQ water were supplemented with 30µl 5M sodium chloride, 5µl 1M Tris pH 7.5 and 1mg BSA. The solution was stirred until completely dissolved and sterilised using a 0.22µm filter.

bFGF stock (10, 000 x, 50µg/ml): The 100µg/ml bFGF stock was diluted 1:1 with sterile 5mM Tris pH 7.5, 150mM sodium chloride, 0.1% BSA. The BSA was added in order to prevent binding of the growth factor to the Eppendorf tubes which would decrease its actual concentration. The aliquots of 50µg/ml can be stored at -20°C for up to 6 months. Once thawed, they are stable at 2-8°C for up to 1 week.

TGF-β1 stock (1, 000 x, 10µg/ml): Lyophilised, recombinant human TGF-β1 was dissolved in sterile PBS containing 4mM hydrochloric acid and 2mg/ml BSA to a final concentration of 10µg/ml. The BSA was added in order to prevent binding of the growth factor to the Eppendorf tubes which would decrease its actual concentration. The aliquots were frozen at -20°C for long-term storage. Once thawed, they are stable at 2-8°C for up to 1 week.

TGF-β2 stock (5, 000 x, 5µg/ml): Lyophilised, recombinant human TGF-β2 was dissolved in MilliQ water to a final concentration of 100µg/ml. Afterwards, it was further diluted to a final concentration of 5µg/ml in sterile PBS containing 4mM hydrochloric acid and 2 mg/ml BSA. BSA was added in order to prevent binding of growth factor to the Eppendorf tubes which would decrease its actual concentration. Aliqouts were frozen at -20°C for long-term storage. Once thawed, they are stable at 2-8°C for up to 1 week.

BMP-2 stock (1, 000 x, 100 µg/ml): Lyophilised, CHO cell-derived, recombinant human BMP-2 was dissolved in sterile PBS containing 4mM hydrochloric acid and 0.1% BSA to a final concentration of 100µg/ml. The BSA was added in order to prevent binding of growth factor to the Eppendorf tubes which would otherwise decrease its actual concentration. The aliquots were frozen, and can be stored at -20°C for up to 3 months. Once thawed, they are stable at 2-8°C for up to 1 month.

PBS for histology (2.32mM sodium phosphate monobasic monohydrate, 8mM sodium phosphate dibasic dihydrate, 154mM sodium chloride): For 5L PBS for histology, 1.6g NaH_2PO_4*H_20, 7.1g Na_2HPO_4*$2H_2O$ and 45g NaCl were completely dissolved in 5L of MilliQ water. The solution can be stored at 2-8°C for up to 1 week.

5% (w/v) Sucrose in PBS for histology: For 100ml, 5g of Sucrose was completely dissolved in 100ml of PBS for histology. The solution can be stored at 2-8°C for up to 1 week.

Weigert´s iron haematoxyline working solution: For 200ml, 100ml of solution A (alcoholic haematoxyline) and 100ml of solution B (hydrochloric acid iron (III) nitrate) were mixed. The solution can be stored at room temperature for up to 1 week.

Safranin-O working solution (0.1%): For 200ml, 0.2g of Safranin-O powder was completely dissolved in 200ml of MilliQ water. The solution is intended for immediate use.

Fast Green stock solution (1%): For 100ml, 1g of powder was completely dissolved in 100ml of MilliQ water. The solution can be stored at room temperature for up to 1 year.

Fast Green working solution (0.02% Fast Green, 0.1% acetic acid): For 250ml, 245ml of MilliQ water (add first!) were mixed with 5ml of 1% Fast Green stock solution and 250µl of glacial acetic acid. The solution is intended for immediate use.

1% acetic acid: For 250ml, 247.5ml of MilliQ water (add first!) were mixed with 2.5ml of glacial acetic acid. The solution is intended for immediate use.

Reduction buffer (50mM Tris, 200mM NaCl, pH 7.35): For 500ml, 3.02g Trizma base and 5.84g NaCl were completely dissolved in 450ml of MilliQ water. Next, pH was adjusted to 7.35 with 1M HCl. Finally, the total volume was adjusted to 500ml with MilliQ water. The solution can be stored at 2-8°C for up to 1 week.

DL-Dithiothreitol (DTT) working solution (10mM DTT in reduction buffer): For 400ml, 0.617g of DTT was completely dissolved in 400ml reduction buffer. The solution is intended for immediate use.

Alkylating solution (40mM iodoacetamide): For 400ml of alkylating solution, 2.95g of iodoacetamide was completely dissolved in 400ml of PBS for histology. The solution is intended for immediate use.

PBS supplemented with 0.1% Tween 20 (PBS-T): For 1L, 1ml of Tween 20 was mixed with 999ml of PBS for histology. The solution can be stored at 2-8°C for up to 1 week.

0.3% H_2O_2 in 100% methanol: For 100ml, 1ml of 30% H_2O_2 was mixed with 99ml of 100% methanol. The solution is intended for immediate use.

Chondroitinase AC working solution (0.025units/ml): For 1ml, 10µl of chondroitinase AC stock solution (2.5units/ml) was mixed with 990µl of PBS-T. The solution is intended for immediate use.

Hyaluronidase working solution (0.05units/ml): For 100ml, 5µl of hyaluronidase stock solution (1unit/µl) was added to 100ml of PBS-T. The solution is intended for immediate use.

Normal horse serum working solution: Normal horse serum was diluted 1 to 20 in PBS-T. The solution is intended for immediate use.

Primary antibodies for immunohistochemistry: Primary antibodies were diluted in PBS-T according to table 2-2. The primary antibodies are intended for immediate use.

Secondary antibody (biotinylated horse anti-mouse IgG) for immunohistochemistry: Concentrated antibody was diluted 1:200 in PBS-T. The solution is intended for immediate use.

Avidin-biotinylated enzyme (AB) complex: Reagent A (avidin) and reagent B (biotinylated enzyme) were diluted 1 to 50 in PBS-T. The complex has to be prepared at least 30 minutes prior to use and stored at 4°C. Subsequently, the solution is intended for immediate use.

3, 3'-diaminobenzidine (DAB) working solution: For 1ml, 1 drop of concentrated DAB was diluted with 1ml of diluent (included in kit). The solution has to be protected from direct light and can be stored at 2-8°C for up to 2 weeks.

Wash buffer 1 and wash buffer 2 for RayBio human cytokine antibody array G-series: The 20x concentrates were diluted in MilliQ water to create the 1x working solutions of wash buffer 1 and wash buffer 2. The diluted buffers can be stored at 2-8°C for up to 4 weeks.

Biotin-conjugated anti-cytokines for RayBio human cytokine antibody array G-series: The concentrated liquid bead was mixed with 300µl of 1x blocking buffer (included in kit). The 1x biotin-conjugated anti-cytokines can be stored at 2-8°C for up to 3 days.

Streptavidin-Fluor for RayBio human cytokine antibody array G-series: First, the 500 x concentrate was mixed with 100µl of 1x blocking buffer and transferred to a fresh Eppendorf tube wrapped in aluminium foil. Next, it was diluted to a 1x working concentration with an additional 1.4ml of 1x blocking buffer. The 1x Streptavidin-Fluor solution can be at 2-8°C for up to 3 days.

2.2.2 Mesenchymal stem cell isolation.

Human bone-marrow aspirates were obtained with the ethical approval and the informed written consent of patients undergoing total hip replacement, spinal fusion or vertebral body isolation. Bone-marrow aspirates were kindly provided by Dr. Sven Hoppe, Dr. Lorin Benneker, (both Inselspital Bern, Bern Switzerland), Dr. Gian Salzmann (Universitätsklinikum Freiburg, Freiburg Germany) and Dr. Markus Loibl (Universitätsklinikum Regensburg, Regensburg, Germany). hMSCs were isolated from BM aspirates under sterile conditions. BM aspirates were diluted 1 to 4 in DMEM containing 5% hMSC FBS. Next, the fat layer was removed by centrifugation for 5 minutes at 170 times gravity (g). All centrifugation steps within this section were conducted with the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany). The cell pellet was re-suspended in an amount of DMEM, containing 5% hMSC FBS, equal to the amount used for the initial dilution step. Next, 2.6ml of room-temperature Ficoll (Sigma, St.Louis, MO), for every 1ml of undiluted BM aspirate, were added to a 5 ml Falcon tube. The diluted BM aspirate was carefully added on top of the Ficoll. Mononuclear cells were separated by centrifugation (20 minutes at 800g with acceleration and brake on the lowest level). The interphase (see figure 2-1), which contains the mononuclear cells, was carefully collected using a 2ml pipette.

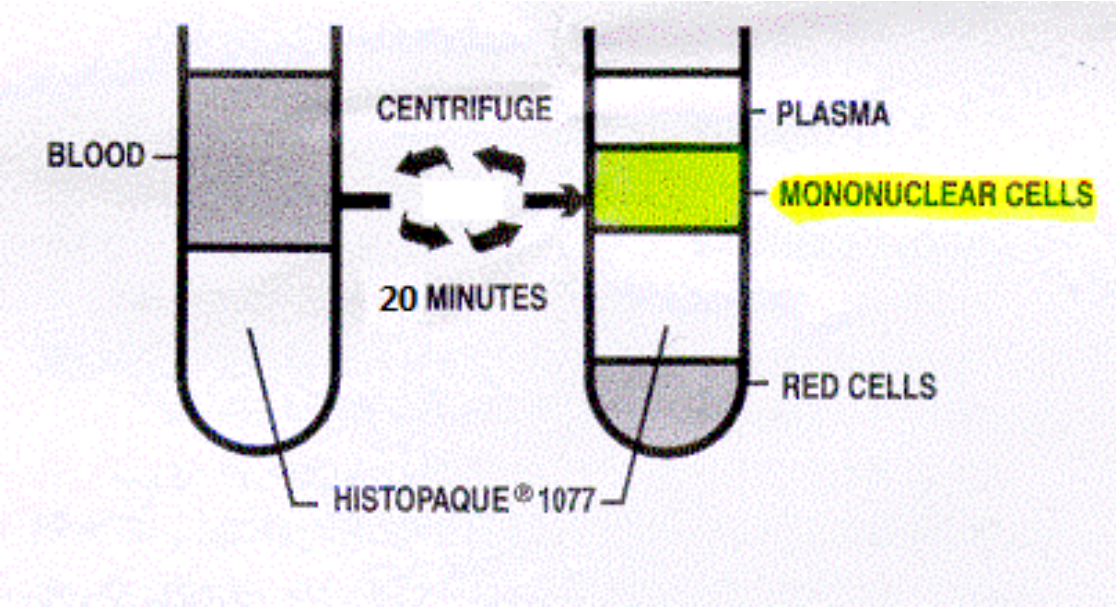

Figure 2-1: Phase-separation using Ficoll (Histopaque 1077). After BM aspirates were centrifuged for 20 minutes at 800g, the mononuclear cells formed an interphase between the Ficoll and the blood plasma. Figure adapted from the standard operation procedure document PRBT 002-04 (AO Research Institute Davos, AO Foundation, Davos, Switzerland).

Next, mononuclear cells were washed twice. For every 1ml of interphase, 5ml of DMEM containing 5% hMSC FBS were added and the mix was centrifuged for 15 minutes at 400g. The supernatant was removed and the cell pellet was re-suspended in 5ml of DMEM containing 5% hMSC FBS for every 1ml of interphase which was initially harvested. The centrifugation step was repeated once. Afterwards, the cell pellet was re-suspended in 10ml of MSC growth medium. Mononuclear cells were counted using a haemocytometer and an Axiovert 25 microscope (Carl Zeiss AG, Oberkochen, Germany). Therefore, 50µl of cells were mixed with 50µl of trypan blue. Trypan blue is a diazo-dye that is not able to pass the cell membrane of living cells. In dead cells however, the membrane is ruptured and trypan blue is able to enter the cell. Thereby, it is possible to distinguish between living and dead cells and exclude the dead cells during analyses. Only big and homogenously stained cells were counted. Four large counting squares (consisting of sixteen small counting squares each) were counted and the average was calculated. The concentration of mononuclear cells, per ml of cell suspension, was calculated by multiplying the average cell number in the four squares by 2 (cells were diluted 1:1 with trypan blue) and 10, 000 (multiplicator for the haemocytometer). Finally, the total cell number was calculated by multiplying the concentration by 10 (total volume). Mononuclear cells were seeded at 13, 333 cells/cm^2 on tissue culture plastic. They were cultured for 4 days in MSC growth medium in order to allow for hMSCs attachment. Standard conditions for cell culture were always 37°C, 5% CO_2, 90% humidity in a CO_2 incubator. After 4 days, MSC growth medium was changed in order to remove any non-adherent or dead cells.

2.2.3 Mesenchymal stem cell propagation.

MSC growth medium was changed three times per week. Once distinct colonies formed, cells were detached using T/E. Briefly, medium was aspirated and cells were washed two times with PBS. The washing step was necessary in order to remove any traces of serum which would otherwise hinder the enzymatic reaction of the trypsin. A working solution of T/E was prepared and 1ml of the solution was used for every 30 cm^2 of tissue culture plastic. Cells were incubated for 10 minutes under standard conditions. Next, MSC growth medium was added (at least 1ml for every 1ml of T/E), hMSCs were dispersed and pooled in a 50ml Falcon tube. Now, cells were centrifuged for 10 minutes at 300g using the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany). Supernatant was removed and the cell pellet re-suspended in MSC growth medium. Cells were sub-cultured and seeded on tissue culture plastic at a density of 13, 333 cells/cm^2. They are now passage (P) 1. Now, cells were further cultured in MSC growth medium with medium changes three times per week under standard conditions. Once sub-confluent (defined as 70-80% confluency), cells were detached and counted as described in section 2.2.2. Finally, 2 x 10^6 cells were suspended in 1ml of 92% sterile hMSC FBS and 8% dimethylsulfoxide which serves as cryopreservant. Prior to cryopreservation, cells were transferred to cryotubes (Nunc, Rochester, NY). After one day at -80°C in a freezing container (Nunc, Rochester, NY), hMSC were transferred to liquid nitrogen for long-term storage. hMSCs underwent a total of approximately 12.54 ± 1.22 PDs before cryopreservation.

For further propagation, 2 x 10^6 hMSC were removed from the liquid nitrogen and plated at cell culture plastic at a density of 6, 666 cells/cm^2. Cells were cultured under standard conditions in MSC growth medium until sub-confluent. Afterwards, they were detached as described above and sub-cultured once. Again, cells were plated at 6, 666 cells/cm^2 and cultured under standard conditions until they were sub-confluent. Finally, cells were harvested and counted as described in section 2.2.2. hMSCs underwent a total of 5.41 ± 1.71 PDs after cryopreservation. In total, hMSCs underwent approximately a combined 18.08 ± 1.92 PDs before they were used for the experiments.

2.2.4 Articular cartilage progenitor cell propagation.

Cartilage samples were obtained from the NHS blood and tissue bank (Liverpool, England, UK). Samples were derived from healthy donors. hACPCs were isolated through differential adhesion to fibronectin (Williams et al., 2010). hACPCs were kindly provided by Dr. Rebecca Williams, Prof. Charles W. Archer and Dr. Ilyas Khan (all University, Cardiff, Wales, UK). Clonal populations of hACPCs, which underwent 25.32 ± 2.12 PDs, were initially plated at 1 x 10^6 cells on one T 75 flask in 15ml of ACPC growth medium. This correlates to a seeding density of 13, 333 cells/cm^2. Medium was partially changed (half of the total volume) three times per week. Once cells were sub-confluent, they were detached using T/E as described in section 2.2.3 (ACPC medium was used instead of MSC growth medium). Next, they were counted as described in section 2.2.2 and sub-cultured. Therefore, they were again plated at 13, 333 cells/cm^2 and cultured under standard conditions until they were sub-confluent. The sub-culture step had to be repeated once in order to obtain sufficient cell numbers for the experiments. Finally, they were harvested and counted as described in section 2.2.2. hACPCs underwent 6.63 ± 2.25 PDs after cryopreservation. In total, hACPCs underwent a combined 33.54 ± 2.60 PDs.

2.2.5 Polyurethane scaffold preparation and sterilisation.

Biodegradable, cylindrical (8 x 4 mm or 8 x 2 mm) and porous (pore size 90-300µm) PU scaffolds (figure 2-2) were prepared as described elsewhere (Gorna and Gogolewski, 2002).

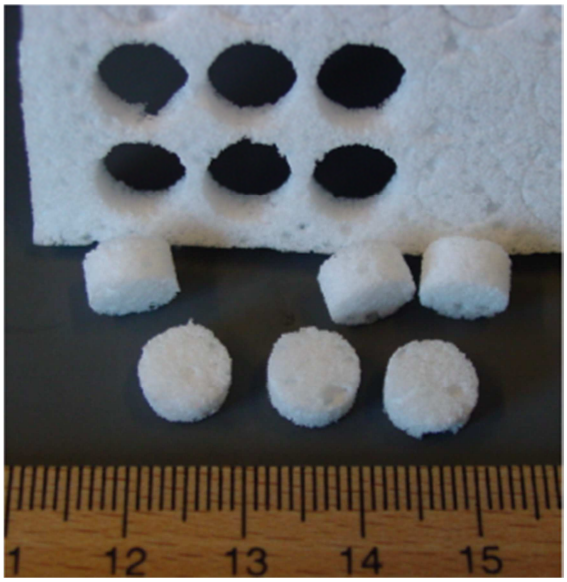

Figure 2-2: Porous, cylindrical PU scaffolds.

Briefly, PU was synthesised in a one-step solution polycondensation reaction from hexamethylene diisocyanate, poly(epsilon-caprolactone) diol and 1,4:3,6-dianhydro-D-sorbitol. Pores were prepared using the salt-leaching phase inverse technique with sodium phosphate heptahydrate dibasic salt as porogen. Subsequently, the porogen was removed by repeated wash steps with water. PU scaffolds and PU rings, surrounding the scaffolds, were cut using the water jet technique. PU scaffolds and rings were sterilised using a cold-cycle (37°C) ethylene oxide process. After sterilisation, the ethylene oxide was removed, for at least 3-4 days, in an evacuation chamber at 45°C and 150 mbar. PU scaffolds were produced by Dr. David Eglin and Markus Glarner, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.6 Propagation of a recombinant adenoviral vector expressing human BMP-2.

Ad.BMP-2, containing a cytomegalovirus (CMV) promoter, from BioLabs (Philadelphia, PA) was further amplified in Ad293 cells. Briefly, Ad293 cells were cultured on tissue culture plastic in DMEM supplemented with 10% FBS until 70-80% confluent. The cells were detached as described in section 2.2.3 and re-plated onto tissue culture plastic. This step was necessary in order to remove the ECM which would otherwise interfere with the adenoviral transduction. Ad293 cells were cultured for 18-24 hours in DMEM supplemented with 10% FBS. The medium was then aspirated and replaced with 1ml of serum-free DMEM per 60 cm^2 of tissue culture plastic. Subsequently, cells were transduced with Ad.BMP-2 (a total of 10^7 infectious viral particles) for 2 hours under standard conditions. During transduction, flasks were gently rocked every 15 minutes in order to ensure that the bottom of the flask was always covered in liquid. After 2 hours, 45ml of serum-free DMEM was added. Ad293 cells were further cultured under standard conditions and regularly monitored. Once they started to detach, medium was collected into 50ml Falcon tubes and centrifuged for 10 minutes at 2, 000g using the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany). The supernatant was aspirated, the Ad293 cell pellet was re-suspended in 3ml of serum-free DMEM, snap-frozen in liquid nitrogen and stored at -80°C until purification.

For purification of the Ad.BMP-2 vector, successive caesium chloride gradients were used. Briefly, Ad293 cells were subjected to three freeze-thaw cycles (snap-freezing in liquid nitrogen followed by thawing at 37°C in a water-bath) in order to break the cells open. Next, 50units/ml of benzonase were added to the cell lysate, followed by incubation for 30 minutes at 37°C in a water-bath. Thereafter, supernatant was always kept on ice. Caesium chloride gradients (1.2g/ml over 1.4g/ml) were prepared in pre-chilled polyallomer tubes (Thermo Fischer Scientific, Waltham, MA). The viral supernatant was carefully added on top of the caesium chloride gradient. The total volume was adjusted to 11.5ml with 10mM Tris-HCl, 0.1mM EDTA, pH 8. Gradients were centrifuged using the ultraspeed centrifuge Sorvall discovery 90 SE (Hitachi, Tokyo, Japan) and a swinging bucket rotor for 90 minutes at 34, 500 g and at 4°C.

After centrifugation, the viral band was harvested with an 18 gauge needle and diluted three-fold with 10mM Tris-HCl, 0.1mM EDTA, pH 8. The caesium chloride density gradient step was repeated once or twice, depending on the intensity of the viral band. Finally, the viral supernatant was put into eliquibrated dialysis bags (Spectrum Labs, Rancho Dominguez, CA) and dialysed against 10mM Tris-HCl, 200mM NaCl, 1mM EDTA, 4% sucrose, pH 7. Dialysis was conducted overnight at 4°C. After the first three hours of dialysis, the dialysis buffer was changed once. On the next day, aliquots of Ad.BMP-2 were prepared, snap-frozen in liquid nitrogen and transferred to -80°C for long-term storage.

For determination of the viral titre, standard plaque assay on Ad293 cells was conducted. Briefly, Ad293 cells were seeded into 6-well plates in 5ml of DMEM supplemented with 10% FBS with 5 x 10^5 cells per well. On the next day, cells were transduced for 2 hours at 37°C with 10-fold dilutions of Ad.BMP-2. Dilutions ranging between 10^5 and 10^{10} were tested. Afterwards, cells were overlaid with 3ml of 1.25% sterile low melting-point agarose in DMEM supplemented with 10% FBS. Ad293 cells were cultured at 37°C, 5% CO_2 and 90% humidity until plaques started to appear. Plaques were counted using an Axiovert 25 microscope (Carl Zeiss AG, Oberkochen, Germany). Finally, viral titres were calculated by multiplying the number of viral plaques with the corresponding dilution factor. For example, five plaques at a 10^8 dilution would correspond to 5 x 10^8 infectious viral particles per 1ml.

2.2.7 Ad.BMP-2 transduction.

For the 2D transduction protocol, hMSCs were propagated as described in section 2.2.3 until the desired cell number was reached. Next, cells were detached using T/E, as described in section 2.2.3, counted as described in section 2.2.2 and re-plated onto tissue culture plastic. This step was necessary in order to remove the ECM which would otherwise hinder adenoviral transduction. After 24 hours, hMSCs were transduced with Ad.BMP-2. Briefly, MSC growth medium was aspirated and replaced with 1ml of serum-free α-MEM per 60cm^2 of tissue culture plastic. Then, 5 or 100 infectious viral particles per cell were added into the medium. This corresponds to a multiplicity of infection (MOI) of 5 or 100. One MOI is defined as one infectious viral particle per cell. Transduction was conducted for 2 hours under standard conditions. Tissue culture flasks were gently rocked every 15 minutes to ensure that the bottom of each flask was evenly covered with medium. Thereafter, 45ml of MSC growth medium was added and cells were cultured overnight under standard conditions. On the next day, cells were detached and counted as described in sections 2.2.2 and 2.2.3. They were then used for cell seeding into fibrin-PU, alginate or agarose as described in section 2.2.11.

For the modified 3D transduction protocol, hMSCs were left untreated prior to the respective seeding procedures as described in section 2.2.11. The adenoviral vector was added directly to the cell/hydrogel mix during the cell seeding. Thereby, the vector was encapsulated in the respective hydrogel. hMSCs that have not been transduced were used as controls. This decision was based on the observation that these cells were not different from cells that have been transduced with an adenovirus coding for a non-bioactive transgene, such as enhanced green fluorescent protein (Kupcsik et al., 2010). Further, it was demonstrated that cells that have been transduced with a control vector do not express elevated levels of BMP-2, TGF-β or IGF-1 (Steinert et al., 2009a).

2.2.8 Retroviral transduction.

For retroviral transduction, 0.666 x 10^6 hMSCs were seeded in a T 75 tissue culture flask. This equals to a seeding density of 8, 888 cells/cm^2. They were cultured in MSC growth medium under standard conditions for 48 hours. Subsequently, they were transduced with the retroviral vectors applying the following protocol. Additionally, a mock-treated control group, where viral vectors were replaced by 1ml of serum-free α-MEM, was carried out. Retroviral vectors and "Polybrene" were thawed on ice. Polybrene" was used to enhance the retroviral transduction efficiency. Next, 8µg/ml of "Polybrene" was added to the retroviral vector or the serum-free α-MEM. Now, MSC growth medium was complete aspirated from the tissue culture flasks and replaced with 1ml of virus (or medium)/"Polybrene" mix. Transduction was conducted for 2 hours under standard conditions. Flasks were gently rocked every 15 minutes to ensure that the bottom of the flasks was completely covered with medium. After transduction, 15ml of MSC growth medium were added and cells incubated for 24 hours under standard conditions. A first generation vesicular stomatitis virus G protein (VSV.G) pseudotyped retroviral vector expressing human BMP-2 (Rv.BMP-2) and a first generation VSV.G pseudotyped retroviral vector expressing enhanced green fluorescent protein (Rv.eGFP) were kindly provided by Dr. Martina Anton, department of experimental oncology, TU München, Germany. The gene expression of both retroviral vectors is driven by the moloney murine leukaemia virus (MMLV) 5´ long terminal repeat (LTR).

2.2.9 hMSC seeding and culture for the effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation study.

This experiment is part of chapter 3. One day after retroviral transduction, cells were detached and counted as described in sections 2.2.2 and 2.2.3. Now, cells were sub-cultured in a 1:9 ratio into T 300 flasks with 50ml of MSC growth medium. Cells were cultured under standard conditions with medium changes every 3-4 days until they were 70-80% confluent.

For the evaluation of the effect of "Polybrene", sub-confluent hMSCs at P 3 were seeded at 1 x 10^5 cells per well in 6-well tissue culture plates. They were cultured in 3ml of MSC growth medium. After 24 hours, cells were exposed to 8µg/ml "Polybrene" in 0.5ml of medium (for 2 hours under standard conditions). In the control group, medium without "Polybrene" was added instead. Afterwards, 2.5ml of fresh medium was added and cells were cultured overnight. On the next day, the medium was changed. The cells were cultured for a total of 10 days with medium changes every 2-3 days. Images at day 1, day 3 and day 6 were taken using the Axiovert 25 microscope (Carl Zeiss AG, Oberkochen Germany) and the Axiovision V.4.8 software (Carl Zeiss AG, Oberkochen, Germany). A total magnification of 100 was used (10 x from the microscope and 10 x from the lens).

For the evaluation of the effect of recombinant human BMP-2, sub-confluent, untransduced hMSCs were seeded at 1 x 10^5 cells per well into 6-well tissue culture plates. They were cultured in 3ml of MSC growth medium with or without (control) the addition of 100ng/ml of recombinant human BMP-2. Cells were cultured for 2 weeks with medium changes twice a week under standard conditions.

2.2.10 Sample collection effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation study.

Once sub-confluent, cells were detached and counted as described in sections 2.2.2 and 2.2.3. This took between 7 and 9 days, depending on the donor. 2 x 10^6 cells were harvested in TRI reagent as described in section 2.2.18. Additionally, after 2 weeks, the hMSCs that have been exposed to 100ng/ml of recombinant human BMP-2 or were left as naïve controls were harvested. Cells were re-suspended in 1ml of TRI reagent supplemented with 5µl of polyacryl carrier. Finally, samples were stored at -80°C for subsequent analysis.

Furthermore, cellular phenotype was regularly monitored using an Axiovert 25 microscope (Carl Zeiss AG, Oberkochen, Germany). Images were taken using a total magnification of 100 (10 x from the microscope and 10 x from the lens) and the Axiovision V.4.8 software (Carl Zeiss AG, Oberkochen, Germany).

2.2.11 hMSC seeding into alginate, agarose or fibrin-PU.

Alginate: hMSCs were encapsulated in sterile 1.2% alginate at a density of 10 x 10^6 cells per ml. Briefly, alginate beads were prepared with a syringe and an 18 gauge needle. First, hMSCs were suspended in alginate. If the 3D transduction protocol was used, the adenoviral vector was additionally added and extensively mixed. Next, the cell/alginate/(virus) mix was slowly dropped into sterile 102 mM calcium chloride that facilitates cross-linking. Alginate beads were allowed to form for 10 minutes at room temperature. Afterwards, alginate beads were washed in order to remove excessive calcium chloride. Briefly, beads were transferred to sterile 0.9% sodium chloride and incubated for 5 minutes at room temperature. Afterwards, a second wash step, for another 5 minutes at room temperature, in serum-free DMEM was conducted. Three alginate beads per sample were used. This procedure correlates to approximately 1.05 x 10^6 cells per sample.

Agarose: hMSCs were encapsulated in sterile 1% low melting-point agarose at a final density of 20 x 10^6 cells per ml. Briefly, 2% low melting-point agarose was shortly heated (700 watts) in a microwave until completely fluid. Afterwards, solution was adjusted to 37°C in a water-bath. Next, cells were re-suspended in sterile, pre-warmed chondrogenic medium and mixed with an equal amount of sterile 2% low melting-point agarose. If the 3D transduction protocol was used, the adenoviral vector was additionally added and extensively mixed. Agarose beads were formed by pipetting 35µl of cell/1% low melting-point agarose/(virus) mix into 24-well plates and allowing them to cool for 30 minutes at room temperature. Gelling of the agarose occurs if temperature is $\leq$ 35°C. One sample correlates to approximately 0.7 x 10^6 cells.

Fibrin-PU: hMSCs were embedded in a fibrin gel (final concentrations 17mg/ml fibrinogen, 0.5units/ml thrombin) and seeded into porous, cylindrical 8 x 2 mm or 8 x 4 mm PU scaffolds. Seeding densities were 2.5 x 10^6 cells per scaffold for the 8 x 2 mm scaffolds and 5 x 10^6 cells per scaffold for the 8 x 4 mm scaffolds. Thereby, one sample correlates to either 2.5 x 10^6 or 5 x 10^6 cells. Before the PU scaffolds were used for seeding, they have been pre-wetted in serum-free DMEM under vacuum for at least one hour. Afterwards, DMEM was completely aspirated from the scaffolds. For the seeding procedure the following protocol was applied. hMSCs were suspended in 15µl of fibrinogen per 1 x 10^6 cells. If adenoviral transduction was conducted in 3D, Ad.BMP-2 was added to the fibrinogen/cell mix and volumes were adjusted accordingly. For each sample, one sterile cap of an Eppendorf tube was put into a 12-well plate. Next, fibrinogen/cell/(virus) mix was pipetted into a sterile Eppendorf cap. Thrombin was diluted 1 to 500 in dilution buffer T and 15µl per 1 x 10^6 cells were added to the fibrinogen/cell/(virus) mix. Solutions were rapidly mixed and one PU scaffold was pressed into the mixture using a sterile forceps. During the recovery of its original dimensions, the cell/fibrinogen/thrombin/(virus) mix was absorbed into the pores of the scaffold. Thereby, a homogeneous cell seeding was achieved. Next, PU scaffolds were incubated for one hour under standard conditions to allow fibrin gel formation. Fibrin components were kindly provided by Dr. Andreas Goessl, Baxter Biosurgery, Vienna, Austria.

2.2.12 hMSC culture enhanced adenoviral transduction in 3D study.

For the enhanced adenoviral transduction in 3D study (part of chapter 3), 2 x 8 mm scaffolds were seeded with 2.5 x 10^6 hMSCs. After fibrin gelation, scaffolds were transferred to 6-well plates and cultured in 3ml of chondrogenic medium under standard conditions. Alginate and agarose beads were prepared, as described in section 2.2.11, and cultured in 24-well plates in 1ml of chondrogenic medium. Thereby, the number of cells that conditioned 1ml of medium was similar between groups. For fibrin these were 0.83 x 10^6 cells, for alginate 1.05 x 10^6 cells and for agarose, 0.7 x 10^6 cells. The cells were cultured for 28 days, under standard conditions, with medium changes three times per week.

2.2.13 Sample collection enhanced adenoviral transduction in 3D study.

Medium was changed three times per week and collected for day 1, days 2-7, days 8-14, days 15-21 and days 22-28. Afterwards, it was stored at -20°C for subsequent analysis. After 28 days, samples were harvested for analysis of DNA content and amount of transgene product retained within each hydrogel.

Alginate: For quantification of cellular DNA from alginate, two samples per group were used. Alginate beads were washed three times with sterile PBS. Next, they were dissolved in sterile 150 mM sodium chloride and 55 mM sodium citrate for 10 minutes at 37°C. Centrifugation steps were done with the 5415D centrifuge (Eppendorf, Hamburg, Germany). The samples were centrifuged for 5 minutes at 500g. After centrifugation, the dissolving step was repeated once using the pellet. Next, the supernatant was removed and the cell pellet was re-suspended in sterile 0.9% sodium chloride. Then, samples were centrifuged for 5 minutes at 500g. The supernatant was discarded and the cell pellet was re-suspended in 1ml of sterile FBS. Afterwards, samples were centrifuged for 5 minutes at 500g. Again, the supernatant was discarded and the cell pellet was re-suspended in 1ml of 0.5mg/ml Proteinase K. Now, samples were digested for 16 hours at 56°C. Afterwards, the Proteinase K was heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Samples were stored at -20°C for subsequent analyses.

For quantification of BMP-2 retained within the hydrogel, one sample per group was analysed. Alginate beads were transferred into 1ml of chondrogenic medium. Afterwards, they were homogenised (3 minutes at 25 Hertz) using a sterile metal ball and a TissueLyser (Retsch, Haan, Germany). In order to pellet the remnants of the hydrogel, samples were centrifuged for 10 minutes at 12, 000g using the 5417R centrifuge (Eppendorf, Hamburg, Germany). The supernatant was transferred to a fresh Eppendorf tube and stored at -20°C for subsequent analysis.

Agarose: For quantification of DNA content from agarose, two samples per group were analysed. Agarose pellets were dissolved at 65°C, re-suspended in 1ml of 0.5mg/ml of Proteinase K and incubated for 16 hours at 56°C. Afterwards, Proteinase K was heat-deactivated as described for the alginate group. Samples were centrifuged for 5 minutes at 1, 500g using the 5415D centrifuge (Eppendorf, Hamburg, Germany). The supernatant was collected and stored at -20°C for subsequent analysis.

For quantification of transgene product retained within agarose, one bead per group was treated as described for the alginate group.

Fibrin-PU: For quantification of cellular DNA, two scaffolds per group were analysed. Scaffolds were put into 1ml of 0.5mg/ml Proteinase K and incubated for 16 hours at 56°C. After heat-deactivation, scaffolds were removed and samples were store at -20°C for subsequent analysis.

For quantification of BMP-2 retained within the scaffold, one scaffold per group was treated as described for the alginate group.

2.2.14 hMSC seeding, Ad.BMP-2 transduction and culture timed Ad.BMP-2 transduction study.

For the timed Ad.BMP-2 transduction study (part of chapter 3), hMSCs were seeded into 8 x 4 mm PU scaffolds at a density of 5 x 10^6 cells per scaffold as described in section 2.2.11. Three different groups, with triplicates each, were investigated. Namely, these were control, week 1 and week 2. After the fibrin was polymerised, the samples were transferred to 6-well plates. They were cultured in 5ml of chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1. Medium was changed three times per week. For the delayed Ad.BMP-2 transduction, samples were transduced with Ad.BMP-2, using a MOI of 7.5, after 1 week (week 1) or 2 weeks (week 2) of culture. Adenoviral titre was determined by standard plaque assay as described in section 2.2.6. For transduction, medium was aspirated and Ad.BMP-2, diluted in chondro-permissive medium to a final volume of 50µl, was added on top of the PU scaffolds. After incubation for 15 minutes at room temperature, 5ml chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1 was added to each sample. Afterwards, they were further cultured under standard conditions. Control samples were left untransduced.

2.2.15 Sample collection timed Ad.BMP-2 transduction study.

Scaffolds were cultured for 3 weeks in 5ml of chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1. The medium was changed three times per week. During medium changes, one and a half ml of each group was aliqouted and stored at -20° C for subsequent analysis. The remaining medium was aspirated. Afterwards, 5ml of fresh chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β was added to each well.

After 3 weeks of culture, PU scaffolds were transferred to an Eppendorf tube containing 1ml of 0.5mg/ml Proteinase K. Samples were incubated for 16 hours at 56°C and afterwards heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Scaffolds were removed and samples were stored at -20°C for subsequent analysis.

2.2.16 hMSC seeding and culture Ad.BMP-2 and load study.

For the Ad.BMP-2 and load study (chapter 4), hMSCs were seeded into 8 x 4 mm PU scaffolds at a density of 5 x 10^6 cells per scaffold as described in section 2.2.11. Half of the groups were transduced with Ad.BMP-2 in 3D with a MOI of 5 as described in section 2.2.7 (BMP-2). The other half was left as untransduced controls (control). After fibrin polymerisation, scaffolds were transferred to 6-well plates and pre-cultured for 3 days under standard conditions in 5ml of chondro-permissive medium. After the pre-culture period, PU scaffolds were embedded in PU rings and transferred into polyetheretherketone (PEEK) control or loading holders containing a PEEK ring (figure 2-3).

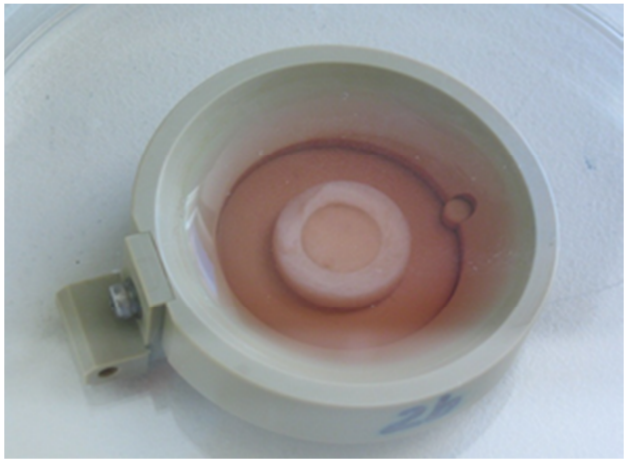

Figure 2-3: Fibrin-PU composite scaffold surrounded by a PU ring in a PEEK loading holder.

Scaffolds were cultured, for 7 (day 7) or 28 days (day 28), in 2.5ml of chondro-permissive medium. The medium was changed and collected three times per week. Medium changes were carried out before the application of mechanical stimulation. Scaffolds were either kept as free-swelling controls (unloaded) or subjected to complex mechanical stimulation, as described in section 2.2.17, for 1 hour per day during 6 days per week (loaded).

2.2.17 Mechanical stimulation.

Complex, mechanical stimulation (compression and shear) was applied using a custom-built "ball-on-pin" bioreactor system (figure 2-4) and a ceramic hip ball with a diameter of 32 mm (Wimmer et al., 2004). Samples were loaded for 1 hour per day during 6 days per week. Unconfined, dynamic compression strain was superimposed on shear stress. A static offset strain of 0.4mm was established first. Unconfined, dynamic compression strain was generated by pressing the ceramic hip ball onto the cell-seeded fibrin-PU scaffold. Sinusoidal strain (0.4mm) was superimposed on the static offset strain with a frequency of 1 hertz. The total strain amplitude applied was 10-20% of the total scaffold height. Shear stresses were generated by rotating the ball around an axis perpendicular to the scaffold axis. A ball oscillation of ± 25° with a frequency of 1 hertz was superimposed on the static offset strain.

Figure 2-4: Incubator-installed four station bioreactor system which is able to generate joint-like movements. A specimen holder with a cell-seeded fibrin-PU scaffolds construct is inserted into each station. Image with courtesy of Dr. Sibylle Grad (AO Research Institute Davos, AO Foundation, Davos, Switzerland).

2.2.18 Sample collection Ad.BMP-2 and load study.

Medium was changed and collected three times per week and pooled for days 1-7, days 8-14, days 15-21 and days 22-28. Additionally, the 5ml of pre-culture medium was collected. Medium was stored at -20°C for subsequent analysis.

After 7 or 28 days of culture, scaffolds were vertically cut into two halves using a sterile scalpel. One half was used for messenger ribonucleic acid (mRNA) isolation and gene expression analyses, the other half was used for biochemical analyses.

The half scaffold that was used for gene expression analyses was transferred to a polymerase chain reaction (PCR) clean Eppendorf tube containing 1ml of TRI reagent. TRI reagent is a complete ready-to-use reagent for the isolation of RNA from biological samples. It combines phenol and guanidine thiocyanate to facilitate immediate inhibition of RNase activity. Additionally, 5µl of polyacryl carrier were added. Polyacryl carrier is designed for use in the isolation of small amounts of RNA from biological samples. Next, a sterile metal ball was added and samples were homogenised for 3 minutes at 25 hertz using a Tissue Lyser (Retsch, Haan, Germany). Afterwards, the remnants of the scaffolds were pelleted by centrifugation (4°C, 12, 000 g, 10 minutes) using the 5417R centrifuge (Eppendorf, Hamburg, Germany). Supernatant was transferred to a fresh PCR clean Eppendorf tube and stored at -80°C for subsequent analysis.

The half scaffold that was used for biochemical analyses was transferred to an Eppendorf tube containing 1ml of 0.5mg/ml Proteinase K. Samples were incubated for 16 hours at 56°C and afterwards heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Scaffolds were removed and samples were stored at -20°C for subsequent analysis.

2.2.19 hACPCs and load study.

For the hACPCs and load study (chapter 7), the exact same parameters as for the Ad.BMP-2 and load study (chapter 4) were applied. The only difference was the cell type used. hACPCs were used instead of hMSCs. Therefore, the response of both cell populations towards adenoviral-mediated over-expression of BMP-2 and complex mechanical stimulation was investigated. hACPC seeding and culture was conducted as described for hMSCs in section 2.2.16. Complex mechanical stimulation was conducted as described in section 2.2.17. Medium samples were collected as described in section 2.2.18. After 7 or 28 days, scaffolds were harvested for biochemical analyses and gene expression analyses as described in section 2.2.18. Seven cycles of mechanical stimulation, in the fourth repeat of the study (between January 22th and January 31th 2013) and samples harvest of the day 7 samples in the fourth repeat of the study were conducted by Oliver Gardner, AO Research Institute Davos, AO Foundation, Davos Switzerland.

2.2.20 Cell labelling, cell seeding and cell culture co-culture of hMSCs and hACPCs study.

In one of the three runs of the co-culture of hMSCs and hACPCs study (chapter 6), cells were labelled with a fluorescent dye prior to the seeding procedure. The PKH26 dye was used for hACPCs and the PKH67 dye was used for hMSCs. Final dye concentration was 6 x 10^{-6}M for each dye. For each cell type, 8.5 x 10^{6} cells were suspended in 10ml of serum-free DMEM. Cells were centrifuged, using the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany), for 10 minutes at 300g. Next, medium was aspirated and the cell pellet was re-suspended in 500µl of diluent C. Further, 494µl of diluent C were mixed with 6µl of the corresponding fluorescent dye. The diluted dye solution was mixed with the cells and incubated for 5 minutes at room temperature with gentle shaking. Cells were protected from direct light during the incubation step. Afterwards, 1ml of sterile FBS (hACPCs) or sterile hMSC FBS (hMSCs) was added and incubated for 1 minute in order to stop the reaction. Next, cells were washed by adding 10ml of ACPC growth medium (hACPCs) or MSC growth medium (hMSCs). Cells were pelleted, using the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany), for 10 minutes at 500 g. Then, medium was aspirated and the wash step was repeated twice.

Finally, cells were re-suspended in 8.5ml of chondrogenic medium and used for cell seeding (final cell concentration was 1 x 10^6 cells per ml). Cells were seeded into 96-well V-bottom plates with a total of 250, 000 cells (hMSCs and hACPCs in different ratios) per samples.

In order to form pellets, 96-well V-bottom plates were wrapped in Parafilm® and centrifuged using the Rotanta 46 centrifuge (Hettich, Tuttlingen, Germany) for 10 minutes at 500 g. Cells were cultured for a total of three weeks. The cell culture during the first run of the study was done by Oliver Gardner, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.21 Sample collection culture co-culture of hMSCs and hACPCs study.

Medium was changed and collected three times per week and pooled for week 1, week 2 and week 3.

For gene expression analyses, three pellets per group were individually transferred to a PCR clean Eppendorf tube, containing 1ml of TRI reagent, 5µl of polyacryl carrier and a sterile metal ball. Samples were homogenised for 3 minutes at 25 hertz using a Tissue Lyser (Retsch, Haan, Germany). Afterwards, the supernatant was transferred to a fresh PCR clean Eppendorf tube and stored at -80°C for subsequent analysis.

For biochemical analyses, three pellets per group were individually transferred to an Eppendorf tube containing 1ml of 0.5mg/ml Proteinase K. Samples were digested for 16 hours at 56°C and afterwards heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Samples were stored at -20°C for subsequent analysis.

For histological analysis, one pellet per group (after 7 days) or two pellets per group (after 21 days) were embedded in O.C.T compound and snap-frozen using isopentane and liquid nitrogen.

All samples within the first run of the study and all histological samples within the study were harvested by Oliver Gardner, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.22 Cell seeding, cell culture and sample harvest secretome analysis of hMSCs and hACPCs.

For the secretome analysis of hMSCs and hACPCs (part of chapter 6), cells were seeded into 12-well tissue culture plates at a density that allowed for confluent culture from the onset of the experiment. As hMSCs are bigger than hACPCs, seeding densities were 33, 000 cells per cm^2 for hMSCs, which were used at P 2, and 125, 000 cells per cm^2 for hACPCs. Cells were initially seeded in 1ml of MSC growth medium (hMSCs) or 1ml of ACPC growth medium (hACPCs) and allowed to attach overnight. On the next day, growth medium was aspirated, wells were washed with PBS and 1ml of serum-free DMEM was added to each well. Cells were grown for 48 or 72 hours and afterwards samples were harvested. The culture medium was collected and stored at -20°C for subsequent analysis. Further, in order to quantify the DNA content, cells were digested in 1ml of 0.5mg/ml Proteinase K. Therefore, wells were washed in PBS and Proteinase K was added to each well. Next, the plate was wrapped in Parafilm and incubated for 16 hours at 56°C. Finally, the Proteinase K was transferred to an Eppendorf tube and heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Samples were stored at -20°C for subsequent analysis.

2.2.23 Cell seeding and cell culture effect of dorsomorphin on hMSC hypertrophy study.

For the effect of dorsomorphin on hMSC hypertrophy study (chapter 5), hMSCs were seeded into 8 x 2 mm PU scaffolds at a density of 2.5 x 10^6 cells per scaffold as described in section 2.2.11. Scaffolds were cultured for 2 weeks in 12-well plates. 2.5ml of chondrogenic medium were added to each well. After 2 weeks, samples were divided into two groups; control and dorsomorphin. The control samples were cultured for another two weeks in chondrogenic medium. In the dorsomorphin group, the chondrogenic medium was supplemented with an additional 10µM of dorsomorphin.

2.2.24 Sample collection effect of dorsomorphin on hMSC hypertrophy study.

The medium was changed three times per week. In the samples for the biochemical analyses, it was pooled for week 1, week 2, week 3 and week 4. In the samples of the PCR and the histology group it was discarded instead.

For gene expression analyses, whole scaffolds where transferred to a PCR clean Eppendorf tube, containing 1ml of TRI reagent, 5µl of polyacryl carrier and a sterile metal ball. Samples were homogenised, for 3 minutes at 25 hertz using a Tissue Lyser (Retsch, Haan, Germany). Afterwards, the remnants of the scaffolds were pelleted by centrifugation (4°C, 12, 000g, 10 minutes) using a 5417R centrifuge (Eppendorf, Hamburg, Germany). Supernatant was transferred to a fresh PCR clean Eppendorf tube and stored at -80°C for subsequent analysis.

For biochemical analyses and histological analysis, samples were vertically cut in halves using a sterile scalpel prior to the harvesting procedure.

For biochemical analyses, half scaffolds were transferred to an Eppendorf tube containing 1ml of 0.5mg/ml Proteinase K. Samples were digested for 16 hours at 56°C and afterwards heat-deactivated (10 minutes at 96°C) on a thermostat 5320 (Eppendorf, Hamburg, Germany). Afterwards, the half scaffolds were removed and samples were stored at -20°C for subsequent analysis.

For histological analysis, half scaffolds were fixed in 99.5% methanol and stored at 4°C for subsequent analysis.

2.2.25 BMP-2 enzyme-linked immunosorbent assay.

For the determination of BMP-2 concentration within the cell culture supernatant, either the Duo Set ELISA Development System for human BMP-2 or the Quantakine BMP-2 Immunoassay was used. There exist several different types of the ELISA. These particular systems both use the so-called "Sandwich ELISA" method. Two different antibodies are used, which bind to different epitopes of the molecule (antigen) that should be detected. A brief summary of the principle is outlined in figure 2-5.

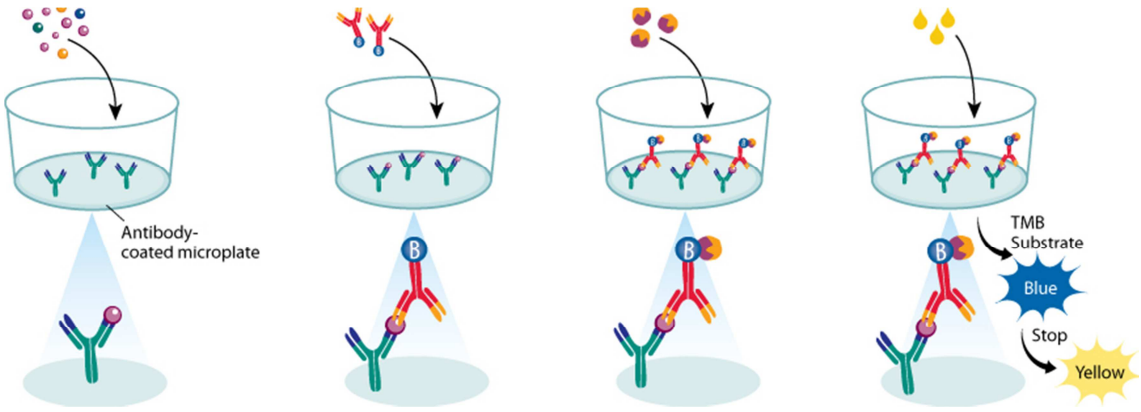

Figure 2-5: Brief summary of the "Sandwich ELISA" method. A microplate is covered with a capture antibody. Free capture antibody is washed away and unspecific binding sites are blocked. Then, samples or standards are added which bind to the immobilised antibody. Unbound samples are washed away and a second biotinylated antibody is added. This detection antibody binds to a different epitope. Next, unbound detection antibody is washed away and a streptavidin-conjugated HRP solution is added, which binds to the detection antibody. Unbound solution is washed away and a tetramethylbenzidine (TMB) substrate solution is added. A blue colour develops in proportion to the amount of antigen present within the sample. Colour reaction is stopped with an acid. If sulphuric acid is used, the colour turns into yellow. Now, absorbance at 450nm is measured using a spectrophotometer. Figure adapted from http://www.rndsystems.com.

For the Duo Set ELISA Development System for human BMP-2, first the capture antibody (mouse anti-human BMP-2) was diluted to a working concentration of 1µg/ml in PBS. A 96-well microplate (R&D Systems, Minneapolis, MN) was coated by adding 100µl of capture antibody to each well. Plate was sealed and incubated overnight at room temperature. Next, plate was aspirated and unbound capture antibody was removed by washing the wells three times with 400µl of wash buffer. After the last wash, the plate was tapped against a clean paper towel in order to completely remove the wash buffer. Now, unspecific binding sites were blocked by adding 300µl of reagent diluent per well. The plate was sealed and incubated for a minimum of 1 hour at room temperature. The wash step was repeated in order to remove the reagent diluent.

A seven-fold standard curve was created with 3, 000pg of BMP-2 per ml as highest and 46.875pg of BMP-2 per ml as lowest standard. Reagent diluent was used to dilute the concentrated standard (190 000pg/ml). A plate design was created and 100µl of blank (reagent diluent), standard or sample were added accordingly. The plate was sealed and incubated for 2 hours at room temperature. During incubation, BMP-2 within samples or standards was allowed to bind to the capture antibody. The wash step was repeated to remove unbound samples. Detection antibody (biotinylated mouse anti-human BMP-2) was diluted in reagent diluent to a final concentration of 1µg/ml and 100µl were added to each well. Again, plate was sealed and incubated for 2 hours at room temperature. Afterwards, the wash step was repeated to remove unbound detection antibody. Streptavidin-conjugated HRP was diluted 1:200 with reagent diluent and 100µl were added to each well. The streptavidin binds to the biotin (on the detection antibody) with high affinity. Plate was sealed and incubated for 20 minutes at room temperature protected from direct light. Afterwards, wash step was repeated in order to remove unbound streptavidin-conjugated HRP. The substrate solution was prepared which contains TMB and hydrogen peroxide. 100µl were added to each well. The TMB acts as chromogenic substrate for the enzyme HRP. HRP oxidises the TMB which leads to the formation of a blue product. The plate was incubated for 20 minutes at room temperature protected from direct light. Finally, enzymatic reaction was stopped by adding 50µl of 1M sulphuric acid to each well. Through addition of the sulphuric acid, TMB changed its colour from blue to yellow. The optical density at 450nm was measured using a 1420 Multilabel counter (Perkin Elmer, Waltham, MA). Wavelength correction was set to 570nm in order to correct for optical imperfections of the plate. Blank correction was conducted using the Work Out2 V.2 software (Perkin Elmer, Waltham, MA). Data were analysed, using software capable of 4 parameter logistics (https://www.readerfit.com). If BMP-2 concentrations within the samples were higher than the highest standard (3, 000pg/ml), the assay was repeated using the same protocol. However, samples were diluted in reagent diluent prior to use. Final BMP-2 concentrations were calculated by multiplying the measured concentration with the dilution factor.

The Quantakine BMP-2 Immunoassay uses the same basic principle (Sandwich ELISA). However, a different protocol was applied. This assay uses a pre-coated (monoclonal mouse antibody against human BMP-2) 96-well polystyrene microplate. First, 100µl of pre-made assay diluent RD1-19 were added to each well. Next, a plate design was created and 50µl of blank (calibrator diluent RD5P), standard or sample, in duplicates, were added accordingly. The plate was sealed and incubated for 2 hours at room temperature on an orbital shaker set to 500 rounds per minutes (rpm). During incubation, BMP-2 within samples or standards was allowed to bind to the primary antibody. Next, plate was aspirated and unbound samples were removed by washing the wells three times with 400µl of wash buffer. After the last wash, the plate was tapped against a clean paper towel in order to completely remove the wash buffer. Then, 200µl of BMP-2 conjugate (monoclonal antibody against BMP-2 conjugated to HRP) were added to the plate. The plate was sealed and incubated for 2 hours at room temperature on an orbital shaker set to 500rpm. Afterwards, the wash step was repeated to remove excess secondary antibody and the substrate solution was prepared. Now, 200µl of substrate solution were added to the plate and the plate was incubated for 30 minutes at room temperature protected from direct light. Finally, 50µl of stop solution (1M sulphuric acid) were added in order to stop the enzymatic reaction. Through addition of the sulphuric acid, TMB changed its colour from blue to yellow. The optical density at 450nm was measured using a 1420 Multilabel counter (Perkin Elmer, Waltham, MA). Wavelength correction was set to 570nm in order to correct for optical imperfections of the plate. Blank correction and calculation of results was conducted using the Work Out2 V.2 software (Perkin Elmer, Waltham, MA). If BMP-2 concentrations within the samples were higher than the highest standard (4 000pg/ml), the assay was repeated using the same protocol. However, samples were diluted in calibrator diluent RD5P prior to use. Final BMP-2 concentrations were calculated by multiplying the measured concentration with the dilution factor.

2.2.26 TGF-β1 enzyme-linked immunosorbent assay.

The Duo Set ELISA Development System for human TGF-β1 uses the same basic principle ("Sandwich ELISA") as both ELISA´s for human BMP-2 (described in section 2.2.25). First, the capture antibody (mouse anti-human TGF-β1) was diluted to a working concentration of 2µg/ml in PBS. A 96-well microplate (R&D Systems, Minneapolis, MN) was coated by adding 100µl of capture antibody to each well. The plate was sealed and incubated overnight at room temperature. Next, the plate was aspirated and unbound capture antibody was removed by washing the wells three times with 400µl of wash buffer. After the last wash, the plate was tapped against a clean paper towel in order to completely remove the wash buffer. Now, unspecific binding sites were blocked by adding 300µl of block buffer per well. Plate was sealed and incubated for a minimum of 1 hour at room temperature. The wash step was repeated in order to remove the reagent diluent. A seven-fold standard curve was created with 2, 000pg of TGF-β1 per ml as highest and 31.25pg of TGF-β1 per ml as lowest standard. Reagent diluent was used to dilute the concentrated standard (220, 000pg/ml). Next, latent TGF- β1 within the samples was activated. 100µl of sample were mixed with 20µl of 1M HCl and incubated for 10 minutes. Then, samples were neutralised with 20µl or 30µl of 1.2M NaOH/0.5M HEPES. A plate design was created and 100µl of blank (reagent diluent), standard or sample were added accordingly. The plate was sealed and incubated for 2 hours at room temperature. During incubation, TGF-β1 within samples or standards was allowed to bind to the capture antibody. The wash step was repeated to remove unbound samples. Detection antibody (biotinylated chicken anti-human TGF-β1) was diluted in reagent diluent to a final concentration of 300ng/ml and 100µl were added to each well. Again, plate was sealed and incubated for 2 hours at room temperature. Afterwards, the wash step was repeated to remove unbound detection antibody. Streptavidin-conjugated HRP was diluted 1:200 with reagent diluent and 100µl were added to each well. The streptavidin binds to the biotin (on the detection antibody) with high affinity. Plate was sealed and incubated for 20 minutes at room temperature protected from direct light. Afterwards, wash step was repeated in order to remove unbound streptavidin-conjugated HRP. The substrate solution was prepared which contains TMB and hydrogen peroxide. 100µl were added to each well.

The TMB acts as chromogenic substrate for the enzyme HRP. HRP oxidises the TMB which leads to the formation of a blue product. Plate was incubated for 20 minutes at room temperature protected from direct light. Finally, enzymatic reaction was stopped by adding 50µl of 1M sulphuric acid to each well. Through addition of the sulphuric acid, TMB changed its colour from blue to yellow. The optical density at 450nm was measured using a 1420 Multilabel counter (Perkin Elmer, Waltham, MA). Wavelength correction was set to 570nm in order to correct for optical imperfections of the plate. Blank correction was conducted using the Work Out2 V.2 software (Perkin Elmer, Waltham, MA). Data were analysed using software capable of 4 parameter logistics (https://www.readerfit.com). Finally, concentrations obtained were multiplied by 1.4 or 1.5 (depending on if 20µl or 30µl of 1.2M NaOH/0.5M HEPES were used for sample neutralisation).

2.2.27 Biochemical analyses.

2.2.27.1 Quantification of sulphated GAG.

The amount of sulphated GAG, within both culture medium and scaffolds, was quantified using the DMMB dye binding assay (Farndale et al., 1986). DMMB is a metachromic dye for the histochemical detection of sulphated GAG. Proteinase K-digested samples were thawed at room temperature. A 6-fold standard curve was prepared by diluting the chondroitinsulfate stock with PBS. The standard ranged from 2.5µg per well to 0.078µg per well. Next, a plate design was prepared and 20µl of blank (PBS), sample or standard were added, in duplicates, onto a Costar 96-well assay plate (Corning, Life Sciences, Lowell, MA). Now, 200µl of DMMB was added to each well. The absorbance at 535nm was immediately measured using a 1420 Multilabel counter (Perkin Elmer, Waltham, MA). For quantification of GAG from cell culture medium, the protocol was slightly modified. Serum-free DMEM was used to create the standard curve and used as blank. Further, 50µl of blank, standard and sample was used. Blank correction and calculation of GAG concentration were conducted using the Work Out2 V.2 software (Perkin Elmer, Waltham, MA). For evaluation, the GAG concentration per ml was calculated from the concentrations obtained from either 20µl or 50µl of sample. The measured values were multiplied by 50 (Proteinase K samples) or 20 (medium samples). Finally, the total amount of GAG in each sample was calculated by multiplying the concentration per ml by the corresponding volume. If half scaffolds were analysed, the obtained number was additionally multiplied by 2.

2.2.27.2 Quantification of cellular DNA.

The total amount of DNA was analysed using the Hoechst 33258 dye (bisbenzimide) binding assay (Labarca and Paigen, 1980). The assay is based on the ability of bisbenzimide to bind to the minor groove of DNA. The binding results in an enhancement of the fluorescence of the dye, which can be detected spectrophotometrically. This fluorescence is related to the AT content of the DNA sample. Therefore, it is important to use a DNA standard with a similar AT content as the sample which is analysed. Calf thymus DNA was used as standard (double-stranded DNA with an AT content of approximately 60%). A 6-fold standard curve was prepared by diluting calf thymus DNA with DPBS. The standard curve ranged from 2, 000ng of DNA in 40µl of volume to 62.5ng of DNA in 40µl of volume. A plate design was prepared and 40µl of blank (DPBS), standard or sample was added in, duplicates, to a 96-well white plate (Becton Dickinson, Franklin Lakes, NY). Next, a 1µg/ml Hoechst 33258 assay solution was prepared by diluting the dye stock with DPBS. 160µl of dye assay solution were added to each well. The plate was wrapped in alumina foil, in order to protect it from direct light, and incubated for 20 minutes at room temperature. Afterwards, the plate was immediately measured on a 1420 Multilabel counter (Perkin Elmer, Waltham, MA). The following parameters were used: 360 m excitation and 465nm fluorescence emission. Blank correction and calculation of DNA concentration were conducted using the Work Out2 V.2 software (Perkin Elmer, Waltham, MA). Finally, the total DNA content in the samples was calculated. First, the DNA concentration within 1ml was obtained by multiplying the measured concentration (in 40µl) by 25. Now, the concentration per ml was multiplied by the total volume in order to obtain the total DNA content within the samples. If half scaffolds were analysed, the obtained number was additionally multiplied by 2.

2.2.28 Gene expression analyses.

2.2.28.1 mRNA isolation.

TRI samples were thawed at room temperature and 100µl of 1-Bromo-3-chloro-propane was added, for every 1ml of TRI reagent initially used. Samples were mixed vigorously for 15 seconds and, afterwards, stored for 12 minutes at room temperature on an orbital shaker (Heidolph Instruments, Schwabach, Germany) with gentle agitation. Centrifugation steps during mRNA isolation were conducted using the 5417R centrifuge (Eppendorf, Hamburg, Germany). Samples were centrifuged at 12, 000g and 4°C for 15 minutes. This process facilitates phase separation into a lower phenol-chloroform phase, an interphase and an upper aqueous phase. The mRNA remains in the aqueous phase, whereas DNA and proteins are sequestered into the organic phase and interphase. The aqueous phase was transferred to a fresh Eppendorf tube and mRNA was precipitated by addition of 250µl of isopropanol and 250µl of high salt precipitation solution, for every 1ml of TRI reagent initially used. Now, samples were stored for 10 minutes at room temperature on an orbital shaker (Heidolph Instruments, Schwabach, Germany) with gentle agitation and afterwards centrifuged for 8 minutes at 4°C and 12, 000g. The high salt precipitation solution was used in order to prevent contamination of mRNA samples with polysaccharides and proteoglycans. After centrifugation, the supernatant was removed and the mRNA pellet was washed by adding 1ml of 75% ethanol in DEPC water and subsequent centrifugation for 5 minutes at 7, 600g and 4°C. Now, the mRNA pellets can be stored at -20°C for up to 1 year or immediately further processed.

Next, mRNA was solubilised. The ethanol was removed and pellets were allowed to air-dry for 5 to 10 minutes. Afterwards, mRNA pellets were dissolved in 40µl of DEPC water by passing the solution through a pipette tip. Then, the mRNA was incubated for 15 minutes at 56°C to achieve complete dissolution. Finally, mRNA content was quantified using a ND-1000 spectrophotometer (Nano Drop, Wilmington, DE). The purity was assessed by measuring the 260/280 and the 260/230 ratio. The 260/280 ratio was expected to be higher than 1.9. Lower values can originate from contaminating protein. The 260/230 ratio is expected to be higher than 1.6. Lower values can originate from reagents used during isolation or purification, such as chaotropic salts or phenol.

2.2.28.2 Reverse transcription.

Reverse transcription (RT) was carried out using TaqMan reagents (Applied Biosystems, Carlsbad, CA). These reagents are extensively screened to be RNAse free. First, a master mix was prepared. It contains 2µl of 10-fold PCR buffer, 4.4µl of magnesium chloride, 4µl of deoxynucleotide triphosphate (dNTP) mixture, 1µl random hexamers, 0.4µl of RNAse inhibitor (equals to 8units) and 0.5µl of reverse transcriptase (equals to 25units). 12.3µl of master mix were added to 200µl thermowell tubes (Corning Incorporated, Corning, NY). Next, 1µg of mRNA was added and the final volume was adjusted to 20µl using DEPC water. The buffer provides the pH and ionic strength which is optimal for RT. Magnesium chloride act as cofactor for the enzyme reverse transcriptase. The random hexamers are short oligonucleotides of random sequences that anneal randomly to complementary sites on DNA or RNA. They act as primer for the enzyme reverse transcriptase. The dNTP mixture provides the enzyme reverse transcriptase with the four dNTPs (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytosine triphosphate and deoxyguanine triphosphate) in equimolar ratios. The RNAse inhibitor was added to inhibit the activity of any contaminating RNAse (enzyme that cleaves RNA). Finally, the enzyme reverse transcriptase synthesises cDNA, using the RNA as template. For RT, a 5700 sequence detector system (Applied Biosystems, Carlsbad, CA) with the following program was used: 10 minutes at 25°C for primer incubation, 30 minutes at 48°C for RT and 5 minutes at 95°C for inactivation of the enzyme reverse transcriptase. Finally, samples were diluted with 60µl of TE buffer and stored at -20°C for long-term storage. If less than 1µg of mRNA had to be used for RT, the volume of TE buffer was adjusted accordingly. For example, if only 600ng of RNA were used for RT, 36µl of TE buffer were used to dilute the cDNA.

2.2.28.3 Real-time polymerase chain reaction.

PCR is a technique in molecular biology which is used to amplify DNA in several orders of magnitude. It is even possible to start with one single molecule of DNA as template. PCR was first described in 1986 by Karry Mullis (Mullis et al., 1986). Almost all PCR applications use thermostable DNA polymerases in order to amplify DNA sequences. Further, the four nucleotides (adenosine, thymidine, cytosine and guanine) and a short primer sequence are needed. A primer is a short sequence of complementary RNA that binds to the DNA and acts a starting point for DNA replication.

Real-time PCR (RT-PCR) is a technique which is able to quantify the initial amount of template specifically and sensitively. It is based on the detection and quantification of fluorescent reporter dyes. The intensity of the fluorescent signal is directly proportional to the amount of PCR product present within the reaction. The fluorescent signal can be measured at the end of every PCR cycle and it is monitored when it crosses a certain threshold level, the so-called C_t value. Some oligonucleotide primers and probes, used within this study (see table 2-1), were previously designed and validated with our group using the primer express oligo design software V.1.5 (Applied Biosystems, Carlsbad, CA). They were synthesised by Microsynth (Balgach, Switzerland). For detection of the remaining genes, the commercial pre-developed TaqMan reagents (Applied Biosystems, Carlsbad, CA) were used.

Gene	Primer forward	Primer reverse	Probe (5´FAM/3´TAMRA)
human ColIA1	5'-CCC TGG AAA GAA TGG AGA TGA T-3'	5'-ACT GAA ACC TCT GTG TCC CTT CA-3'	5'-CGG GCA ATC CTC GAG CAC CCT-3'
human ColIIA1	5'-GGC AAT AGC AGG TTC ACG TAC A-3'	5'-GAT AAC AGT CTT GCC CCA CTT ACC-3'	5'-CCT GAA GGA TGG CTG CAC GAA ACA TAC-3'
human ColXA1	5'-ACG CTG AAC GAT ACC AAA TG-3'	5'-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3'	5'-ACT ACC CAA CAC CAA GAC ACA GTT CTT CAT TCC-3'
human Aggrecan	5'-AGT CCT CAA GCC TCC TGT ACT CA-3'	5'-CGG GAA GTG GCG GTA ACA-3'	5'-CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT-3'
human Runx2 (cbaf1)	5'-AGC AAG GTT CAA CGA TCT GAG AT-3'	5'-TTT GTG AAG ACG GTT ATG GTC AA-3'	5'-TGA AAC TCT TGC CTC GTC CAC TCC G-3'
human Sp7 (Osterix)	5'-CCT GCT TGA GGA GGA AGT TCA-3'	5'-GGC TAG AGC CAC CAA ATT TGC-3'	5'-TCC CCT GGC CAT GCT GAC GG-3'
human BMP-2	5'-AAC ACT GTG CGC AGC TTC C-3'	5'-CTC CGG GTT GTT TTC CCA C-3'	5'-CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG-3'

Table 2-1: Self-designed forward primers, reverse primers and probes used for RT-PCR. The primers and probes were designed and validated within our group using the primer express design software V.1.5 and were synthesised by Microsynth.

The self-designed probes contain a fluorescent dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end. When the probe is intact, the fluorescent dye and the quencher dye are in close proximity. Therefore, when excited, the fluorescence of FAM is quenched by a phenomenon called fluorescence resonance energy transfer. The probe binds to the DNA between forward and reverse primers. During amplification of the DNA, the DNA polymerase cleaves the probe through its 5' exonuclease activity. Now, the fluorescent dye at the 5' end is released from the DNA and thereby separated from its quencher. Hence, the fluorescent dye is now able to emit fluorescence and this fluorescence can be detected. For RT-PCR, a mastermix was prepared first. For every reaction, 10µl of TaqMan gene expression master mix was mixed with 1µl of 20-fold pre-developed TaqMan assay reagent or 2µl of 10-fold primer/probe mix and 7µl (TaqMan reagents) or 6µl (self-designed primers and probes) of DEPC water. Final concentrations, for self-designed primers and probes, were 900nM for forward primer and reverse primer and 250nM for probe. 18µl of mastermix was added to a thermo-fast 96 PCR detection plate (Thermo Fischer Scientific, Waltham, MA).

Afterwards, 2µl of cDNA was added resulting in a final reaction volume of 20µl. The plate was covered with absolute QPCR seal (Thermo Fischer Scientific, Waltham, MA) and briefly centrifuged (impulse up to 800g) using the Rotanta 46 centrifuge (Hettich, Tuttlingen, Germany). RT-PCR was conducted using the 7500 real time PCR system (Applied Biosystems, Carlsbad, CA). The following program was used: 95°C for 10 minutes to activate DNA polymerase, followed by 40 cycles of amplification at 95°C for 15 seconds (denaturation) and 60°C for 1 minute (annealing and extension). Gene expression was detected at the end of every cycle. C_t values were automatically derived using the 7500 system software V. 1.4 (Applied Biosystems, Carlsbad, CA). Gene expression analyses were conducted using the comparative $\Delta\Delta C_t$ method. Eighteen s ribosomal RNA (18s rRNA) was used as internal control. For evaluation, first the ΔC_t value for each sample was calculated by subtracting the corresponding C_t value of the house keeping gene (18s rRNA) from the C_t value of the gene of interest. Next, the $\Delta\Delta C_t$ value was calculated. Therefore, the C_t value of the reference sample was subtracted from the ΔC_t value of each sample. Finally, the n-fold changes in gene expression, relative to the reference sample, were calculated using the formula $2^{\wedge -\Delta\Delta Ct}$.

2.2.29 Histology and Immunohistochemistry.

2.2.29.1 Cryo-sectioning of fibrin-PU scaffolds (fixed).

First, scaffolds were transferred to 5% Sucrose in PBS and incubated overnight at 4°C. This step allows the replacement of methanol and infiltration of the porous scaffold with the viscous, isoosmolar solution. On the next day, scaffolds were transferred to O.C.T compound and incubated for at least 20 minutes at room temperature. Now, they were placed centred on a specimen holder, embedded in O.C.T compound and frozen in the HM 560 cryostat microtome (Carl Zeiss AG, Oberkochen, Germany). Once the scaffolds were completely frozen, sections were cut. Therefore, the specimen was put behind a N35HR microtome blade. Samples were trimmed (40µm) until a smooth scaffold surface was visible. Now, 12µm sections were cut. Consequently, the sections were placed on superfrost plus microscope slides (two sections per slide). Finally, slides were stored at -20°C until histological or immunohistochemical analysis.

2.2.29.2 Cryo-sectioning of pellets (unfixed).

For harvesting (section 2.2.21), pellets were snap-frozen in O.C.T compound and stored at -80°C. For sectioning, they were directly placed centred on a specimen holder and sections were cut as described in section 2.2.29.1. Briefly, the specimen was put behind a N35HR microtome blade and samples were trimmed (40µm) until a smooth surface was visible. Afterwards, 10µm sections were cut and sections were placed on superfrost plus microscope slides (two sections per slide). Finally, slides were stored at -20°C until histological or immunohistochemical analysis. Sectioning of pellets was done by Oliver Gardner, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.29.3 Safranin-O/Fast Green staining.

Safranin-O is a cationic dye that can be used to stain acidic proteoglycans. When bound to proteoglycans, it shows an orange colour. Fast Green is used as contrast stain for Safranin-O. It is an acidic substrate that contains a sulphate group, which binds to the amino group of proteins and stains them green. First, slides were allowed to acquire room temperature. Now, unfixed samples were fixed for 10 minutes in 70% methanol. For already fixed samples, O.C.T compound was removed by washing the sample in distilled water for 10 minutes. The water was changed every 2-3 minutes. Afterwards, the nuclei of the cells were stained black with Weigert´s iron haematoxyline (for 12 minutes). Consequently, a step called "blueing" had to be conducted as they dye itself does not develop a colour unless it has reacted with ions. Therefore, slides were incubated in normal tab water (contains ions) for 10 minutes. The water was changed every 2-3 minutes. Subsequently, slides were rinsed with distilled water and sections were stained for 6 minutes in the Fast Green working solution. Next, slides were briefly put (10-15 seconds) in 1% acetic acid. Afterwards, the sections were stained for 6 minutes with the Safranin-O working solution. The colour ratio between the two dyes was differentiated by incubating the slides in 96% ethanol for two minutes. Now, slides were completely dehydrated by incubation in 100% ethanol for a minimum of two minutes and subsequent transfer to fresh 100% ethanol for at least another 2 minutes.

For cover-slipping, slides were transferred to a fume hood and incubated in xylene for a minimum of two minutes. Then, they were transferred to fresh xylene and again incubated for a minimum of two minutes. Sections were mounted using Eukitt® quick-hardening mounting medium. A crucible was used to cover pellets or scaffolds with mounting medium. Next, they were carefully cover-slipped. Polymerisation of the mounting medium was induced by incubation for 1 hour at 37-40°C on a heated stretching table (Medite Medizintechnik GmbH, Burgdorf, Germany). Next, the polmyerisation process was allowed to finish by incubating the samples for a minimum of 12 hours at room temperature.

Technical assistance during Safranin-O/Fast Green staining was provided by Nora Goudsouzian, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.29.4 Immunohistochemistry.

For immunohistochemistry, three different primary antibodies were used (see table 2-2). Namely, these were the 1-C-6 antibody for the aggrecan protein, the CIICI antibody for the Col II protein and the Col-10 antibody for the Col X protein. The 1-C-6 antibody detects the hyaluronic acid binding region at the N-terminal end of proteoglycans. The CIICI antibody detects the Col II protein in hyaluronidase-treated samples. The Col-10 antibody specifically detects native and denatured Col X protein.

Antigen	Blocking reagent	Primary antibody	Secondary antibody
Hyaluronic acid binding region	1:20 diluted normal horse serum	1-C-6, monoclonal IgG2a mouse anti-rat, cross-reacts with human, 8.167µg/ml	Concentrated, biotinylated horse anti-mouse IgG, diluted to 7.5µg/ml in PBS-T
Col II	1:20 diluted normal horse serum	CIICI, monoclonal IgG mouse anti-chicken, cross-reacts with human, 1.67µg/ml	Concentrated, biotinylated horse anti-mouse IgG, diluted to 7.5µg/ml in PBS-T
Native and denatured Col X	1:20 diluted normal horse serum	Col-10, monoclonal IgM mouse anti-porcine, cross-reacts with human, 0.5µg/ml	Concentrated, biotinylated horse anti-mouse IgG, diluted to 7.5µg/ml in PBS-T

Table 2-2: Overview of the primary antibodies, secondary antibodies and the blocking reagent which were used for immunohistochemistry.

Before immunohistochemical analysis, sections were pre-treated as described in section 2.2.29.3 (fixation or removal of O.C.T compound). For each group, one section was treated with the corresponding primary antibody. Further, one sections per group was treated as negative control. As the CIICI and the Col-10 antibody have the same enzymatic pre-treatment and the same secondary antibody, the negative control section could be used for both antibodies. For the 1-C-6 antibody (reduction and alkylation step plus a different enzyme pre-treatment), an additional section per group was treated and used as negative control. For detection of the aggrecan protein, samples had to be reduced and alkylated (breaks and stabilises disulphide bonds) prior to the treatment with the primary antibody. This was necessary in order to expose the epitope which is recognised by the 1-C-6 antibody. Pre-treated sections were first incubated for 2 hours at 37°C in 10 mM DTT (reduction step). Afterwards, they were transferred to 40 mM iodoacetamide and incubated for 1 hour at 37°C. This step alkylates and thereby stabilises the reduced (broken) disulphide bounds. Finally, slides were transferred to a "moist chamber" and stored overnight at 2-8°C.

For immunohistochemical labelling, slides were allowed to acquire room temperature. Next, they were incubated for 30 minutes in 0.3% peroxidase in 100% methanol. This step is necessary to block endogenous peroxidase activity which could otherwise lead to false positive results (the DAB detections system is peroxidase based). Then, slides were allowed to completely air-dry and a Dako pen was used to draw a hydrophobic border around the scaffolds/pellets. This procedure saves material and prevents drying of the sections. Now, slides were washed for 2 x 5 minutes in PBS-T. PBS-T was always changed in between washes. This step re-hydrates the section and further reduces the surface tension on the slides. All incubation steps (with enzyme, blocking reagent, antibodies, AB complex and DAB solution) were conducted in a "moist chamber". Subsequently, sections were treated, for 30 minutes at 37°C, with either 0.025units/ml of chondroitinase AC (1-C-6) or 0.05units/ml of hyaluronidase (CIICI and Col-10).

The chondroitinase AC degrades chondroitines A and C and exposes the epitope onto which the 1-C-6 antibody binds. The hyaluronidase hydrolyses linkages in hyaluronate, chondroitin and dermatan. This dissociates the ECM of native tissue and tissue-engineered constructs and is a requirement for the binding of the CIICI and the Col-10 antibody. After enzyme treatment, slides were washed for 3 x 5 minutes in PBS-T in order to remove enzyme and thereby stop the enzymatic reaction. Up next, slides were blocked, for a minimum of 1 hour at room temperature, with 150µl diluted normal horse serum. This step masks non-specific binding sites and thereby prevents non-specific binding of the secondary antibody. The appropriate blocking diluent always has to be chosen according to the species in which the secondary antibody was raised. Afterwards, serum was removed (no wash step) and the slides were treated with 150µl of diluted primary antibody or PBS-T (negative controls) and incubated for 30 minutes at room temperature. Now, slides were washed for 3x5 minutes in PBS-T in order to remove any non-specifically bound primary antibody. Then, slides were incubated with 150 µl of diluted secondary antibody for 30 minutes at room temperature. Again, slides were washed for 3 x 5 minutes in PBS-T in order to remove any non-specifically bound secondary antibody.

The "Vectastain elite ABC kit mouse IgG" and the "ImmPACT DAB peroxidase substrate" were used as detection system. Slides were treated with 150µl of AB complex and incubated for 30 minutes at room temperature. The avidine in the AB complex binds with high affinity to the biotinylated secondary antibody. Further, the AB complex contains biotinylated HRP. Avidine has for binding sites for biotin and can thereby enhance signal intensity. After the 30 minute incubation step, the antigen is effectively labelled with HRP. Now, the slides were washed for 3 x 5 minutes in PBS-T in order to remove any excess AB complex. Thereafter, 150µl of DAB working solution were added to each slide and they were incubated for 4 minutes at room temperature, protected from direct light. The "ImmPACT DAB peroxidase substrate" is a new diaminobenzidine based peroxidase substrate which forms a brown substrate in a reaction which is catalysed by the enzyme HRP. Following incubation with DAB, the slides were washed for a minimum of 10 minutes in distilled water. The distilled water was changed every 2-3 minutes.

Now, the cell nuclei were counterstained for 15 seconds with haematoxyline according to Mayer. The haematoxyline was filtered prior to use. Subsequently, "blueing" was conducted for a minimum of 5 minutes as described in section 2.2.29.4. Before samples were ready for cover-slipping, sections were dehydrated in a graded series of ethanol with increasing concentrations (50%, 70%, 96%, 100% and again 100%). Minimum incubation time for each ethanol concentration was 2 minutes. Finally, samples were transferred to xylene and cover-slipped in a fume hood as described in section 2.2.29.3.

Technical assistance during immunohistochemistry was provided by Nora Goudsouzian, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.30 Microscopy.

Image acquisition was conducted with the Axioplan 2 microscope, the AxioCamHR camera and the Carl Zeiss AxioCamHR V.5.07.03 software (all Carl Zeiss AG, Oberkochen, Germany). For visualisation of fluorescent labels in the co-culture of hMSCs and hACPCs experiment, images were taken with a total magnification of 12.6 (0.63 x from the camera and 20 x from the lens). The following channels were used; DAPI (for cell nuclei stained with DAPI), FITC (for PKH67-labelled hMSCs) and Cy3 (for PKH26-labelled hACPCs). Before image acquisition, cell nuclei were labelled, using the ProLong® antifade reagent with DAPI, and cover-slipped. This step was conducted by Nora Goudsouzian, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

In the effect of dorsomorphin on hMSC hypertrophy study, for visualisation of Safranin-O/Fast Green staining, aggrecan protein immunolabelling, Col II protein immunolabelling and Col X protein immunolabelling (in one donor; ♂ age 24), images were taken with a total magnification of 3.15 (0.63 x from the camera and 5 x from the lens). In the same experiment, for visualisation of Col X protein immunolabelling in the other two donors, a total magnification of 6.3 (0.63 x from the camera and 10 x from the lens) was used. For visualisation of Safranin-O/Fast Green staining, aggrecan protein immunolabelling, Col II protein immunolabelling and Col X protein immunolabelling, in the co-culture of hMSCs and hACPCs experiment, images were taken with a total magnification of 6.3 (0.63 x from the camera and 10 x from the lens). Images were taken by Dr. Martin Stoddart, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

2.2.31 RayBio human cytokine antibody array G-series.

In order to analyse the secretome of hMSCs and hACPCs, the RayBio human cytokine antibody array G-series was used. With this assay, it is possible to detect 174 different cytokines (secreted cell-cell signalling proteins) from medium samples at the same time. The assay uses three different sub-arrays named G-Array 6, G-Array 7 and G-Array 8. Each array is able to detect 60 (G-Array 6 and G-Array 7) or 54 (G-Array 8) different cytokines in one well. The different array maps can be found in the appendix (section A 3). For analysis, only the 48 hours samples were taken. This decision was based on the observation that cells started to change their phenotype (they rounded up) and further began to die between the 48 and the 72 hour time-point, under serum-free conditions. The basic principle of the RayBio human cytokine antibody array G-series assay is similar to a "Sandwich ELISA" and summarised in figure 2-6.

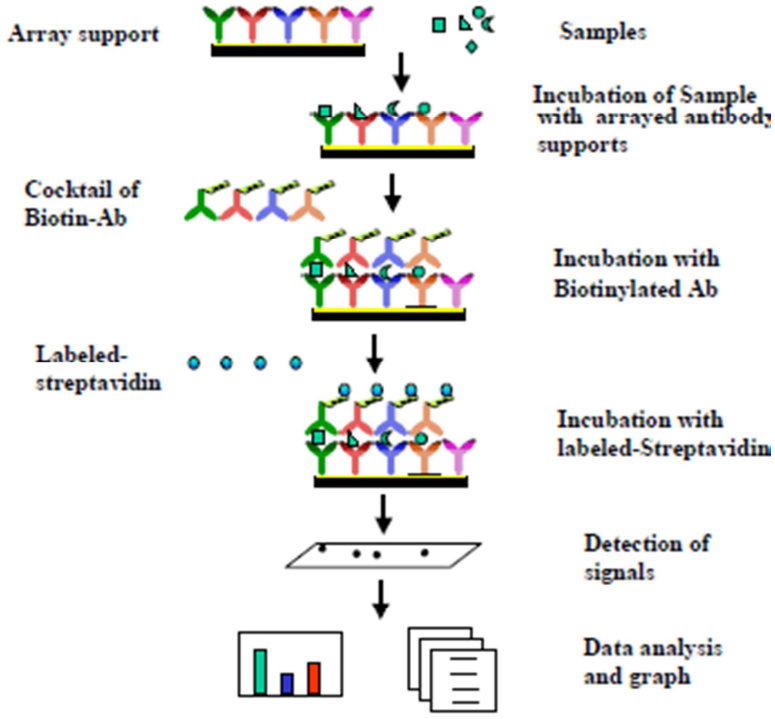

Figure 2-6: Basic principle of the RayBio human cytokine antibody array. The array is pre-coated with different primary antibodies against specific cytokines. Samples are incubated and the respective cytokine, within the sample, binds to its corresponding primary antibody. Then, a mix of biotinylated specific secondary antibodies is added and they bind, using a different epitope, to their respective cytokine (which is bound to the primary antibody). Next, labelled (HiLyte Plus Fluor 532) streptavidin is added which specifically binds to the biotin on the secondary antibody. Finally, the signal can be obtained by a laser scanner using the Cy3 channel. Image adapted from the official user manual provided by RayBiotech.

First, sub-arrays were allowed to acquire room temperature and subsequently air-dried for 2 hours in a laminar-flow hood. Next, 100µl of 1x blocking buffer was added to each well and incubated for 30 minutes at room temperature. All incubation and wash steps were conducted with gentle agitation (30rpm) on an orbital shaker (Heidolph Instruments, Schwabach, Germany). In the meantime, samples were centrifuged for 10 minutes at 12, 000rpm using the 5417R centrifuge (Eppendorf, Hamburg, Germany). The blocking buffer was completely removed and 100µl of sample was added to each sub-array. Now, chambers were covered with adhesive film and incubated overnight at 4°C with gentle agitation. On the next day, samples were completely removed and chambers were washed. Therefore, 150µl of wash buffer 1 were added to each well and incubated for 2 minutes at room temperature. Wash buffer was changed and fresh wash buffer was added to each well. This procedure was repeated once for a total of three washes. Now, chambers were transferred to a clean pipette tip box and completely submerged in wash buffer 1. They were washed for 10 minutes at room temperature. The wash buffer was changed and the wash step was repeated once. Next, the chambers were transferred to a new clean pipette tip box and the two 10 minute wash steps were repeated using wash buffer 2. Afterwards, wash buffer was completely removed and 70µl of Biotin-conjugated anti-cytokines were added to each well on the corresponding sub-arrays. Chambers were covered with adhesive film and incubated for 2 hours at room temperature. Now, Biotin-conjugated anti-cytokines were completely aspirated and the wash steps (using both wash buffer 1 and 2) were repeated as described above. Subsequently, 70µl of 1x Streptavidin-Fluor were added to each well, the chambers were covered with adhesive film, wrapped in alumina foil and incubated for 2 hours at room temperature. Next, 1x Streptavidin-Fluor was completely removed and the wash steps were repeated as described above. After washing, the glass slides were removed from their frames and transferred to a 30ml Falcon tube containing glass slide holders (included in the kit). The tube was filled with wash buffer 1 and incubated for 10 minutes at room temperature. The wash buffer was decanted and replenished with fresh wash buffer 1. Again, the slides were incubated for 10 minutes at room temperature. Hereafter, slides were gently rinsed with MilliQ water and transferred to a new 30ml Falcon tube containing glass slide holders. In order to remove water droplets, the slides were centrifuged for 3 minutes at 1, 000rpm using the Rotanta, RP centrifuge (Hettich, Tuttlingen, Germany).

The slides were protected from direct light and allowed to air-dry (at least 30 minutes) in a laminar-flow hood. Finally, they were sent to THP Medical Products Vertriebs GmbH (Vienna, Austria) for laser scanning and processing of the raw data. Background normalisation was conducted by subtracting the measured value of the negative control sample from each sample. The negative control spots are printed with a protein containing buffer and detect unspecific binding of either biotin-conjugated anti-cytokines and/or Streptavidin-Fluor. The data were afterwards expressed as n-fold signal intensity compared to positive control samples (know amount of biotinylated IgGs which are printed directly onto each array). For final analysis, one medium sample of the 48 hour time-point for each donor was used. The processed raw data, obtained from THP Medical Products Vertriebs GmbH (Vienna, Austria), were normalised to the DNA content of the respective sample. The average of both samples was calculated for both the hMSC and the hACPC group. In order to calculated n-fold differences between both groups, the average of the hMSC group was normalised to the average of the hACPC group. As suggested by the manufacturer, n-fold changes higher than 2 were considered as higher cytokine content in hMSCs and n-fold changes lower than 0.5 were considered as lower cytokine content in hMSCs.

2.2.32 Statistical analyses.

Statistical analysis was performed with the SPSS software package V.19 (IBM, Armonk, NY).

For the enhanced adenoviral transduction in 3D study (part of chapter 3), normality of each group was tested with the Shapiro-Wilk test. Levene's Test of equality of error variances was conducted to test for equal variances between groups. The significance of differences between the groups was tested with a general linear model and repeated measures analysis of variance with a Games Howell Post-hoc analysis for unequal variances. These particular analyses were carried out by Dr. Martin, AO Research Institute Davos, AO Foundation, Davos, Switzerland.

For the effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation study, the timed Ad.BMP-2 transduction study (both part of chapter 3) and the effect of dorsomorphin on hMSC hypertrophy (chapter 5) study, normality of each group was tested with the Shapiro-Wilk test. Levene's Test of equality of error variances was conducted to test for equal variances between groups. The significance of differences between the groups was determined with an independent samples t-test.

For the Ad.BMP-2 and load study (chapter 4) and the ACPC and load study (chapter 7), normality of each group was tested with independent samples Kruskal-Wallis test. Levene's Test of equality of error variances was conducted to test for equal variances between groups. The significance of differences between the groups was determined with a general linear model analysis of variance with a Games Howell Post-hoc analysis for unequal variances. For the gene expression analyses, data transformation by natural logarithm was applied to normalise skewed data.

For the co-culture of hMSCs and hACPCs study (chapter 6), normality of each group was tested with the independent samples Kruskal-Wallis test. Levene's Test of equality of error variances was conducted to test for equal variances between groups. The significance of differences between the groups was determined with a general linear model analysis of variance with a Games Howell Post-hoc analysis for unequal variances.

For each chapter, all descriptive results are either displayed as mean ± standard deviation or mean + standard deviation. Significance was defined as $p \leq 0.05$.

Chapter 3: Viral gene transfer.

3.1 Effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation – introduction.

Retroviruses (family: *retroviridea*) belong to the so-called RNA viruses. As a consequence, their genome has to be converted to DNA before it can be integrated into the host genome. This process is mediated by the enzyme reverse transcriptase. Figure 3-1 outlines the life-cycle of a wild-type retrovirus.

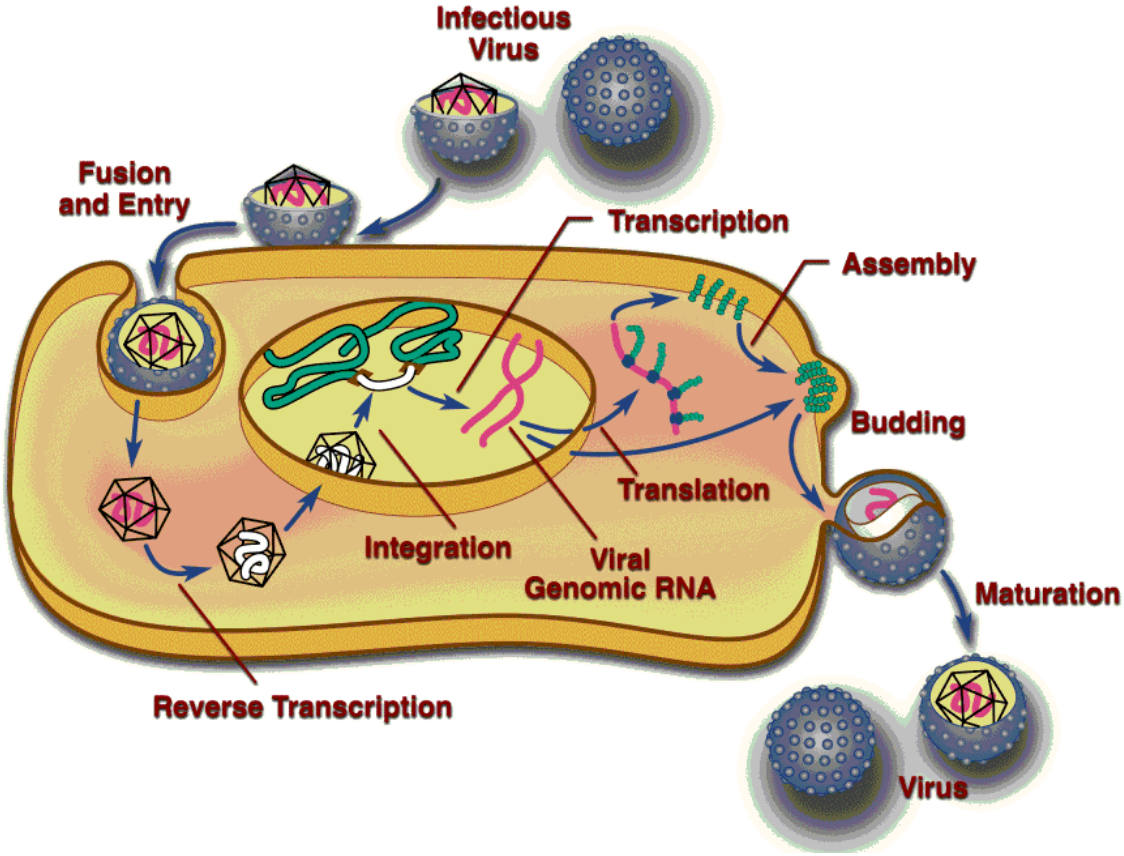

Figure 3-1: Life-cycle of a wild-type retrovirus. The life-cycle starts with the receptor-mediated entry of the retrovirus into the cytoplasm of the host cell. Now, viral RNA is reverse transcribed into DNA. This process is mediated by the viral encoded enzyme reverse transcriptase. Next, the DNA copy enters the nucleus and is integrated into the host genome. This process is mediated by the viral encoded enzyme integrase. The transcriptional and translational machinery of the host cell then produces viral RNA and proteins. Finally, new viral particles are formed in the cytoplasm which then leave the host cell through budding from the cell membrane. Figure adapted from http://www.home.ncifcrf.gov.

The life-cycle starts with the entry of the retrovirus into the cytoplasm of the host cell. This entry is mediated through interaction of viral envelope proteins and a receptor on the membrane of the host cell. Now, the genome of the virus is converted into DNA. This process is mediated through the viral encoded enzyme reverse transcriptase. The DNA copy enters the nucleus of the host cell and integrates into the host genome. Once integrated, the viral genome is steadily transmitted to subsequent cell generations. This so-called provirus exploits the host cells transcriptional and translational machinery for the production of viral RNA and proteins. Finally, new viral particles are assembled in the cytoplasm and leave the host cell through budding from its membrane.

Early retroviral vectors, for gene therapy applications, were based on the MMLV. This kind of virus is only able to infect dividing cells, as it cannot cross the nuclear membrane (Miller et al., 1990). In retroviral gene transfer vectors, almost all viral genes are deleted. Only genes necessary for packaging the RNA genome into the capsid, reverse transcription and integration remain. A pre-requisite for the production of retroviral vectors is the so-called packaging cell. The packaging cell provides crucial viral genes (gag, pol, env) in *trans*. Thus, no infectious viral particles can be produced in the absence of the packaging cell. This feature increases the safety of retroviral vectors for gene therapy applications. However, as these viruses are integrated into the host genome, the risk of insertional mutagenesis is ever present. Once cells have been transduced with most first generation retroviral vectors, the expression of the transgene product is constitutively active. It is permanently produced, at a certain rate. Its production cannot be exogenously controlled (with the exception of inducible retroviral vectors) and is neither dependent on its concentration nor on a specific physiological demand. Retroviral vectors are frequently used for gene transfer in basic research and represent an effective way to provide the sustained delivery of bioactive factors (explicitly reviewed in: (Hu and Pathak, 2000)). The maximum packaging capacity of these vectors is 8 kilo-bases (kb) (Thomas et al., 2003).

For basic research applications, cells that have been transduced with retroviral vectors *ex vivo* are usually further amplified for a period of time. This amplification step is carried out in order to obtain sufficient cell numbers for the given experimental setup. The gene of interest generally encodes for a bioactive factor and control cells are usually transduced with a retroviral vector expressing a non-bioactive factor, such as β-galactosidase or enhanced green fluorescent protein (Kuroda et al., 2006; Salzmann et al., 2009). As most retroviral vectors are constitutively active, the cells are already exposed to the transgene product during the initial monolayer proliferation step. However, its effect on the cells during this step is rarely, if ever, investigated. It is natural to assume that bioactive transgene products will influence the cells during proliferation. Further, stem/progenitor cells are particularly sensitive to bioactive stimuli. Therefore, the current study wanted to investigate if transduction with Rv.BMP-2 influences the gene expression profile of hMSCs during monolayer proliferation. Rv.eGFP was used as control vector. The transgene expression of both vectors was driven by the MMLV 5´LTR. Further, it was evaluated if retroviral transduction or exposure to 8µg/ml of "Polybrene" alters the phenotype or the proliferation rate of hMSCs. "Polybrene" is routinely used to enhance retroviral transduction efficiency. Therefore, cells exposed to 8µg/ml of "Polybrene" were compared to naïve cells. Further, Rv.BMP-2 and Rv.eGFP transduced cells were compared to mock-treated cells. Finally, the effect of exposure to 100ng/ml of exogenous BMP-2, on the gene expression profile of hMSCs during monolayer proliferation, was investigated. This work was published in the Electronic Journal of Biotechnology (Neumann et al., 2013b).

3.2 Effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation – results.

The experiment was independently repeated with cells from 4 donors (♀ age 81, ♀ age 55, ♂ age 56 and ♂ age 54). Three samples for every treatment group were used. The mock-treatment control has only been conducted with cells from three donors (♀ age 55, ♂ age 56 and ♂ age 54). Population doublings after cryopreservation were not tracked within this experiment.

Additionally, the effect of recombinant human BMP-2 or exposure to 8µg/ml of "Polybrene", on monolayer-expanded hMSCs, was monitored in one donor (♂ age 56). Triplicate samples were carried out in each group.

In two out of four donors (♂ age 56 and ♂age 54), 1ml of medium was collected on day 2, 4, 6 and 7 after transduction with Rv.BMP-2. The medium was stored at -20°C for subsequent quantification of BMP-2 concentration.

3.2.1 Exposure to 8µg/ml of "Polybrene" for 2 hours has no effect on hMSC phenotype or confluency.

In order to exclude the possibility that exposure to 8µg/ml of "Polybrene" has a negative effect on hMSCs (such as an altered phenotype or a reduced confluency), cells in the treated group were exposed to 8µg/ml of "Polybrene", in 0.5ml of medium, for 2 hours. Control cells were cultured in 0.5ml of medium without addition of "Polybrene". Afterwards, an additional 2.5ml of medium was added and cells were cultured overnight under standard conditions. Next, the medium was changed and cells were cultured for a total of 10 days with additional medium changes at day 3 and day 6. Cells were regularly monitored and images were taken at day 1, 3 and 6 after exposure to "Polybrene". For image acquisition, an Axiovert 25 microscope (Carl Zeiss AG, Oberkochen, Germany), the Axiovision V.4.8 software (Carl Zeiss AG, Oberkochen, Germany) and a total magnification of 100 (10 x from the microscope and 10 x from the lens) were used. There were no obvious differences in phenotype or confluency between both groups. At day 1, cells were about 90% confluent at the side of the wells and about 60% confluent in the middle of the wells. At day 3 and day 6, cells were 100% confluent in both groups. The cells exposed to 8µg/ml of "Polybrene" showed the normal elongated, fibroblastic phenotype of hMSCs. There were no detectable differences between the side and the middle of the wells. Images of representative samples can be found in the Appendix (section A 1). After 10 days, cells were still 100% confluent and there were no obvious differences between conditions. As a result, the experiment was stopped and samples were discarded.

3.2.2 Neither hMSC phenotype nor confluency is affected by retroviral transduction.

Next, the effect of retroviral transduction on hMSC phenotype and confluency was examined. Hence, Rv.eGFP transduced hMSCs, Rv.BMP-2 transduced hMSCs and mock-treated hMSCs were compared. The cells were regularly monitored and images were taken at day 3, 5, 7 and 8 after treatment. For image acquisition, an Axiovert 25 microscope (Carl Zeiss AG, Oberkochen, Germany), the Axiovision V.4.8 software (Carl Zeiss AG, Oberkochen, Germany) and a total magnification of 100 were used. After 8 days, cells were sub-confluent and harvested for gene expression analyses. In three out of four donors (♀ age 55, ♂ age 56 and ♂ age 54), no obvious differences in phenotype or confluency, between the different groups, was observed. Albeit, cells of one donor (♀ age 81) responded differently after retroviral transduction. Within this particular donor, the cellular phenotype was changed and they were less confluent, when compared to control cells. Representative images, from the three matching donors, can be found in the Appendix (section A 2).

3.2.3 BMP-2 production, in the two Rv.BMP-2 transduced hMSCs donors that were investigated, starts between day 4 and day 6 of culture.

Moreover, it was essential to confirm that Rv.BMP-2 transduced hMSCs produced detectable amounts of BMP-2 during monolayer proliferation. Therefore, 1ml of medium was collected at day 2, 4, 6 and 7 after Rv.BMP-2 transduction and the concentration of BMP-2 was quantified using the Duo Set ELISA Development System for human BMP-2. Cells from two different donors were used for this analyses (♂ age 56 [Pat 28] and ♂ age 54 [Pat 34]). Results are displayed in figure 3-2. Control cells, which were transduced with Rv.eGFP, did not produce detectable levels of BMP-2. Therefore, they were not included in figure 3-2.

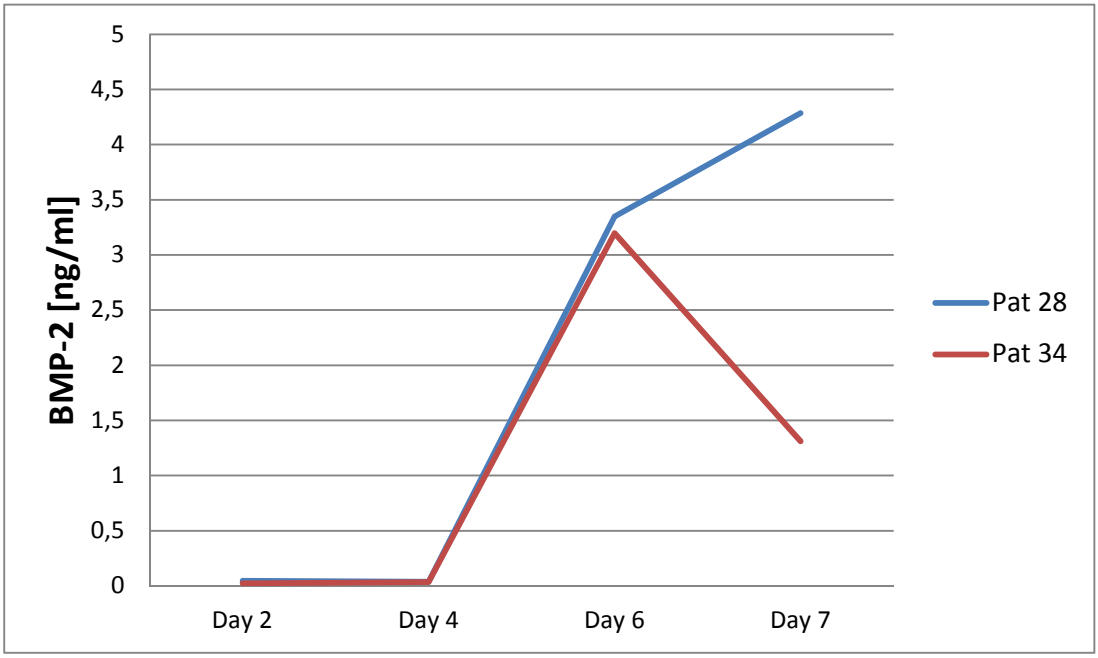

Figure 3-2: BMP-2 concentration within the culture medium of Rv.BMP-2 transduced hMSCs. hMSCs were transduced with Rv.BMP-2 and, subsequently, cultured in monolayer until they reached 70-80% confluency. The medium was changed once, after 3 days. One ml of medium was collected at day 2, 4, 6 and 7 after Rv.BMP-2 transduction. BMP-2 concentration within the culture medium was determined using a quantitative ELISA kit for human BMP-2. The experiment was conducted with cells from two donors (male age 54 [Pat 28] and male age 56 [Pat 34]). Results are derived from single samples per time-point, as only one tissue culture flask per donor was cultured.

During the first four days of culture, medium BMP-2 levels were below 0.05ng/ml for both donors (Rv.BMP-2 transduced). After day 4, the concentration of the transgene product started to rise. It peaked at 3.19ng/ml (Pat 34 on day 6) respectively 4.28ng/ml (Pat 28 on day 7).

3.2.4 The gene expression profile of Rv.BMP-2 transduced hMSCs is altered during monolayer proliferation.

Next, it was determined if Rv.BMP-2 transduction alters the gene expression profile of hMSCs during monolayer proliferation. Therefore, the gene expression of Rv.eGFP and Rv.BMP-2 transduced hMSCs was compared. Gene expression was monitored using RT-PCR and the comparative $\Delta\Delta C_t$ method. 18s rRNA was used as endogenous control. The gene expression of Rv.BMP-2 transduced cells was normalised to the gene expression of Rv.eGFP transduced cells. N-fold changes in gene expression, between groups, were calculated for the genes Col I (fibroblastic marker), Col X (hypertrophic marker), aggrecan (chondrogenic marker) and Runx2 (osteogenic marker) (figure 3-3). Col II message was not detected and, therefore, not included in figure 3-3.

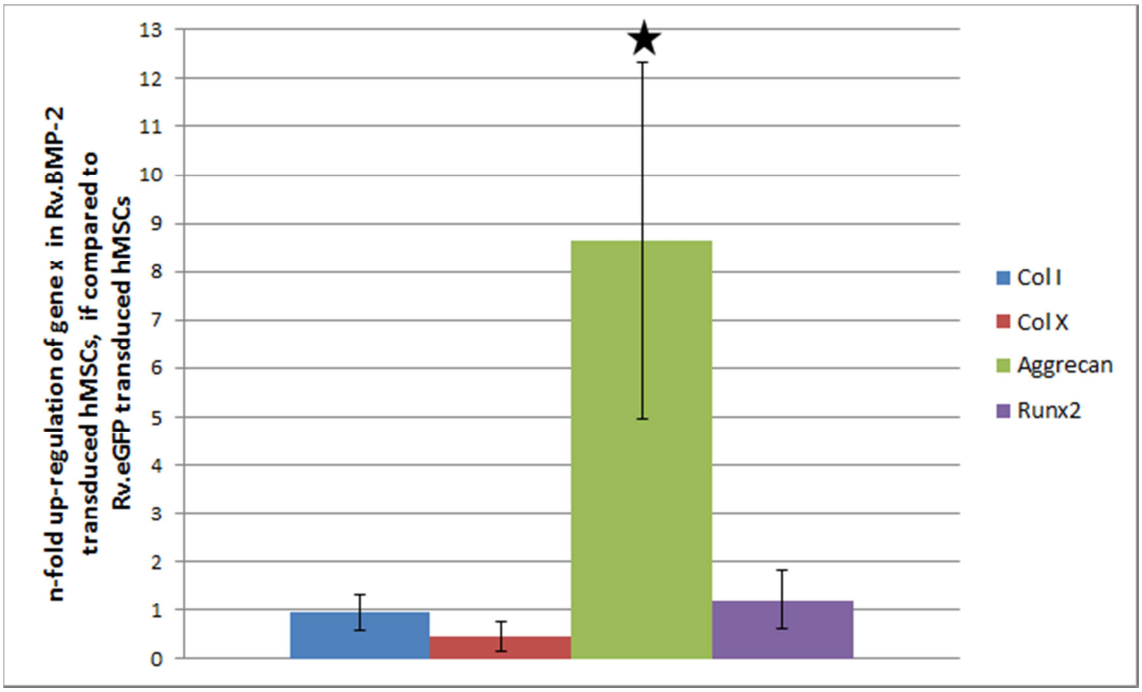

Figure 3-3: Relative gene expression of Rv.BMP-2 transduced hMSCs after monolayer proliferation. Gene expression analyses were conducted using the comparative $\Delta\Delta C_t$ method for genes Col I, Col X, aggrecan and Runx2. 18s rRNA was used as an internal control. Rv.eGFP transduced hMSCs were used as calibrator. Results, in n-fold changes in gene expression compared to Rv.eGFP transduced cells, are displayed as average ± standard deviation. The experiment was repeated with cells from four donors with n =3.★significantly different from Rv.eGFP transduced hMSCs ($p \leq 0.05$).

Col I expression and Runx2 expression were almost identical in both cell populations. The hypertrophic marker Col X displayed a trend towards a down-regulation in gene expression (45.98% ± 29.43%), when compared to Rv.eGFP transduced hMSCs. Yet, this trend did not reach significance. On the other hand, the chondrogenic marker gene aggrecan was the only gene investigated that was up-regulated in Rv.BMP-2 transduced cells. Its expression level was significantly elevated almost 9-fold (864% ± 365%, *p=0.004*), when compared to Rv.eGFP transduced cells.

3.2.5 Exposure to 100ng/ml of recombinant human BMP-2 alters the gene expression profile of hMSCs during monolayer proliferation.

Finally, the effect of exposure to 100ng/ml of exogenous recombinant human BMP-2, on the gene expression profile of hMSCs during monolayer proliferation, was examined. hMSCs were cultured for two weeks in MSC growth medium. Additionally, cells were either exposed to 100ng/ml of recombinant human BMP-2 or left as untreated controls. After 2 weeks, cells were harvested in TRI reagent and gene expression analyses were conducted using the comparative $\Delta\Delta C_t$ method. 18s rRNA was used as endogenous control. The gene expression of hMSCs, exposed to 100ng/ml of recombinant human BMP-2, was normalised to the gene expression of untreated control cells. N-fold changes in gene expression, between both cell populations, were calculated for the genes Col I (fibroblastic marker), Col X (hypertrophic marker), aggrecan (chondrogenic marker) and Runx2 (osteogenic marker) (figure 3-4). Col II message was not detected and, therefore, not included in figure 3-4.

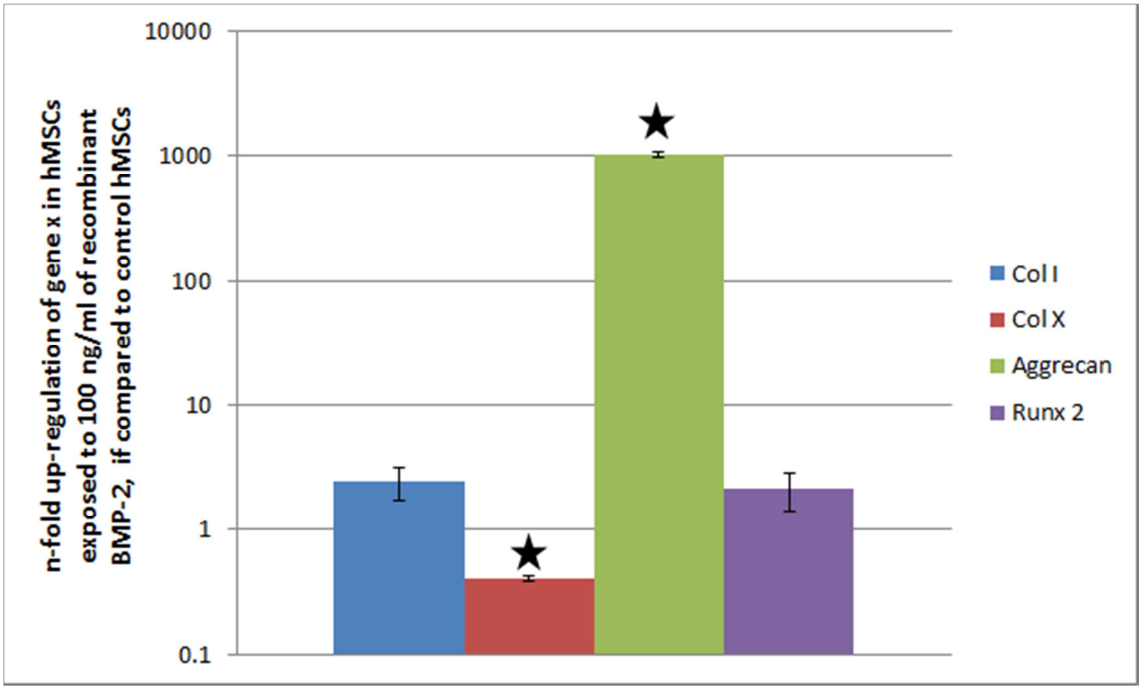

Figure 3-4: Relative gene expression of hMSCs which have been exposed to 100ng/ml of recombinant human BMP-2 for 14 days, during monolayer proliferation. Gene expression analyses were conducted using the comparative ΔΔ C_t method for genes Col I, Col X, aggrecan and Runx2. 18s rRNA was used as an internal control. Control cells cultured in hMSCs growth medium, without exogenous BMP-2, were used as calibrator. Results, in n-fold changes in gene expression compared to control cells, are displayed as average ± standard deviation. The experiment was conducted with cells from one donor with n=3.★significantly different from Rv.eGFP transduced hMSCs ($p \leq 0.05$).

For the genes Col I and Runx2, no significant differences between hMSCs, which have been exposed to 100ng/ml recombinant human BMP-2, and control cells were detected. The gene Col X was significantly down-regulated in these cells. Its expression level reached only 41% ± 2.5% of control cells (*p=0.001*). By contrast, aggrecan gene expression was markedly and significantly increased in hMSCs exposed to 100ng/ml recombinant human BMP-2, when compared to control cells. An up-regulation of over 1000-fold (1029 ± 45-fold, *p=0.001*) was detected.

3.3 Effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation – discussion.

First, it was confirmed that the harsh conditions during retroviral transduction (exposure to 8µg/ml of "Polybrene" and a small volume of culture medium) had no negative effect on hMSCs phenotype or confluency . Therefore, hMSCs were either exposed to 8µg/ml of "Polybrene" in 0.5ml of medium (for 2 hours under standard conditions) or left as controls. In the control group, 0.5ml of medium without additional "Polybrene" was used. Over the course of ten days, no obvious differences (confluency and phenotype) between both cell populations were detected. As expected, this result confirmed that the conditions during retroviral transduction were not harmful for hMSCs. "Polybrene" is commonly used to enhance transduction efficiency of retroviral and lentiviral vectors (Morgenstern and Land, 1990; Wubbenhorst et al., 2010).

It was further investigated if retroviral transduction negatively influenced hMSCs. Rv.eGFP and Rv.BMP-2 transduced cells were compared to mock-treated control cells. In three out of four donors, it was confirmed that transduction with a retroviral vector was not harmful for hMSCs. The cells did not show a change in phenotype or confluency, after transduction with either vector (when compared to mock-treated hMSCs). However, hMSCs from the fourth donor (♀ age 81) responded differently towards retroviral transduction. Within this particular donor, the cellular phenotype was changed and the cells were less confluent, if compared to mock-treated hMSCs. The reason for these conflicting observations cannot be definitively determined. There exist several possible explanations, the most likely being the age of this particular donor. When working with hMSCs, donor variation is a widely known phenomenon. Further, it has been demonstrated that hMSCs from old donors have a diminished proliferation rate and survival when compared to cells from younger donors (Veronesi et al., 2011). These features might make them more susceptible to retroviral transduction. Another possible explanation might be that the viral vector dose was different within this particular experiment. Both Rv.eGFP and Rv.BMP-2 are first generation retroviral vectors based on the plasmid pBullet. This vector lacks an antibiotic resistance gene. As a consequence, it is not possible to quantify the viral titre by means of the colony forming unit assay. Thus, it was not 100% possible to guarantee that comparable vector doses were used, in the different repeats of the experiment.

Further, the question if transduction with Rv.BMP-2 will change the gene expression profile of hMSCs, during monolayer proliferation, was addressed. hMSCs were transduced with Rv.eGFP or Rv.BMP-2. They were then sub-cultured and allowed to grow until sub-confluent (70-80% confluency). Once sub-confluent, gene expression analyses were conducted using the $\Delta\Delta C_t$ method with 18s rRNA as internal control. The gene expression of Rv.BMP-2 transduced cells was normalised to the gene expression of Rv.eGFP transduced cells. Thereby, any differences of the monitored genes (Col I, Col X, aggrecan and Runx2), were detected. Additionally, 1ml of culture medium was harvested at day 2, 4, 6 and 7 after transduction and analysed using a quantitative ELISA for human BMP-2. Thereby, it was confirmed that Rv.BMP-2 transduced hMSCs, from the two donors that were investigated, started to secrete detectable amounts of BMP-2 by day 4 of monolayer culture. Interestingly, the gene expression profile of both cell populations was different following monolayer proliferation. The expression level of the fibroblastic marker Col I and the osteoblastic marker Runx2 were comparable between both cell populations. The hypertrophic marker gene Col X showed a trend towards a down-regulation (by approximately 50%), yet this trend did not reach significance. On the other hand, the message of the chondrogenic marker gene aggrecan was significantly elevated around 9-fold.

Finally, it was examined how hMSCs respond towards exposure to 100ng/ml of recombinant human BMP-2. For this purpose, hMSCs were cultured for 14 days in monolayer. Control cells were grown in regular hMSCs growth medium. Treated cells were exposed to an additional 100ng/ml of exogenous recombinant human BMP-2. After 2 weeks, cells were harvested and gene expression analyses were conducted. The gene expression of BMP-2 treated cells was normalised to the gene expression of control cells. Interestingly, the genes Col I and Runx2 were not influenced by exposure to exogenous BMP-2, even at the higher dose. The effect on the chondrogenic marker gene aggrecan was massive, in this experimental setup. In hMSCs, exposed to 100ng/ml of recombinant BMP-2, aggrecan message was elevated over 1, 000-fold, when compared to control cells (significant). Interestingly, the decrease in hypertrophic marker expression (Col X) was almost identical between both experimental setups. Again, its message was approximately halved, when compared to control cells (significant).

The combined results of both experiments make it tempting to speculate that the aggrecan response is dose-dependent, while the Col X response appears to be threshold-dependent. This assumption is based on the observation that exposure to 25-times higher concentrations of BMP-2 (100ng/ml vs. 3-4ng/ml) resulted in a massive up-regulation of the gene aggrecan. Yet, the gene Col X responded similarly to both concentrations of the bioactive factor. Summarised, the PCR data showed that exposure to human BMP-2 influenced the hMSCs population during the initial monolayer proliferation step. This is not surprising as BMP-2 is a known bioactive factor, with several distinct biological functions. In research it is, among other things, used for maintaining the articular chondrocyte phenotype as well as for chondrogenic or osteogenic induction of stem/progenitor cells (Jorgensen et al., 2004; Majumdar et al., 2001; Meinel et al., 2006; Sailor et al., 1996; Schmitt et al., 2003). It, therefore, indicates that, when using integrating vectors and expanding the cells after transduction, controls need to be carefully planned to ensure that the results obtained during the 3D experiments are not due to artefact created in response to the different growth conditions (exposure to a bioactive factor), employed. Initially, one aim of the thesis was to investigate the effect of retroviral mediated over-expression of BMP-2 and mechanical stimulation on the chondrogenesis of monolayer-expanded hMSCs. Rv.eGFP transduced hMSCs were planned to serve as controls within this experimental setup. Yet, as the gene expression between Rv.eGFP and Rv.BMP-2 transduced hMSCs already differed after monolayer proliferation, it is not possible to ascribe differences in gene expression, after 3D culture, to the different experimental conditions applied. Further, it is difficult to analyse how the combination of over-expression of BMP-2 and mechanical stimulation influence the chondrogenesis of hMSCs during 3D culture, as these cells were already conditioned during the monolayer expansion phase.

In conclusion, the effect of Rv.BMP-2 transduction on monolayer-expanded hMSCs was monitored. Rv.eGFP transduced cells and mock-treated hMSCs served as controls. The harsh conditions during retroviral transduction (exposure to 8µg/ml of "Polybrene" and small amount of medium) had no negative effect on hMSCs phenotype or confluency. Also, in three out of four donors, no harmful effects, such as an altered phenotype or a decrease in confluency, were detected after transduction with either retroviral vector. Albeit, the investigated transgene product (BMP-2) itself had a pronounced effect on hMSCs. The gene expression profile of Rv.BMP-2 and Rv.eGFP transduced cells differed markedly. These observations were confirmed in an additional experiment, where the effect of exposure to recombinant BMP-2 on hMSC during monolayer proliferation was investigated. Again, the gene expression profile of hMSCs, exposed to exogenous recombinant BMP-2, was distinctly different, when compared to the gene expression profile of untreated hMSCs.

3.4 Enhanced adenoviral transduction in 3D - introduction.

The adenovirus (family: *adenoviridae)* is a non-enveloped DNA virus. Its genome contains of linear, double stranded DNA. The genome is surrounded by a protein capsid, containing a penton base and a fiber knob. Figure 3-5 outlines the life-cycle of a wild-type adenovirus.

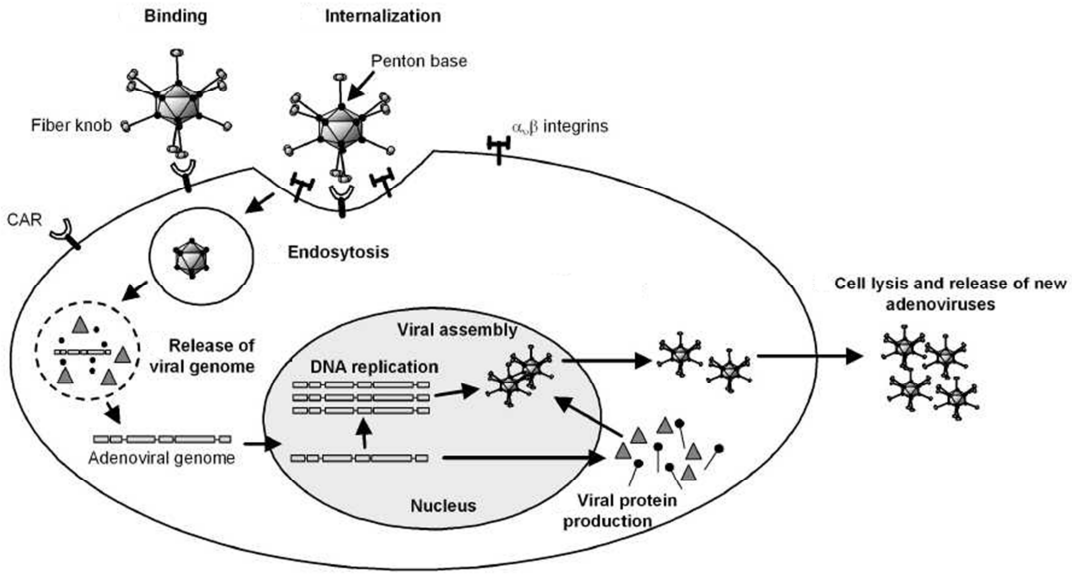

Figure 3-5: Life-cycle of a wild-type adenovirus. The life cycle starts with the binding of the virus to the host cell. Next, the adenovirus is internalised by endocytosis. This process is mediated by the coxsackie-adenovirus receptor (CAR) as well as member of the integrin family. The viral genome is released and enters the nucleus of the cell *via* the nuclear pores. The adenoviral genome does not integrate into the host genome but stays episomal. The viral DNA is replicated and viral proteins are produced within the cytoplasm. Finally, assembly of viral particles takes place and the new adenoviral particles are released *via* lysis of the host cell. Figure adapted from http://www.ssg-adenovirus.blogspot.ch/.

The life-cycle of an adenovirus starts with the initial binding of the virus to the host cell, followed by its internalisation *via* endocytosis. The process of internalisation is mediated by CAR and by binding of the penton base to members of the integrin family. Subsequently, the protein coat is destroyed and the adenoviral genome is released into the cytoplasm of the host cell. The adenoviral genome enters the nucleus of the host cell through the nuclear pores. As opposed to retroviruses, the genome of the adenovirus does not integrate into the host genome but stays episomal. The nuclear machinery of the host cell is used for viral DNA replication and viral mRNA is translated within the cytoplasm of the host cell. Viral proteins enter the nucleus of the host cell and new viral particles are assembled. They accumulate within the cytoplasm of the cell. Finally, the host cell is lysed and, thereby, the adenoviral particles leave the cell and are able to start over with the viral life-cycle.

Adenovirus can be used as a gene transfer vector in musculoskeletal regeneration research (Gelse et al., 2003; Hao et al., 2008; Meinel et al., 2006; Palmer et al., 2005; Steinert et al., 2009a; Steinert et al., 2009b). The maximum size of cDNA that can be inserted depends on the specific kind of adenoviral vector. It covers a range of 7.5 kb (first generation vectors), over 14 kb (second generation vectors) and up to 37 kb for gutless vectors (Danthinne and Imperiale, 2000). Adenovirus, as a gene transfer vector, possesses several advantages. Namely, these are the ability to infect both dividing and non-dividing cells, a high level of transgene expression, a high efficiency of transduction and its simple and relatively cheap production. Furthermore, adenoviral vectors, where the DNA does not integrate into the host genome, are considered to be safer in comparison to integrating viruses. Last, but not least, adenoviral vectors have already been approved for clinical trials (Peng, 2005; Penny and Hammond, 2004).

Gene transfer, using adenoviral vectors, can be used as an effective way to provide a local, albeit transient, production of bioactive factors. It might be particularly helpful in the treatment of musculoskeletal defects, where only a localised and short-term (up to weeks) expression of a desired therapeutic factor is required, in order to induce a repair response or to enhance the endogenous repair. Yet, the protocols which are currently available require further optimisation. Especially, if they are to be transferred into the clinic. One major improvement would be a more efficient transduction protocol. Hence, the amount of adenoviral vector needed for a given application could be reduced. This is desirable, as the adenovirus itself and transduced cells can be immunogenic. Furthermore, adenovirus is cytotoxic when used at high doses (Brunetti-Pierri et al., 2004). Thus, an improved adenoviral transduction protocol might help to overcome the safety concerns which are present, when applying adenoviral vectors, especially for the treatment of non-lethal diseases.

Regarding a clinical application, the development of a transduction protocol that can be conducted directly within the operating theatre is highly desirable. Consequently, this would eliminate the need for a cell culture step. Routinely, adenoviral transduction takes place on cell culture plastic (2D environment) (Gelse et al., 2001; Hao et al., 2008; Meinel et al., 2006; Palmer et al., 2005; Steinert et al., 2009a; Steinert et al., 2009b; Zachos et al., 2007).

In TE and regenerative medicine, hydrogels are commonly used to encapsulate and culture different cell types, such as chondrocytes and MSCs (Ho et al., 2010; Huang et al., 2004; Li et al., 2009a; Steinert et al., 2003; Weisser et al., 2001). A possible approach might be to apply the adenovirus at the same time the cells are encapsulated within the 3D hydrogel. It is hypothesised that this procedure will not only enhance gene transfer efficiency but also reduce the amount of viral vector necessary to generate the desired amount of transgene product. Furthermore, it makes the procedure quicker and easier to apply.

The present study aimed to directly compare the efficiency of standard 2D versus 3D adenoviral-mediated transduction of hMSCs. For both approaches, different commonly used hydrogels, namely alginate and agarose, were compared. Furthermore, a clinically relevant composite scaffold, consisting of cells embedded in a fibrin hydrogel and seeded into a biodegradable PU scaffold, was investigated. Ad.BMP-2 was used at a MOI of 5 or 100. The transgene expression of this vector is driven by a CMV promoter. The efficiency of the different transduction protocols was compared by measuring the concentration of transgene product within the culture medium and calculating the amount of transgene product produced per cell. Furthermore, the amount of transgene product (BMP-2) that was retained within the hydrogels was quantified. Finally, the DNA content of the different groups was determined. This study was published in Molecular Biotechnology (Neumann et al., 2013c).

3.5 Enhanced adenoviral transduction in 3D - results.

For this study, five groups were tested. Namely, these were untransduced control, 2D 5 MOI, 2D 100 MOI, 3D 5 MOI and 3D 100 MOI. Three samples were prepared per hydrogel and group. The experiment was independently repeated with cells from 4 different donors (♀ age 81, ♀ age 55, ♂ age 71, ♀ age 67).

3.5.1 Transgene product production is enhanced when applying the 3D transduction protocol.

First, the efficiency of 3D transduction versus the efficiency of routine transduction in 2D was investigated. Therefore, hMSCs were transduced in either 2D or 3D with a MOI of 5 or 100. Control cells were left untransduced. Afterwards, the cells were encapsulated in alginate, agarose or fibrin-PU composite scaffolds. Subsequently, the samples were cultured for 28 days in defined chondrogenic medium. The medium was changed three times a week, pooled and collected for five time-points (day 1, day 2-7, day 8-14, day 15-21 and day 22-28). The amount of transgene product within the culture medium was quantified using a quantitative ELISA for human BMP-2. For evaluation, the BMP-2 content within the medium was calculated by multiplying the transgene product concentration per ml with the total medium volume for each group. Next, the total BMP-2 content was normalised to the cell number within each group (2.5×10^6 for fibrin, 1.05×10^6 for alginate and 0.7×10^6 for agarose). Thereby, the skewing effect of different cell numbers between groups was eliminated, allowing for a better comparison between groups. Results for all hydrogels are displayed in figures 3-6, 3-7 and 3-8. Statistical significances are included within the text and omitted from the figures in order to increase their clarity.

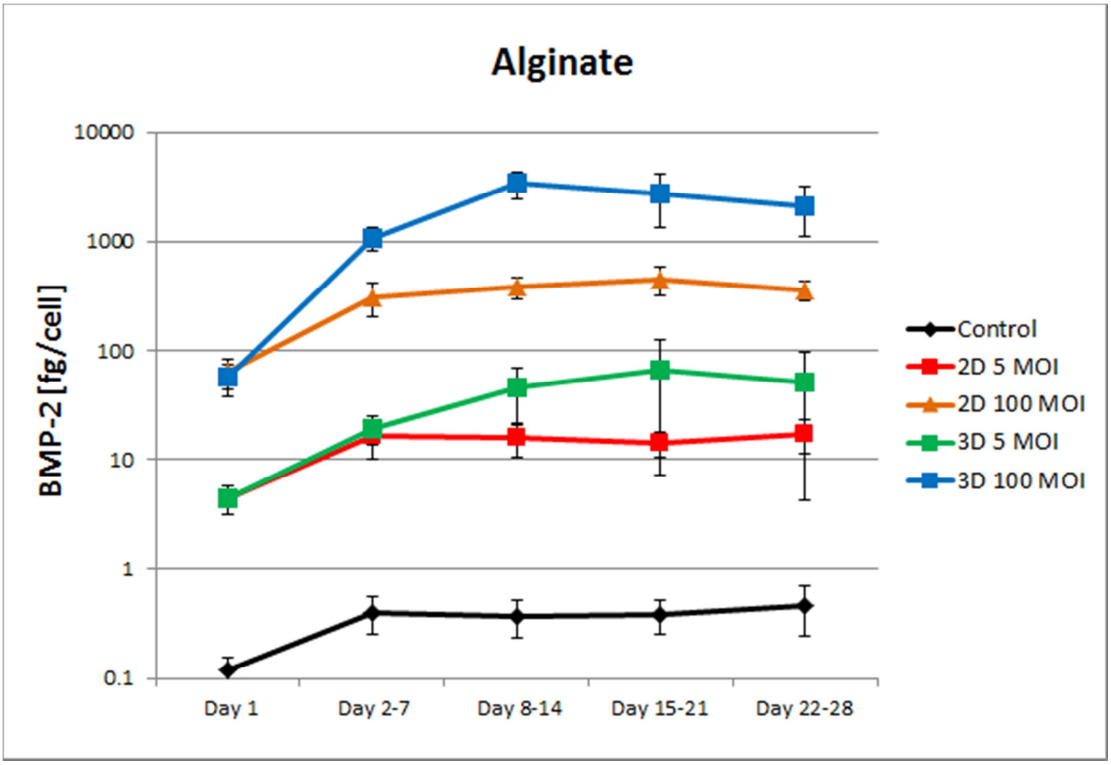

Figure 3-6: Quantitative comparison of the amount of BMP-2 produced in hMSCs, after applying 2D or 3D Ad.BMP-2 transduction, in alginate. The BMP-2 production was monitored for 28 days and quantified from cell culture medium using a BMP-2 ELISA kit. Results are shown as average +/- standard deviation from 4 donors with n=3. Starting from day 2-7, the 3D transduction protocol showed an increased efficiency, when compared to the standard 2D transduction protocol. This result is true for transduction with both the lower (5 MOI) and the higher (100 MOI) amount of adenoviral vector. Statistical significant differences between groups are described within the text.

In alginate, the transgene product production was lowest among all hydrogels investigated. Its expression peaked between day 8-14 (3D 100 MOI), day 15-21 (2D 100 MOI, 3D 5 MOI) or day 22-28 (2D 5 MOI) and started to decline thereafter. Transduction in 3D, using a MOI of 5, led to a trend towards an increased peak BMP-2 expression (17.3 ± 6.1 v 67 ± 60fg per cell), when compared to routine transduction in 2D. This increased efficiency of 3D vs. 2D transduction was even higher at 100 MOI. Here, a 7.4-fold increase in peak expression was monitored (456 ± 139 v 3380 ± 947fg per cell, *p=0.000*). Each of the groups were significantly different to each other, except for 2D 5 MOI and 3D 5 MOI where the trend towards increased efficiency did not reach statistical significance. However, when applying the higher vector dose (100 MOI), hMSC generated significantly more transgene product after transduction in 3D, when compared to transduction in 2D.

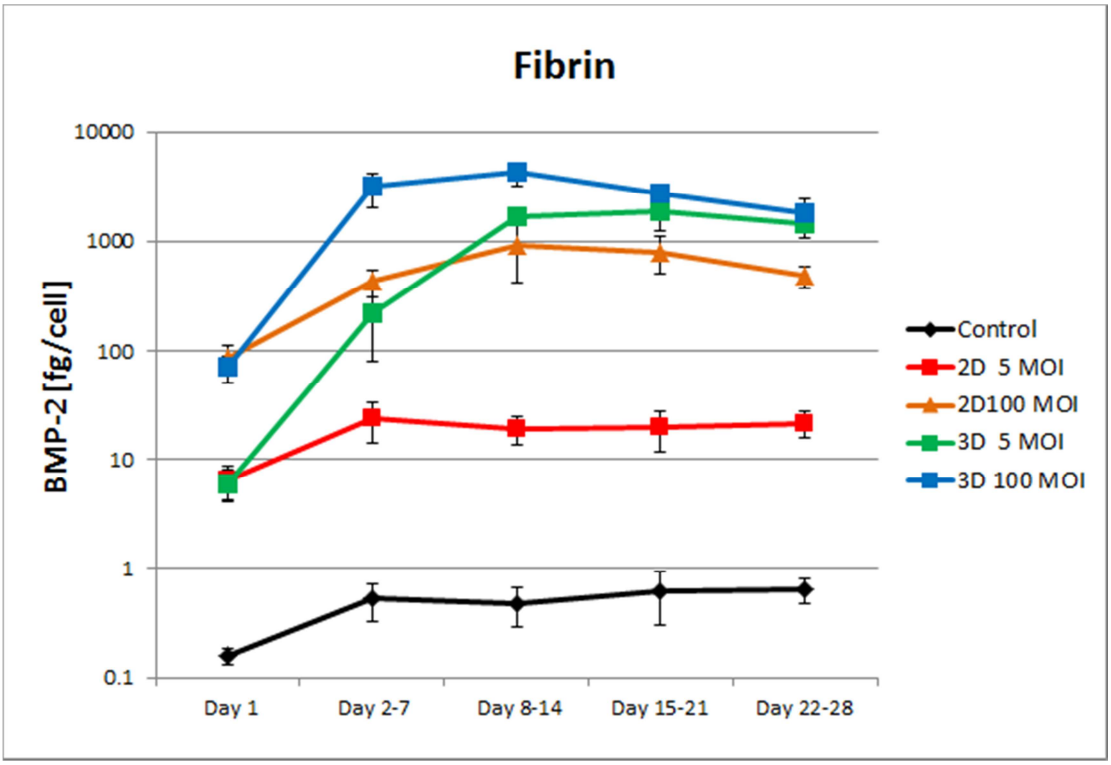

Figure 3-7: Quantitative comparison of the amount of BMP-2 produced in hMSCs, after applying 2D or 3D Ad.BMP-2 transduction, in fibrin. The BMP-2 production was monitored for 28 days and quantified from cell culture medium using a BMP-2 ELISA kit. Results are shown as average +/- standard deviation from 4 donors with n=3. Starting from day 2-7, the 3D transduction protocol showed an increased efficiency, when compared to the standard 2D transduction protocol. This result is true for transduction with both the lower (5 MOI) and the higher (100 MOI) amount of adenoviral vector. Statistical significant differences between groups are described within the text.

In the fibrin-PU composite scaffold, BMP-2 production was higher than in alginate but lower than in agarose. The transgene expression started to decline after day 2-7 (2D 5 MOI), day 8-14 (2D 100 MOI, 3D 100 MOI) or day 15-21 (3D 5 MOI). Within the fibrin-PU composite scaffolds, the 3D transduction protocol led to an increased transgene product production, when compared to routine transduction in 2D. This was true for both vector doses tested. 3D transduction at 5 MOI led to a 77.66-fold increase in peak BMP-2 expression (24 ± 9.8 v 1865 ± 640fg per cell, $p=0.048$). At 100 MOI, the increase was only 4.52-fold (933 ± 523 v 4218 ± 1030fg per cell, $p=0.016$). For both vector doses investigated, the enhanced transduction efficiency in 3D reached statistical significance ($p \leq 0.048$). Interestingly, the control and the 2D 5 MOI group were significantly different from the other groups ($p \leq 0.048$). Further, 3D 5 MOI was not significantly different from either the 2D 100 MOI or the 3D 100 MOI group.

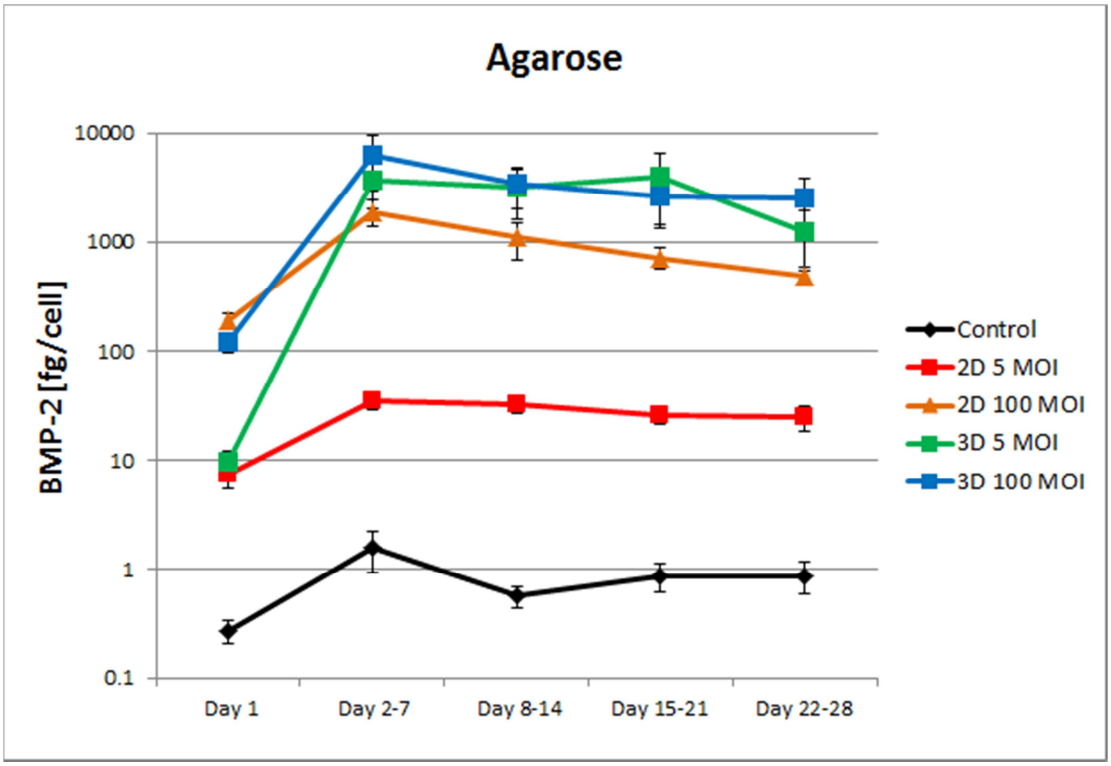

Figure 3-8: Quantitative comparison of the amount of BMP-2 produced in hMSCs, after applying 2D or 3D Ad.BMP-2 transduction, in agarose. The BMP-2 production was monitored for 28 days and quantified from cell culture medium using a BMP-2 ELISA kit. Results are shown as average +/- standard deviation, from 4 donors with n=3. Starting from day 2-7, the 3D transduction protocol showed an increased efficiency, when compared to the standard 2D transduction protocol. This result is true for transduction with both the lower (5 MOI) and the higher (100 MOI) amount of adenoviral vector. Statistical significant differences between groups are described within the text.

In agarose, the transgene product production per cell was highest between all hydrogels investigated. Additionally, the BMP-2 production peaked sooner. All groups reached their peak production at day 2-7 except for the 3D 5 MOI group. This group peaked later on day 15-21. Again, in agarose, transduction in 3D was superior, when compared to standard 2D transduction. At 5 MOI, it led to a 113-fold increase in BMP-2 expression (35 ± 5.8 v 3944 ± 2600fg per cell, *p=0.048*). Contrary to alginate, transduction with 100 MOI only led to a 3.28-fold increase (1898 ± 508 v 6231 ±3335fg per cell, *p=0.016*) in BMP-2 production. Yet, absolute expression was two to four times higher in agarose, when compared to alginate. Both the control group and the 2D 5 MOI group were significantly different from the other groups ($p \leq 0.048$). While 3D 100 MOI was significantly higher than 2D 100 MOI ($p = 0.016$), there was no significant difference between 3D 5 MOI and 2D 100 MOI or 3D 5 MOI and 3D 100 MOI.

3.5.2 Transgene product production reaches concentrations higher than 100ng/ml.

Next, it was investigated when transgene product concentration, within the culture medium, generally peaked between the different groups. Therefore, the different time-points were compared between groups and the BMP-2 concentrations, in ng/ml within the culture medium, were used for evaluation. It was of particular interest if the transgene product production crossed a certain threshold level. This level was chosen to be 100ng/ml as it is a concentration commonly used when applying recombinant human BMP-2 exogenously.

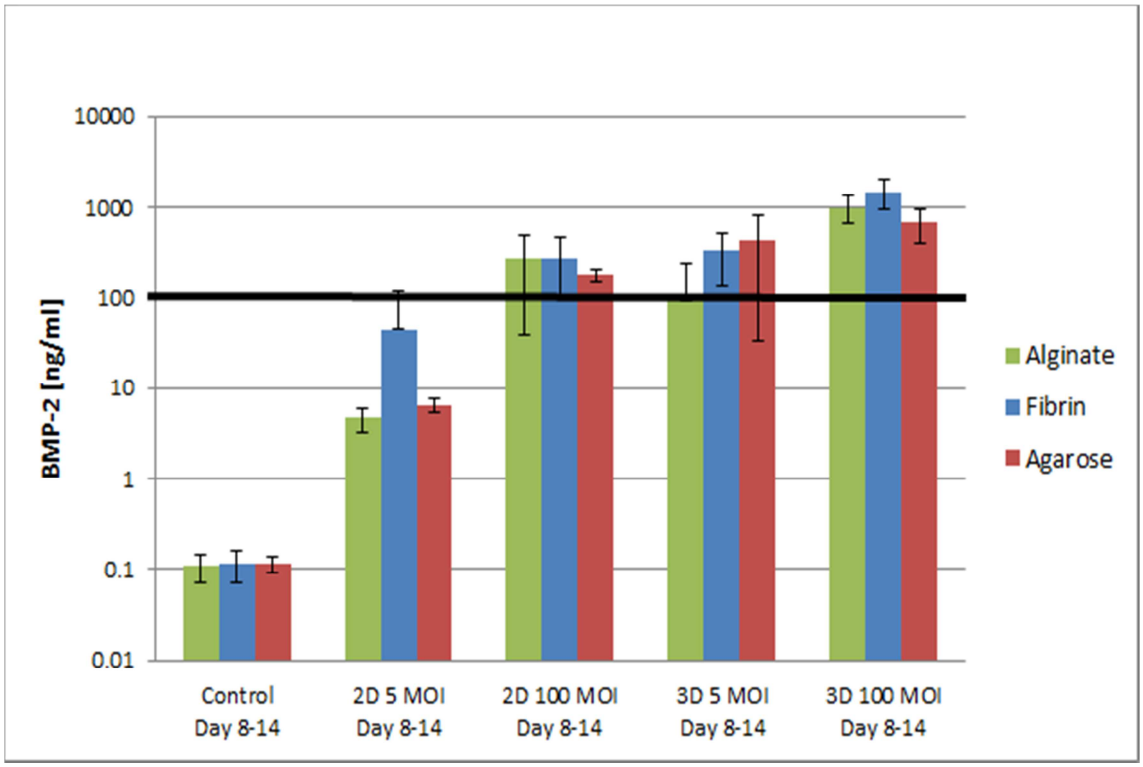

Figure 3-9: Quantitative comparison of the amount of BMP-2 produced in hMSCs, after applying 2D or 3D Ad.BMP-2 transduction in alginate, fibrin and agarose. The transgene product production was monitored between days 8-14 and quantified from cell culture medium using a BMP-2 ELISA kit. Results are shown as average +/- standard deviation from 4 donors with n=3. The BMP-2 concentrations were higher than 100ng/ml in all 3D 5 MOI groups (except for alginate) and in all 100 MOI groups. The black line indicates a concentration of 100ng/ml. This concentration is commonly used when BMP-2 is applied exogenously.

Generally, most groups reached a peak in transgene product production by day 8-14 (figure 3-9). Yet, the concentration of BMP-2, within the culture medium, varied depending on the group investigated. Starting from day 2-7 (data not shown), and even more pronounced from day 8-14 (fibrin and agarose), 3D 5 MOI (except in alginate) and both of the 100 MOI groups reached a BMP-2 concentration higher than 100ng/ml, a concentration commonly added exogenously in studies involving the BMP-2 protein.

3.5.3 After 28 days of culture, only a small proportion of the transgene product is retained within the hydrogels.

Furthermore, the amount of BMP-2 retained within each hydrogel, after 4 weeks of culture, was quantified. This was done in order to ensure that any differences seen in media BMP-2 concentration were not a result of varying protein retention (figure 3-10). The hydrogels were homogenised and the amount of BMP-2, retained within each hydrogel, was quantified using a quantitative ELISA for human BMP-2. Subsequently, the total transgene product production (medium + amount retained within the hydrogels) was calculated. Finally, the percentage of BMP-2 retained (in comparison to the total amount produced) within each hydrogel was determined.

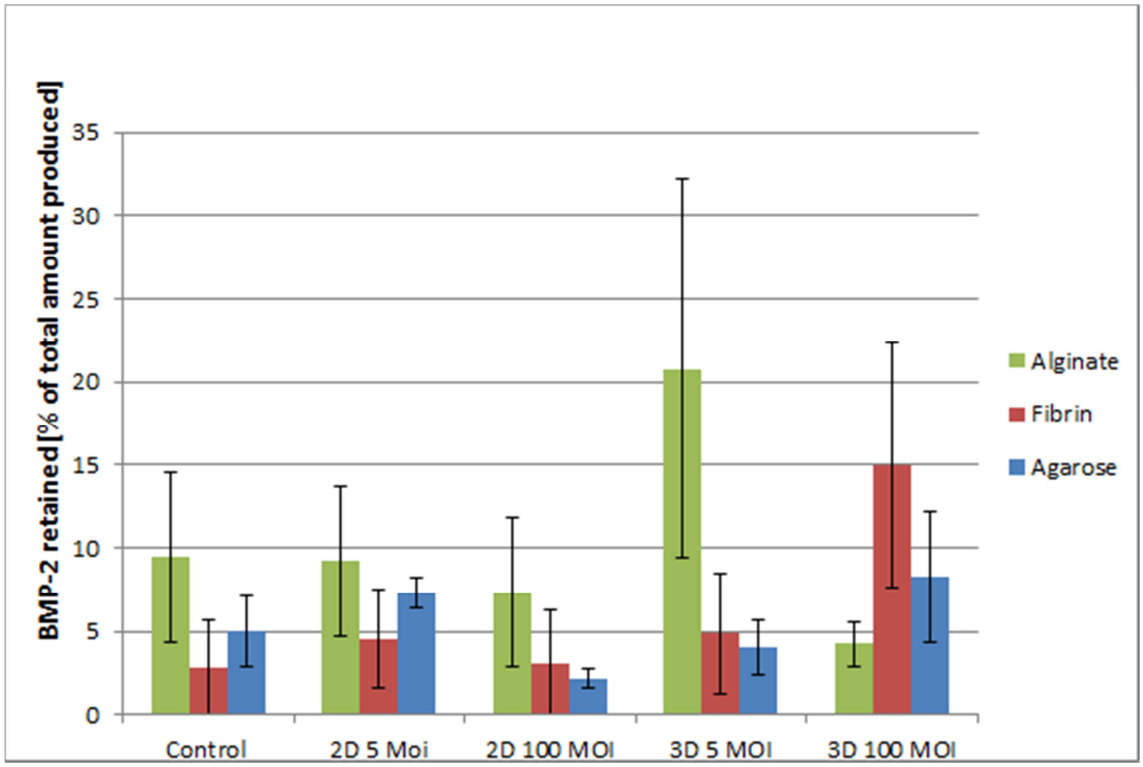

Figure 3-10: The amount of BMP-2, retained within each hydrogel, after 28 days was quantified. Therefore, hydrogels were homogenised and the amount of BMP-2 retained in each hydrogel was quantified using a quantitative ELISA for human BMP-2. Subsequently, this amount was used to calculate the percentage of transgene product retained in comparison to the total amount (medium + hydrogel) of transgene product produced. Results are displayed as average +/- standard deviation from 4 donors with n=1. For most groups, less than 10% of the total BMP-2 produced was present within the hydrogels. There were two exceptions; 3D transduction in fibrin with a MOI of 100 (14.9% ± 7.4%) and 3D transduction in alginate with a MOI of 5 (20.8% ± 11.4%).

In most cases, the amount of BMP-2 retained was under 10% of the total amount of BMP-2 produced. There were two exceptions. The 3D 100 MOI group in fibrin (14.9% ±7.4%) and the 3D 5 MOI group in alginate (20.8% ± 11.4%). When each of the groups was monitored individually, the amount of transgene product retained lay in a range of 2.2% ± 0.6% to 8.2% ± 3.9% in agarose, 2.8% ± 2.8% to 14.9% ± 7.4% in fibrin and 4.2% ± 1.3% to 20.8% ± 11.4% in alginate.

3.5.4 In fibrin and agarose, Ad.BMP-2 transduction in 3D with a MOI of 5 is more efficient than Ad.BMP-2 transduction in 2D with a MOI of 100.

To further verify the increased efficiency of the modified 3D transduction protocol, it was investigated how efficient 3D transduction with a MOI of 5 is, when compared to 2D transduction with a MOI of 100. Therefore, for every time-point investigated, the BMP-2 medium concentration obtained using the 3D transduction protocol with a MOI of 5 was divided by the BMP-2 medium concentration obtained using the 2D transduction protocol with a MOI of 100. Consequently, the results of this calculation were multiplied by one hundred in order to obtain the efficiency, in per cent, of 3D transduction with a MOI of 5, when compared to 2D transduction with a MOI of 100. Results are displayed in figure 3-11.

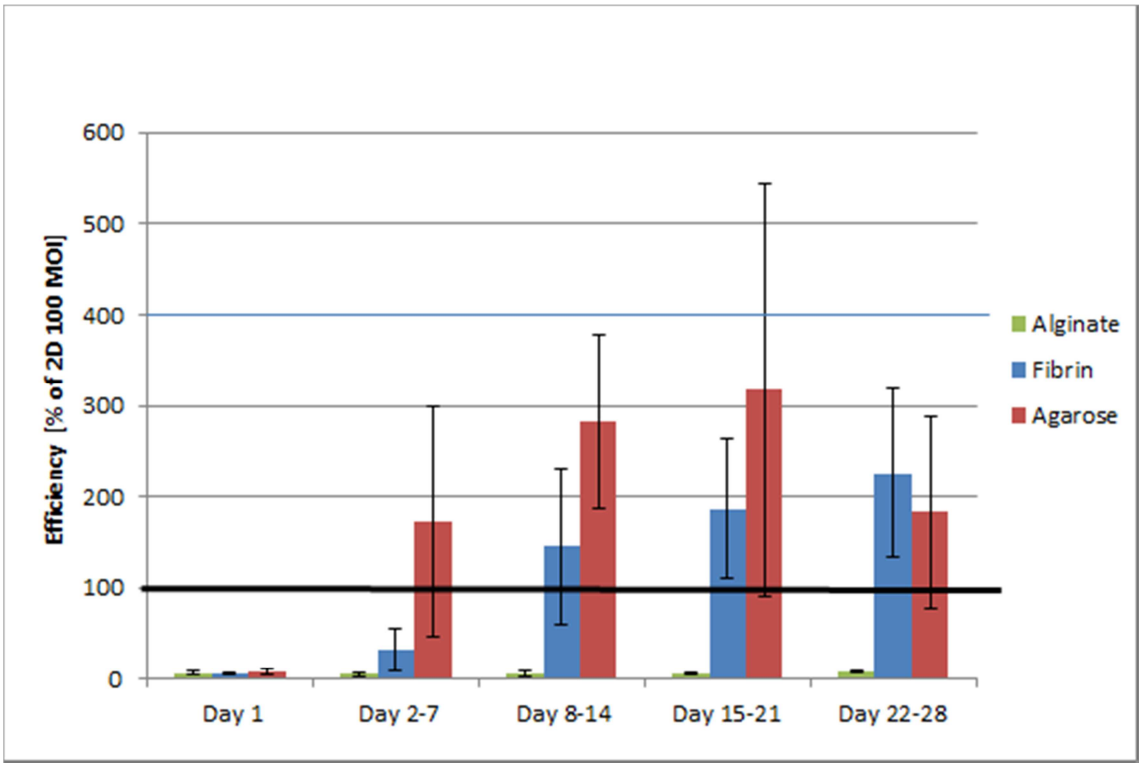

Figure 3-11: The efficiency of adenoviral transduction in 3D using a MOI of 5 was compared to adenoviral transduction in 2D using a MOI of 100. For each time-point, the medium BMP-2 concentration of the 3D 5 MOI group was divided by the corresponding BMP-2 medium concentration of the 2D 100 MOI group. Subsequently, these results were multiplied with one hundred in order to obtain the efficiency, in percent, compared to transduction in 2D with a MOI of 100. Results are displayed as average +/- standard deviation from 4 donors with n=3. In alginate, 3D transduction with only 5% of the viral vector failed to generate similar amounts of transgene product, when compared to the 2D 100 MOI group. For the other two hydrogels investigated, starting from week 1 (agarose) or week 2 (fibrin), efficiency of 3D transduction with a MOI of 5 was comparable or even superior, when compared to transduction in 2D using a MOI of 100. The black line indicates 100% efficiency, when compared to 2D transduction with a MOI of 100.

In the alginate hydrogel, it was not possible to generate comparable amounts of transgene product, when transduction was conducted in 3D with only 5% of the viral vector dose. The efficiency of 3D transduction with a MOI of 5 was lower, when compared to routine transduction in 2D with a MOI of 100. It reached only between 5.4% ± 2.2% and 7.9% ± 0.8% of 2D 100 MOI. For agarose and fibrin, starting from week 1 (agarose) or week 2 (fibrin), 3D transduction with a MOI of 5 was more efficient, when compared to 2D transduction using 100 MOI. The efficiency was more than 2-fold elevated (226% ± 92%) in fibrin and more than 3-fold (318% ± 227%) elevated in agarose.

3.5.5 DNA content does not change significantly after 2D or 3D transduction with Ad.BMP-2.

In order to determine whether transduction with Ad.BMP-2 influences cell viability or cell growth, DNA content was quantified after 28 days using the Hoechst 33258 dye binding assay (figure 3-12). The values obtained after 2D or 3D transduction with Ad.BMP-2, for both vector doses investigated, were compared to untransduced controls. Furthermore, the DNA content on day 0 was quantified within the control group and compared to the control group after 28 days of culture. This was done in order to investigate if pronounced changes in total cell number occured during the 4 weeks of culture.

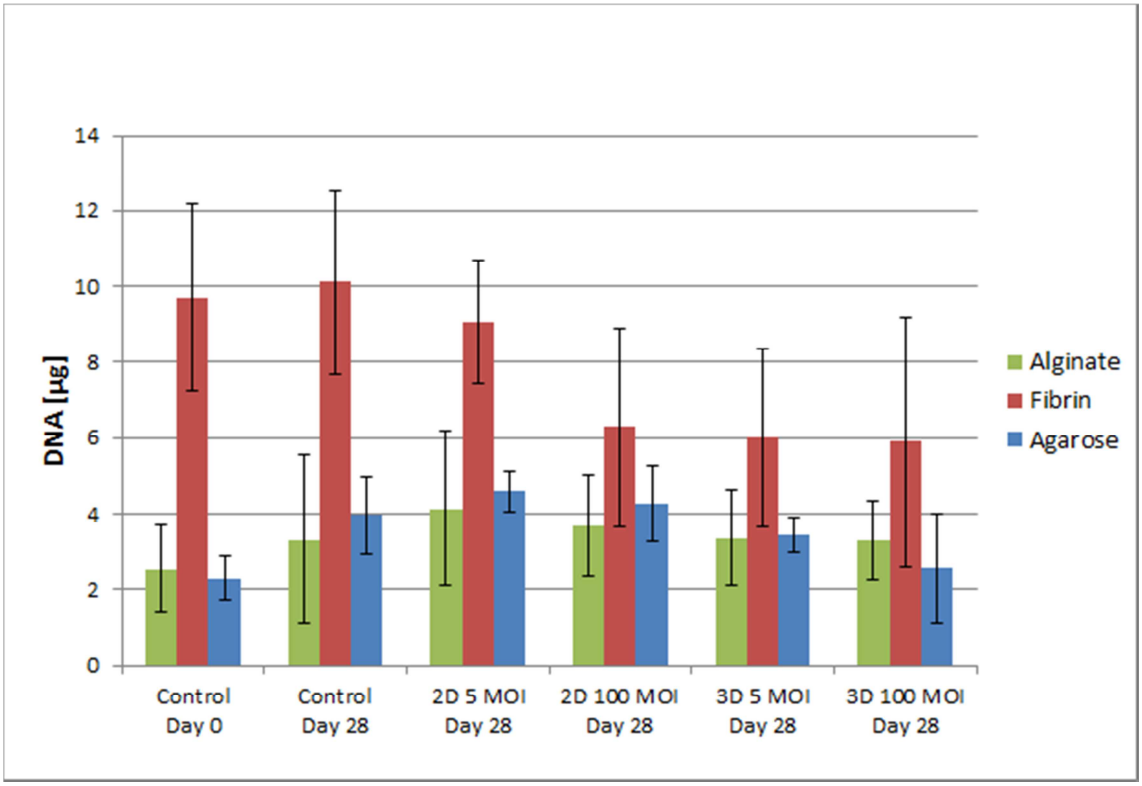

Figure 3-12: The DNA content after 28 days within each hydrogel. The Hoechst 33258 dye binding assay was used to quantify the total DNA content. The Ad.BMP-2 transduced groups were compared to untransduced controls. Furthermore, the untransduced control group on day 28 was compared to the untransduced control group on the day of seeding (day 0). Results are displayed as average +/- standard deviation from 3 donors with n=2. There were no significant differences in DNA content within the control between day 0 and day 28. In the fibrin hydrogel, a trend towards a decline in DNA content, after adenoviral transduction, was observed. Yet, this trend did not reach statistical significance. The DNA content in alginate and agarose was rather equal and statistically not different between groups.

Between day 0 (day of seeding) and day 28, no statistical significant differences in DNA content were observed within the control group for each hydrogel. The reason for the initial differences in DNA content, between the different hydrogels, is that in fibrin more cells (2.5 x 10^6) were seeded, when compared to alginate (approximately 1,05 x 10^6) and agarose (approximately 0.7 x 10^6). In the fibrin hydrogel, a trend towards a decline in DNA content after adenoviral transduction was detected. This trend was more pronounced, when cells were transduced with a MOI of 100. Yet, this trend was statistically not significant. After 28 days, the DNA content within the fibrin group was 10.11µg ± 2.43µg (control), 9.04µg ± 1.62µg (2D5 MOI), 6.29µg ± 2.61µg (2D 100 MOI), 6µg ± 2.37µg (3D 5 MOI) and 5.9µg ± 3.29µg (3D 100 MOI).

In the alginate and agarose hydrogels, the DNA content was rather similar between groups and no significant differences were detected. Absolute values were 3.3µg ± 2.23µg (control), 4.1µg ± 2.03µg (2D 5 MOI), 3.69µg± 1.34µg (2D 100 MOI), 3.35µg ± 1.26µg (3D 5 MOI) and 3.29µg ± 1.04µg (3D 100 MOI) for alginate and 3.95 µg ± 1.03µg (control), 4.58µg ± 0.55µg (2D 5 MOI), 4.26µg ± 1µg (2D 100 MOI), 3.43µg ± 0.46µg (3D 5 MOI) and 2.56µg ± 1.45µg (3D 100 MOI)for agarose.

3.6 Enhanced adenoviral transduction in 3D - discussion.

The combination of a hydrogel and a gene transfer agent offers an attractive approach for TE applications. Their combination in a way that allows for a single rapid procedure would provide a further advantage. Viral vectors remain the most effective tools for gene transfer as non-viral vectors still fail to show comparable efficiency. Yet, their application in gene therapy is still impeded. Two of the main hurdles remaining are safety issues and specificity of transgene delivery (Thomas et al., 2003). Adenoviral vectors possess the highest efficiency but also the highest immunogenicity of all viral vectors. Nevertheless, it has been suggested that MSCs are immunosuppressive, which may counteract any negative effect of the adenovirus (Beyth et al., 2005; Maitra et al., 2004). Possible approaches to tackle adenoviral immunogenicity could be, for instance, the development of less-immunogenic vectors, the development of alternate viral serotypes, which are able to circumvent the existing immune responses or the development of a more efficient transduction protocol.

This study focussed on the last option. 2D versus 3D transduction with adenoviral vectors was conducted, in order to assess which one is more efficient. Using hMSCs in monolayer (2D), or during encapsulation within three different hydrogels (3D), different vector doses were tested. The cells which were transduced in 2D were then later encapsulated. In the 3D system, all components were mixed together at the same time. Afterwards, all groups were cultured for 28 days in defined chondrogenic medium. Subsequently, an analysis of transgene concentration within both the culture medium and the hydrogels was conducted.

It was demonstrated that the application of the 3D transduction protocol led to a markedly enhanced production of the transgene product. A significantly enhanced expression was reproducibly seen in all three hydrogels. This would suggest the response is independent of cell density or gel. Interestingly, for agarose and fibrin-PU composites, transduction in 3D with the lower vector dose was comparable or more efficient than transduction in 2D with the higher vector dose, whereas this was not the case for alginate. However, even in alginate, 3D transduction led to an enhanced expression, when compared to 2D transduction. These findings suggest that the efficiency of adenoviral transduction is enhanced, when conducted in a 3D environment. There are several possible explanations for these observations.

First of all, in the 3D environment, adenoviral transduction takes place in a smaller volume, leading to a higher local concentration of the vector. This might increase transduction efficiency. Also, fibrin scaffolds have already been demonstrated to be efficient delivery systems for viral vectors (Schek et al., 2004; Teraishi et al., 2003). Furthermore, in 2008 and 2009, Breen et al., were able to demonstrate that fibrin is able to retain adenoviral vectors at the wound site, leading to an enhanced exposure of infiltrating cells to the viral vector (Breen et al., 2009a; Breen et al., 2008). In the 3D system, this feature will also lead to enhanced adenoviral transduction efficiency. The findings in the current study indicate that alginate and agarose also might have the potential to retain the adenoviral vector and, therefore, enhance adenoviral transduction efficiency.

It is generally accepted that adenovirus type 5 requires the presence of CAR as well as αV integrins for entry into the cell. However, it is known that hMSCs do not express CAR but do express αV integrin (Olmsted-Davis et al., 2002). Even when lacking CAR, a transduction efficiency of 83% was obtained, when using type 5 adenovirus, albeit with low levels of expression (Olmsted-Davis et al., 2002). The 3D transduction method described here led to an increased expression using type 5 adenovirus. It is possible to re-target vectors by substituting the fiber protein of Ad5 with one from an adenovirus which infects cells in a CAR-independent fashion, such as type 35 (Shayakhmetov et al., 2000). This would, in itself, lead to a more efficient transduction and, hence, further minimise the dose of vector. As the 3D transduction method is likely to function by maintaining a longer physical presence between the virus and the cell, this will provide an advantage independent of the receptor used for cellular access.

The concentration obtained within the medium with 3D 5 MOI and all the 100 MOI groups was greater than 100ng/ml, a concentration commonly added exogenously in studies involving the BMP-2 protein (Majumdar et al., 2001; Sailor et al., 1996; Toh et al., 2007). This would suggest that, within certain hydrogel carriers, a viral dose lower than 5 MOI could be used to obtain clinically relevant expression levels. This finding also had direct implications for further work within the thesis. This feature allowed using a MOI of 5, instead of the planned MOI of 100, for the Ad.BMP-2 and load study (chapter 4) and the hACPCs and load study (chapter 7).

The results further demonstrated that the release of the transgene product from the hydrogels was very effective. After 28 days, the amount of transgene released was generally high (over 90% for almost all groups). This property is particularly important if transduced cells should produce a paracrine bioactive factor.

The cytotoxic effect of adenoviral vectors is dose-dependent and only occurs once a certain threshold level is crossed (Morral et al., 2002; Thomas et al., 2001). As expected, the DNA data indicated that adenoviral transduction of hMSCs, using a MOI of 5 or 100, did not reach this threshold level and, therefore, did not lead to a significant decline in total cell number.

In addition to its increased efficiency, the proposed 3D transduction protocol for adenoviral vectors has further benefits. It is less labour-intensive, when compared to transduction in 2D. Further, a whole monolayer passaging step can be conserved. This feature is beneficial, while working with hMSCs. Increased passage number of MSCs has been associated with reduced potential for stem cell activation and differentiation (Crisostomo et al., 2006; Kretlow et al., 2008). Further, as 3D transduction takes place during cell encapsulation, it is no longer necessary to perform the transduction in advance or within a cell culture laboratory. This feature makes it, in theory, possible to perform transduction in 3D directly within the operating theatre. A time- and money-consuming cell culture step is no longer obligatory. This represents a desirable feature for a possible application within clinical practice but would require the transduction of freshly isolated cells, as opposed to monolayer-expanded cells. The feasibility of this approach has already been demonstrated by transducing rabbit whole marrow clots intra-operatively with adenoviruses encoding for certain marker genes. The cells were successfully transduced and the transgene could be seen within an osteochondral defect (Pascher et al., 2004).

In conclusion, the present study aimed to directly compare 2D and 3D adenoviral transduction protocols in several hydrogels relevant for TE and regenerative medicine. The 3D protocol was shown to be more efficient for the adenoviral-mediated transduction of hMSCs, in every experimental condition investigated. This was indicated by higher amounts of transgene product produced per cell. Within agarose or fibrin-PU composite scaffolds, cells that were transduced in 3D were able to generate comparable or even higher amounts of transgene, with only 5% of the vector dose used for standard 2D transduction. These results suggest that it might be possible to further decrease the amount of viral vector needed, in order to generate clinically significant amounts of transgene product. In summary, the proposed 3D transduction protocol, for adenoviral vectors, has many benefits and might help to increase its administration in TE and clinical applications. Further, this procedure does no longer rely on a cell culture step and, in theory, can be conducted within the operating theatre.

3.7 Timed Ad.BMP-2 transduction – introduction.

The effect of many bioactive factors is dependent on a huge number of variables. Some of these variables can be, for instance, the type of cell that responds to the specific factor, the concentration of the specific factor, concentration gradients of the factor, interplay with other factors and, further, timing of its application. In an *in vitro* experiment, the aspect of timing can be easily addressed, if the desired factor is applied exogenously. For instance, factors can be added to the culture medium or omitted from the culture medium (following medium changes), after a certain amount of time. If gene transfer is used in an *in vitro* setup, it is usually more complicated to address the aspect of timing. Most viral vectors (both integrating and non-integrating) are constitutively active. This means that the transduced cell produces the transgene product independent of necessity or concentration. In other words, the production of the desired factor cannot be controlled. Inducible viral vectors are an exception to this rule. With this kind of vector system, it is possible to control the production of the transgene product. Usually, a specific factor has to be added to, or removed from, the culture medium to induce or repress the expression of the transgene. Yet, these vectors are usually more expensive than constitutively active vectors. Further, for many candidate gene products inducible vectors are commercially not available. In this case, the choice is either limited to constitutively active vectors or they have to be produced using commercially available kits. Yet, this procedure is very time- and money-consuming and, therefore, not applicable for every experimental design.

In a previous study, it was demonstrated that 3D transduction of hMSCs during encapsulation in different hydrogels (section 3.4 – 3.6; (Neumann et al., 2013c)) is possible. The 3D transduction of cells was performed during encapsulation in the respective hydrogels (alginate, agarose or fibrin were mixed with hMSCs and Ad.BMP-2). If the adenoviral vector is able to penetrate the respective hydrogel, it should be possible to conduct Ad.BMP-2 transduction after encapsulation of the cells. The current study focussed on the clinically relevant fibrin-PU composite system. It was hypothesised that Ad.BMP-2 transduction can be conducted after cells have already been encapsulated in fibrin and seeded into the PU scaffold. Therefore, hMSCs were seeded into fibrin-PU scaffolds at a density of 5×10^6 cells per scaffold. Scaffolds were cultured in 6-well plates, for a total of 3 weeks, with 5ml of chondro-permissive medium supplemented with an additional 5ng/ml of exogenous TGF-β1 per sample. The medium was changed three times per week and collected for each individual time-point. Cells were transduced with Ad.BMP-2 (by dripping the vector on top of the scaffold), using a MOI of 7.5. Transduction was conducted after 7 (week 1) or 14 (week 2) days. Control cells were left untransduced. After 21 days, samples were harvested in PK for quantification of cellular DNA. Furthermore, the BMP-2 content in the culture medium was determined, for each of the individual time-points.

3.8 Timed Ad.BMP-2 transduction – results.

This experiment was separately repeated with cells from 3 different donors (♀ age 40, ♀ age 65 and ♀ age 55). The control group was only included in 2 out of 3 donors (♀ age 40, ♀ age 65). The number of population doublings was not tracked for this short experiment.

3.8.1 DNA content is not influenced by timed Ad.BMP-2 transduction.

First, it was of interest to confirm that timed Ad.BMP-2 transduction does not change total cell number. Therefore, the DNA content within the scaffolds, after 21 days of culture, was quantified using the Hoechst 33258 dye binding assay (figure 3-13).

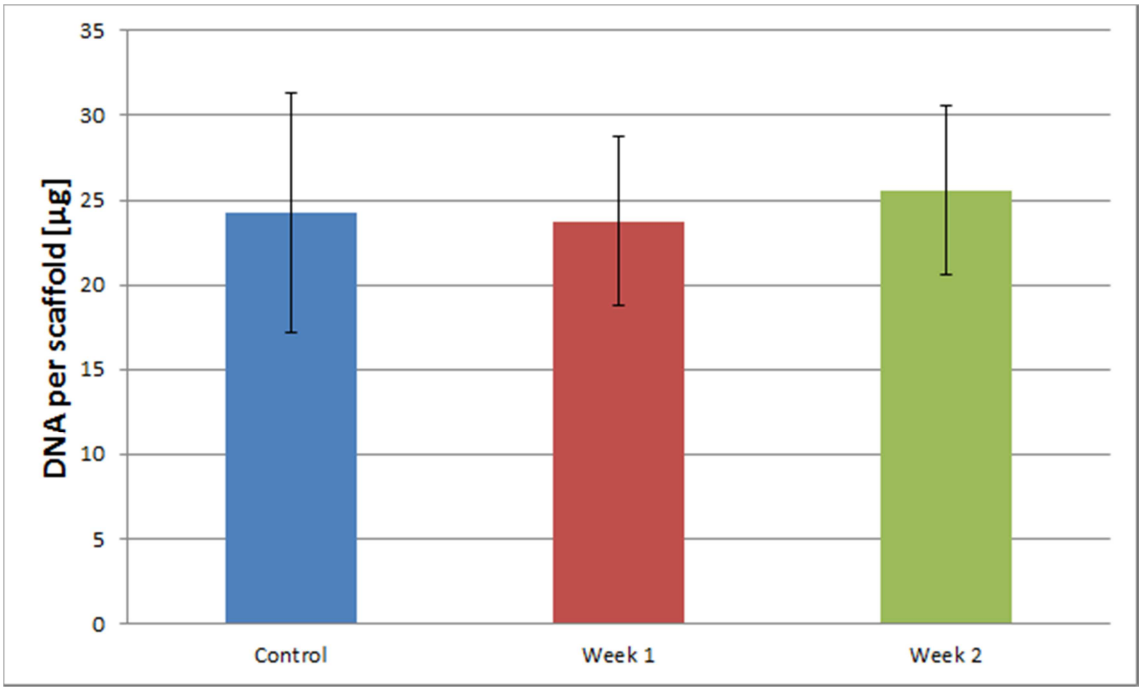

Figure 3-13: DNA content after timed Ad.BMP-2 transduction. For each sample, 5 x 10^6 hMSCs were seeded into fibrin-PU composite scaffolds. They were cultured for 3 weeks, in 5ml of chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1. After 7 (week 1) or 14 (week 2) days, cells were transduced with Ad.BMP-2 using a MOI of 7.5, by dripping the viral vector on top of the scaffold. Control cells were left untransduced. DNA content, after 3 weeks of culture, was determined with the Hoechst 33258 dye binding assay. Results are shown as average +/- standard deviation from 2 donors (control group) or 3 donors (groups week 1 and week 2) with n=3.

There were no significant differences in DNA content between the three groups. The total DNA content, after 3 weeks of culture, was 24.25µg ± 7.03µg (control), 23.75µg ± 5µg (week 1) and 25.57µg ± 4.96µg (week 2).

3.8.2 A rapid onset of BMP-2 production is monitored following timed Ad.BMP-2 transduction.

Next, it was investigated if cells produced and released BMP-2 into the culture medium, following timed Ad.BMP-2 transduction (figure 3-14). Medium was changed three times per week and collected for each individual time-point (day 2, day 4, day 7, day 9, day 11, day 14, day 16, day 18 and day 21). The BMP-2 concentration within the culture medium was quantified using a quantitative ELISA for human BMP-2.

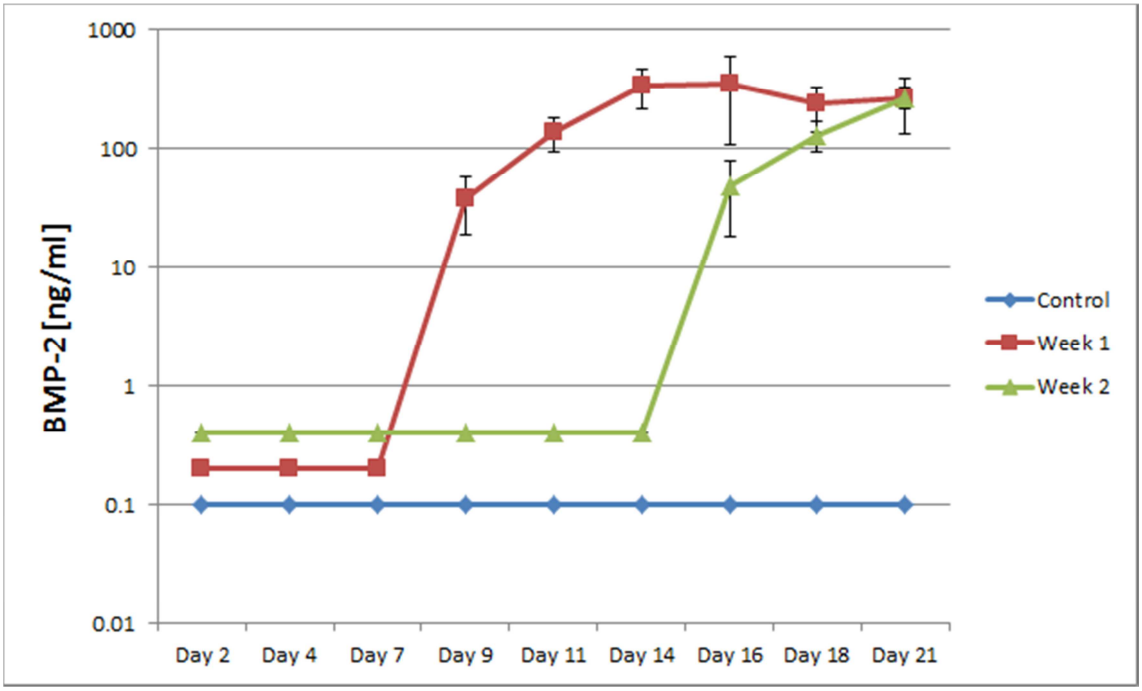

Figure 3-14: Quantitative comparison of the amount of BMP-2 produced after timed Ad.BMP-2 transduction of hMSCs, seeded into fibrin-PU composite scaffolds. For each sample, 5 x 10^6 hMSCs were seeded into fibrin-PU composite scaffolds. They were cultured for 3 weeks in 5ml of chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1. After 7 (week 1) or 14 (week 2) days, cells were transduced with Ad.BMP-2 using a MOI of 7.5 by dripping the viral vector on top of the scaffold. Control cells were left untransduced. The BMP-2 production was monitored for 21 days and quantified from cell culture medium using a BMP-2 ELISA kit. Results are shown as average +/- standard deviation from 2 donors (control group) or 3 donors (groups week 1 and week 2) with n=3. The BMP-2 concentration in the control group was always below the detection level of the assay (46.875pg/ml). In order to increase clarity of the figure, BMP-2 values of the control group were set to 0.1ng/ml. For both the week 1 group and the week 2 group, the BMP-2 medium levels were below the detection level of the assay before timed Ad.BMP-2 transduction was conducted. In order to increase clarity of the figure, BMP-2 values were set to 0.2ng/ml (week 1) respectively 0.4ng/ml (week 2). After timed Ad.BMP-2 transduction, BMP-2 was detected within the cell culture medium and the measured concentrations were used for the figure.

In the control group, the BMP-2 concentration within the culture medium was always below the detection level of the assay (46.875pg/ml). The same observation was made for the groups week 1 and week 2, in the time-period before timed Ad.BMP-2 transduction was conducted. In order to increase clarity of figure 3-14, the BMP-2 values were set to 0.1ng/ml (control), 0.2ng/ml (week 1) and 0.4ng/ml (week 2). For the control group, this was done for each time-point investigated. For the week 1 group and the week 2 group, this was done for the time-points prior to timed Ad.BMP-2 transduction (day 2, day 4 and day 7 in the week 1 group and day 2, day 4, day 7, day 9, day 11 and day 14 in the week 2 group). After timed Ad.BMP-2 transduction, a fast onset (2 days after transduction) of BMP-2 production was monitored in both groups and detectable BMP-2 medium concentrations were generated. For the week 1 group, the BMP-2 medium concentration was 37.8ng/ml ± 19.2ng/ml of BMP-2 on day 9. For the week 2 group, the BMP-2 medium concentration was 48.6ng/ml ± 30ng/ml of BMP-2 on day 16. Afterwards, the BMP-2 medium concentration increased in both groups. The peak concentrations generated were 355.7ng/ml ± 248ng/ml (group week 1) and 271.1ng/ml ± 57.5ng/ml (group week 2). In the week 1 group, the BMP-2 medium concentration started to plateau after day 16 (9 days after timed Ad.BMP-2 transduction). In the week 2 group, this phenomenon was not observed.

3.9 Timed Ad.BMP-2 transduction – discussion.

The effect of many bioactive factors is, among others, dependent on the timing of its administration (Balcom et al., 2012; Kim et al., 2012). Viral vectors are generally constitutively active. That means that the targeted cell, after it has been transduced with the viral vector, constantly produces the respective transgene product. This process cannot be regulated, with the exception of inducible vectors, such as tetracycline-regulated lentiviral vectors (Wubbenhorst et al., 2010). Yet, inducible vectors possess other disadvantages, such as low expression levels, price or availability. As a consequence, it is difficult to address the issue of timing, when viral vectors are used in studies which aim to investigate the effect of a bioactive factor. For some experimental designs, it is necessary to analyse the effect of a specific bioactive factor after a definite period of *in vitro* culture. Usually, for this kind of experiment, the application of a constitutive active viral vector is not a valid option. Therefore, it was investigated if a timed transduction with a constitutively active adenoviral vector is theoretically possible. In the current study, hMSCs were seeded into fibrin-PU composite scaffolds. They were cultured for 3 weeks in chondro-permissive medium with an additional 5ng/ml of exogenous TGF-β1. The cells were either left as untransduced controls or transduced with Ad.BMP-2, using a MOI of 7.5 (by dripping the viral vector on top of the scaffolds), after 7 or 14 days. The culture medium was changed three times per week and collected for single time-points. After 3 weeks, the scaffolds were harvested in PK. Outcome measurements were DNA content and BMP-2 concentration within the cell culture medium.

The DNA content, after 3 weeks of culture, was comparable between all groups investigated. No significant differences were detected, after timed Ad.BMP-2 transduction was conducted. This was true if the groups week 1 and week 2 were compared to the control group and, also, if the groups week 1 and week 2 were compared to each other. This indicates that, as expected, neither timed Ad.BMP-2 production nor the presence of the BMP-2 protein (in the medium of the transduced groups) had an effect on total cell number. These data were consistent with the data that were generated when the 3D Ad.BMP-2 transduction protocol was established (enhanced adenoviral transduction in 3D study section 3.4 – 3.6 and (Neumann et al., 2013c)).

In the control group, the BMP-2 medium concentration was always below the detection level of the assay (46.875pg/ml). The same was true for the week 1 group and the week 2 group, at all time-points prior to timed Ad.BMP-2 transduction. Afterwards, there was a fast onset of BMP-2 production. Already two days after timed Ad.BMP-2 transduction, BMP-2 was detected within the cell culture medium. A BMP-2 concentration of 100ng/ml or above was generated and released into the culture medium. This is a concentration commonly used when applying BMP-2 exogenously (Majumdar et al., 2001; Sailor et al., 1996; Toh et al., 2007). It is worth noticing that, in the week 1 group, BMP-2 concentration seemed to plateau around day 16 (nine days after Ad.BMP-2 transduction). In the week 2 group, samples were already harvested 7 days after Ad.BMP-2 transduction. Therefore, this observation could not be confirmed within this group.

The possibility to transduce hMSCs, after encapsulation within a hydrogel, after a certain time in *in vitro* culture, represents an useful tool for certain basic research applications. It opens the possibility to investigate the effect of a timed administration of a certain factor without the need to apply it exogenously. Yet, the feasibility of this approach has only been demonstrated for the Ad.BMP-2 vector and hMSCs within a fibrin-PU composite scaffold. The question if this is possible for other cells types, viral vectors and hydrogels was beyond the scope of this study and, therefore, will have to remain open. Based on the findings that 3D Ad.BMP-2 transduction is also possible in alginate and agarose (Neumann et al., 2013c), it is likely, but not certain, that this approach will also work for these hydrogels.

The timed Ad.BMP-2 transduction protocol still has some limitations that should not be concealed. First, as the investigated Ad.BMP-2 vector is constitutively active, it is not possible to stop transgene product production after timed transduction has been conducted. For some studies, this might be desirable and for this kind of study it would be preferable to use an inducible vector or to apply the desired factor exogenously. Secondly, as already discussed, the feasibility of this approach will have to be confirmed for other commonly used viral vectors, such as AAV, retrovirus or lentivirus, other cell types, such as chondrocytes or osteocytes, and other hydrogels or synthetic scaffolds, such as collagen, hyaluronic acid or polyethylene glycol. Last, but not least, the application of this procedure would prove difficult in an *in vivo* setup.

In conclusion, the current study wanted to investigate if a timed Ad.BMP-2 transduction is possible within the fibrin-PU composite system. Therefore, hMSCs were encapsulated in fibrin-PU composite scaffolds and transduced with Ad.BMP-2, using a MOI of 7.5, after 1 week or 2 weeks of culture. Control cells were left untransduced. It was demonstrated that this procedure does not influence total cell number, as evidenced by measurement of cellular DNA content. Further, it was shown that hMSCs did not produce any BMP-2 before Ad.BMP-2 transduction was conducted. After timed Ad.BMP-2 transduction, a fast onset of BMP-2 production was monitored. Biologically relevant amounts of BMP-2 (100ng/ml or above) were produced. In conclusion, the timed Ad.BMP-2 transduction protocol represents an useful tool to investigate the effect of a timed administration of a bioactive factor in an *in vitro* setup.

Chapter 4: Ad.BMP-2 and load study.

4.1 Ad.BMP-2 and load study – introduction.

Hyaline, articular cartilage covers the osseous ends in diarthrodial joints. Its function is to absorb forces, generated through loads and, thereby, allowing for a smooth, near frictionless motion between the articulating surfaces. Once articular cartilage is damaged, it possesses a limited intrinsic repair capacity. Currently, several different approaches are applied in clinical practice in order to treat articular cartilage defects. Examples are microfracture (Steadman et al., 2002), soft-tissue grafts, such as periosteum or perichondrium (Homminga et al., 1990), mosaicplasty (Szerb et al., 2005), and ACT (Brittberg et al., 1994). Unfortunately, no procedure can yet demonstrate an ability to reproducibly regenerate articular cartilage and, thereby, provide a long-term solution for every patient (Getgood et al., 2009).

TE is believed to be a promising alternative for the treatment of articular cartilage defects. It consists of three major building blocks; cells, a scaffold and stimulating factors. hMSCs are a favourable cell source for cartilage TE. They can be isolated from several different tissues, such as adipose tissue (Zuk et al., 2002), BM (Friedenstein et al., 1976), muscle (Kuroda et al., 2006) or synovium (De Bari et al., 2001), and it is possible to differentiate them into different cell types, for example chondrocytes (Johnstone et al., 1998).

A scaffold should mimic the 3D environment in which cells normally reside. In TE of articular cartilage, a plethora of different scaffold systems are applied (reviewed e.g. in: (Balakrishnan and Banerjee, 2011; Frenkel and Di Cesare, 2004; Stoddart et al., 2009)). Our group focuses on a composite scaffold, consisting of a fibrin hydrogel and a PU porous scaffold (Lee et al., 2005; Li et al., 2009a). This system combines the advantages of a natural hydrogel cell carrier (e.g. homogeneous cell encapsulation, preservation of the cellular phenotype and nutrient exchange) and a synthetic, porous sponge (resilience and mechanical stability).

In TE of articular cartilage, stimulating factors could have different scopes of application They can be used to preserve the chondrocyte phenotype, to induce, chondrogenesis of hMSCs or enhance the chondrogenic response of hMSCs, which was induced by another bioactive factor. The most commonly applied stimuli are bioactive factors and mechanical forces. Bioactive factors, such as growth hormones, transcription factors or differentiation factors play pivotal roles in embryogenesis and they influence a variety of functions within the human body. For cell-based cartilage TE, the most routinely applied factors include IGF-1 (Gelse et al., 2003; Madry et al., 2013), fibroblast growth factors (Weisser et al., 2001), SOX9 (Kupcsik et al., 2010) and members of the transforming growth factor beta family, such as TGF-β1 (Yeoh and de Haan, 2007; Yoo et al., 1998), TGF-β2 (Jin et al., 2008) or TGF-β3 (Fan et al., 2008; Miyanishi et al., 2006) and different bone morphogenetic proteins (Majumdar et al., 2001; Salzmann et al., 2009; Sekiya et al., 2005).

Gene transfer is an attractive alternative to the exogenous application of recombinant bioactive factors. The concept is based on the delivery of cDNA (encoding a specific transgene) to a target cell. This procedure enables the cell to produce the desired transgene. As the half-life of most bioactive factors is short, their repeated administration is usually necessary. This feature is especially obstructive in a clinical environment and can be circumvented by the application of gene transfer. Generally, gene transfer vectors can be separated into two major classes; viral vectors and non-viral vectors. Non-viral vectors are relatively safe to apply. However, they are considered to be less efficient, when compared to viral vectors (Dinser et al., 2001; Steinert et al., 2008; Thomas et al., 2003). On the other hand, viral vectors are much more efficient but their application raises safety concerns (Steinert et al., 2008; Thomas et al., 2003). Adenoviral vectors, which do not integrate into the host genome, can be used as effective tools to provide a local, albeit transient, production of a bioactive factor. This might be particularly helpful for the treatment of musculoskeletal defects, where only a localised and short-term expression (days to weeks) of a therapeutic factor is needed, in order to initiate a repair response or to enhance the endogenous repair.

Although, adenovirally transduced cells have the potential to be immunogenic, adenovirus has many advantages for proof of principle studies. These include; low production cost, high transduction efficiency, their ability to infect both dividing and non-dividing cells, high levels of transgene production and increased safety, when compared to integrating viruses (e.g. retroviruses). Last, but not least, adenoviral vectors have already been approved for clinical trials (Peng, 2005; Penny and Hammond, 2004).

Healthy articular cartilage is subjected to mechanical forces on a daily basis, and, it is widely accepted that they play a pivotal role for both the maintenance and the development of this tissue. Therefore, the concept of functional TE implies mechanical stimulation (e.g. compression (Angele et al., 2004; Buschmann et al., 1995), fluid-flow (Freed et al., 1998), shear stress (Frank et al., 2000) or hydrostatic pressure (Miyanishi et al., 2006)) as additional stimuli. The physiological movement of the joint is kinematically very complex. Thus, the application of a single stimulus will not adequately reflect the complex *in vivo* situation. For that reason, a bioreactor that is able to reproduce the kinematics of the joint more precisely was designed (Wimmer et al., 2004). With this bioreactor system, several different stimuli (compression, shear and flexion) can be applied solely or simultaneously. This innovative system has been used in several studies that showed promising results (Kupcsik et al., 2010; Li et al., 2009b; Li et al., 2009c; Schatti et al., 2011). It also has been demonstrated that retroviral-mediated over-expression of BMP-2, in combination with mechanical stimulation, synergistically enhanced the re-differentiation of de-differentiated bovine chondrocytes (Salzmann et al., 2009).

These encouraging results led to the development of the present study where it was opted to investigate the effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2, alone and in combination, on the differentiation of monolayer-expanded hMSCs. No exogenous growth factor was applied, which makes the procedure easier to apply in clinical practice, as it does not depend on the repetitive administration of an exogenous factors or the use of an integrating viral vector. It was hypothesised that mechanical stimulation will enhance the paracrine production of TGF-β1 which will induce chondrogenesis (Li et al., 2009b). Transduction with Ad.BMP-2 will lead to a temporary production of BMP-2. This production was hypothesised to further enhance chondrogenesis. hMSCs were encapsulated in a clinically relevant fibrin-PU composite scaffold. They were transduced with Ad.BMP-2 in 3D (using a novel protocol for enhanced adenoviral transduction (Neumann et al., 2013c)) or left as untransduced controls. After pre-culture, they were either subjected to mechanical stimulation, for 7 or 28 days, or left as free-swelling controls. Production of biologically relevant amounts of transgene product (BMP-2) was validated using a quantitative BMP-2 ELISA. Biochemical analyses (GAG, DNA and the amount of GAG normalised to the DNA content (GAG/DNA)) and gene expression analyses were conducted. This work was published in Tissue Engineering (Neumann et al., 2013a).

4.2 Ad.BMP-2 and load study – results.

In total, eight different groups were analysed during the study (table 4-1). Triplicate samples were done per group. The experiment was separately repeated with cells from 5 different donors (♀ age 65, ♀ age 78, ♂ age 55, ♀ age 73 and ♂ age 41).

Group	Viral Transduction (in 3D with 5 MOI)	Mechanical stimulation	Time in 3-D culture (+ 3 days of preculture)
1	-	No	7
2	-	Yes	7
3	-	No	28
4	-	Yes	28
5	Ad.BMP-2	No	7
6	Ad.BMP-2	Yes	7
7	Ad.BMP-2	No	28
8	Ad.BMP-2	Yes	28

Table 4-1: Overview of the eight different experimental groups of the Ad.BMP-2 and load study.

4.2.1 Transduction of hMSCs, in 3D with a MOI of 5, leads to the production of biologically relevant amounts of BMP-2.

The feasibility of Ad.BMP-2 transduction in 3D within the fibrin-PU composite system has already been demonstrated (see enhanced adenoviral transduction in 3D study (section 3.4 – 3.6) and (Neumann et al., 2013c)). Nevertheless, it was of fundamental interest to confirm that biologically relevant amounts of BMP-2 were generated over the course of the study. Hence, BMP-2 concentration within the culture medium was quantitatively determined using an ELISA kit for human BMP-2. In all untransduced samples, under both loaded and unloaded conditions, BMP-2 medium concentrations were below 0.1ng/ml. Thus, these groups were not included in figure 4-1.

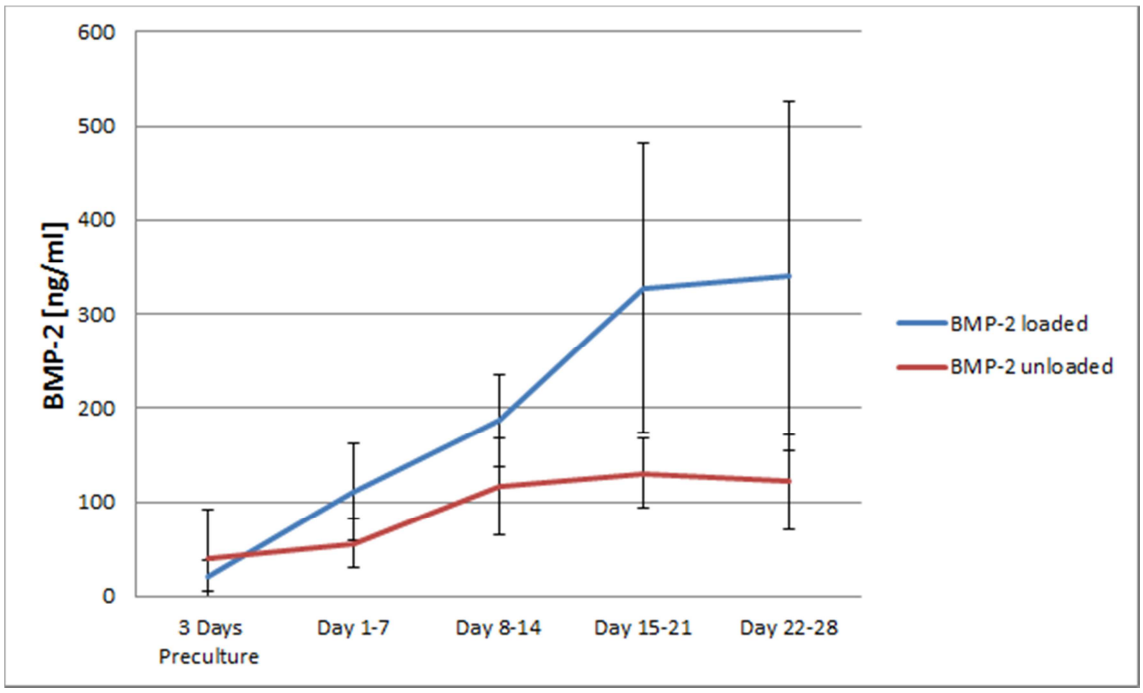

Figure 4-1: BMP-2 concentration within the cell culture medium of Ad.BMP-2 transduced hMSCs, which were cultured in fibrin-PU composite scaffolds. hMSCs were transduced with Ad.BMP-2 in 3D (5 MOI). Subsequently, they were cultured for up to 28 days. Either static culture conditions were applied (unloaded) or cells were cultured with application of 1 hour of mechanical stimulation each day for 6 days per week (loaded). Medium was changed and collected three times per week. Additionally, the medium of the 3 day pre-culture period was collected. Transgene concentration within the culture medium was determined using a quantitative ELISA for human BMP-2. Results are displayed as average ± standard deviation of triplicates from three donors.

There were no significant differences in BMP-2 concentration, within the culture medium, between the unloaded and the loaded group. In the unloaded group, BMP-2 concentrations stayed relatively constant over the course of the study. In the loaded group, a trend towards increasing medium levels was detected. Yet, this trend failed to reach statistical significance. The peak BMP-2 concentrations were 130ng/ml ± 37.3.4ng/ml in the unloaded group and 339.8ng/ml ± 186ng/ml in the loaded group. It is worth noting that, starting from week 1, BMP-2 concentrations were above 100ng/ml (a concentration commonly used in studies applying recombinant BMP-2 exogenously) for all groups, except BMP-2 unloaded at week 1.

4.2.2 Mechanical stimulation leads to an up-regulation of chondrogenic genes, when compared to free-swelling controls. Transduction with Ad.BMP-2 is the predominant stimulus for the gene aggrecan and it leads to an up-regulation of SOX9 message at the early time-point.

The effect of mechanical stimulation and transduction with Ad.BMP-2, on the gene expression profile of hMSCs, was investigated. Gene expression analyses were conducted using the comparative $\Delta\Delta C_T$ method with 18s rRNA as internal control. The unloaded control group at day 7 was used as normaliser and, therefore, set to 1 (figure 4-2).

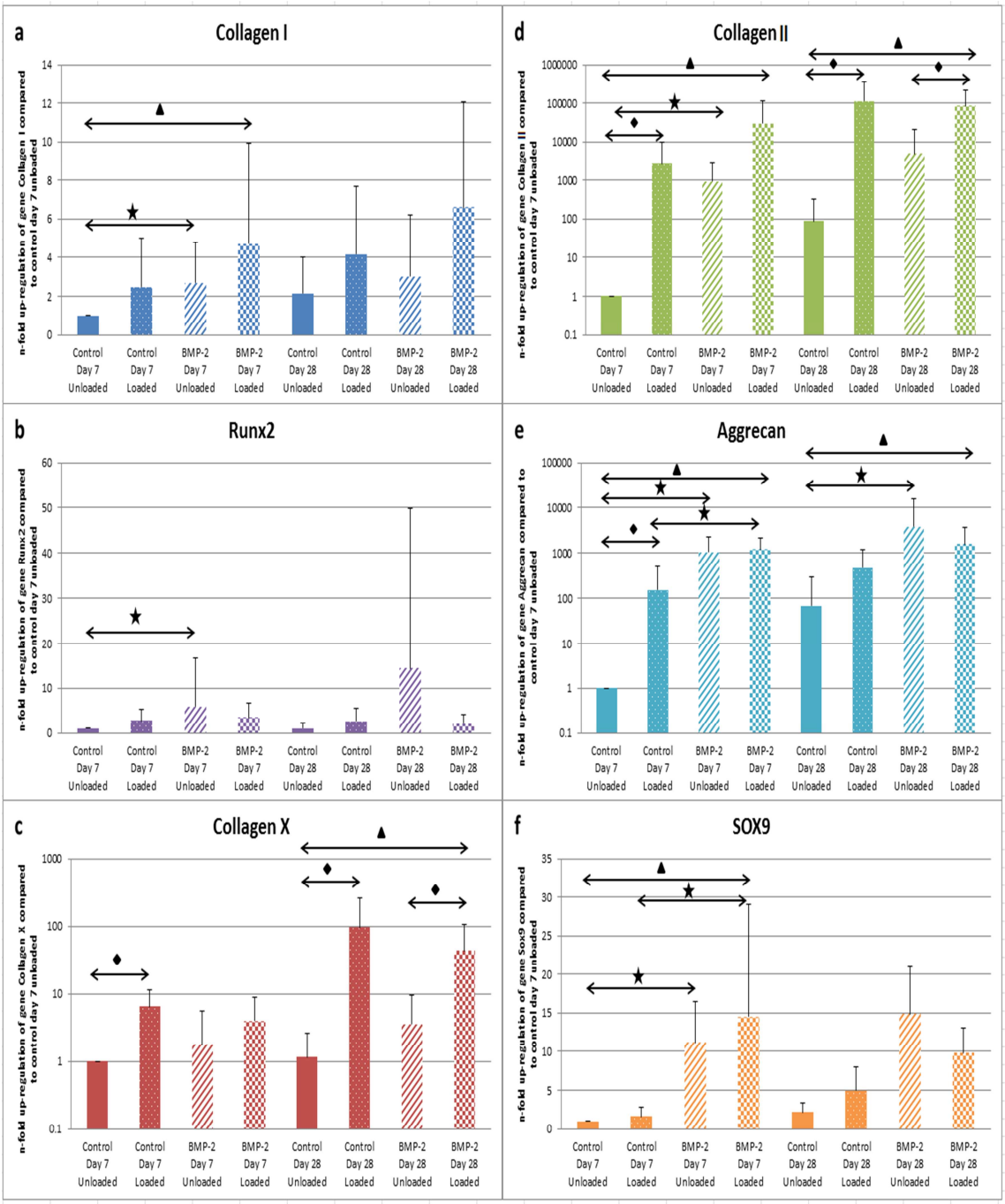

Figure 4-2: Relative gene expression of hMSCs, which have been cultured in fibrin-PU composite scaffolds for 7 or 28 days. They have been transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or were left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Col I (a), Runx2 (b), Col X (c), Col II (d), aggrecan (e) and SOX9 (f). 18s rRNA was used as internal control. The unloaded control group at day 7 was used as calibrator and therefore set to 1. Results are displayed as average + standard deviation of triplicates from five donors.★ Significantly different in the control vs. the Ad.BMP-2 transduced group ($p \leq 0.05$);◆significantly different in the unloaded vs. the loaded group ($p \leq 0.05$); ▲significantly different in the control unloaded vs. the Ad.BMP-2 transduced loaded group ($p \leq 0.05$).

The genes Col I (figure 4-2 a) and Runx2 (figure 4-2 b) were almost un-responsive towards both stimuli, with slight variation due to a small response seen in one of the five donors (♀ age 65). The only significant changes observed were an up-regulation for both genes in Ad.BMP-2 transduced vs. control samples within the day 7 unloaded group (*p=0.041* for Col I and *p=0.006* for Runx2) and an up-regulation for Col I on day 7 (*p=0.017*), when both stimuli were simultaneously applied. The gene Col X (figure 4-2 c) was significantly up-regulated through mechanical stimulation in all groups (*p=0.000* for control on day 7, *p=0.000* for control on day 28 and *p=0.000* for BMP-2 on day 28), except the Ad.BMP-2 transduced group on day 7. Interestingly, at the early time-point, if both stimuli were combined, Col X expression was not significantly different from the unloaded control group. The gene Col II (figure 4-2 d) was massively up-regulated through mechanical stimulation in all groups (*p=0.014* for control on day 7, *p=0.000* for control on day 28 and *p=0.000* for BMP-2 on day 28), except the Ad.BMP-2 transduced group on day 7. In the Ad.BMP-2 transduced groups, a statistically significant up-regulation in Col II message was only detected in the unloaded group at day 7 (*p=0.001*). Further, a synergistic effect of both stimuli was detected on day 7. The n-fold up-regulation in Col II message was 2655 ± 7417 (control loaded, *p=0.014*), 936 ± 1785 (BMP unloaded, *p=0.001*) and 29878 ± 86410 (BMP-2 loaded, *p=0.000*). The response of the gene aggrecan (figure 4-2 e) was the most complex. At the early time-point (day 7), both load (*p=0.050*) and transduction with Ad.BMP-2 (*p=0.000*) led to a significant increase in aggrecan message. Yet, transduction with Ad.BMP-2 seemed to be the predominant stimulus, as aggrecan message was significantly elevated in the BMP-2 loaded vs. the control loaded group (*p=0.000*). Further, the combination of both stimuli did not lead to an elevated response in comparison to the unloaded Ad.BMP-2 transduced group. At the late time-point (day 28), the same general response was detected. Yet, the application of mechanical stimulation had no significant influence on aggrecan gene expression. Further, transduction with Ad.BMP-2 only led to a significantly higher aggrecan message in the unloaded group (*p=0.000*). Finally, at the early time-point, transduction with Ad.BMP-2 led to a significant up-regulation of SOX9 message (figure 4-2 f). This was true in both the unloaded (*p=0.000*) and the loaded group (*p=0.000*).

4.2.3 The DNA content did not differ statistically between groups.

The DNA content was quantified using the Hoechst 33258 dye assay. Neither transduction with Ad.BMP-2 nor mechanical stimulation had a statistically significant effect on total DNA content (figure 4-3). Figure 4-4 displays the average of day 7 and day 28 samples for each group. These values were used for the calculation of the GAG/DNA values in section 4.2.5.

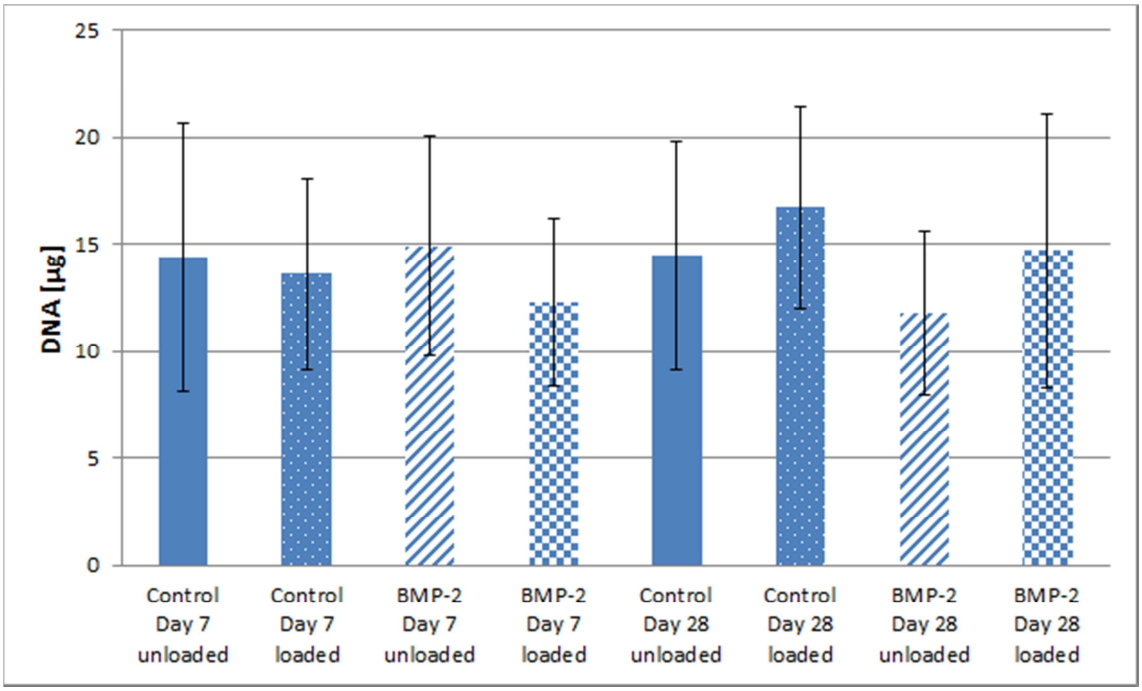

Figure 4-3: Total DNA content of hMSCs after 7 or 28 days. hMSCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). DNA content was quantified from scaffolds using the Hoechst 33258 dye assay with calf thymus DNA as standard. Results are displayed as average ± standard deviation of triplicates from five donors.

As already mentioned, there were no significant differences in DNA content between the groups. Total DNA content for each group was 14.35µg ± 6.27µg (control day 7 unloaded), 13.59µg ± 4.44µg (control day 7 loaded), 14.92µg ± 5.15µg (BMP-2 day 7 unloaded), 12.27µg ± 3.91µg (BMP-2 day 7 loaded), 14.47µg ± 5.35µg (control day 28 unloaded), 16.72µg ± 4.71µg (control day 28 loaded), 11.81µg ± 3.83µg (BMP-2 day 28 unloaded) and 14.69µg ± 6.38µg (BMP-2 day 28 loaded).

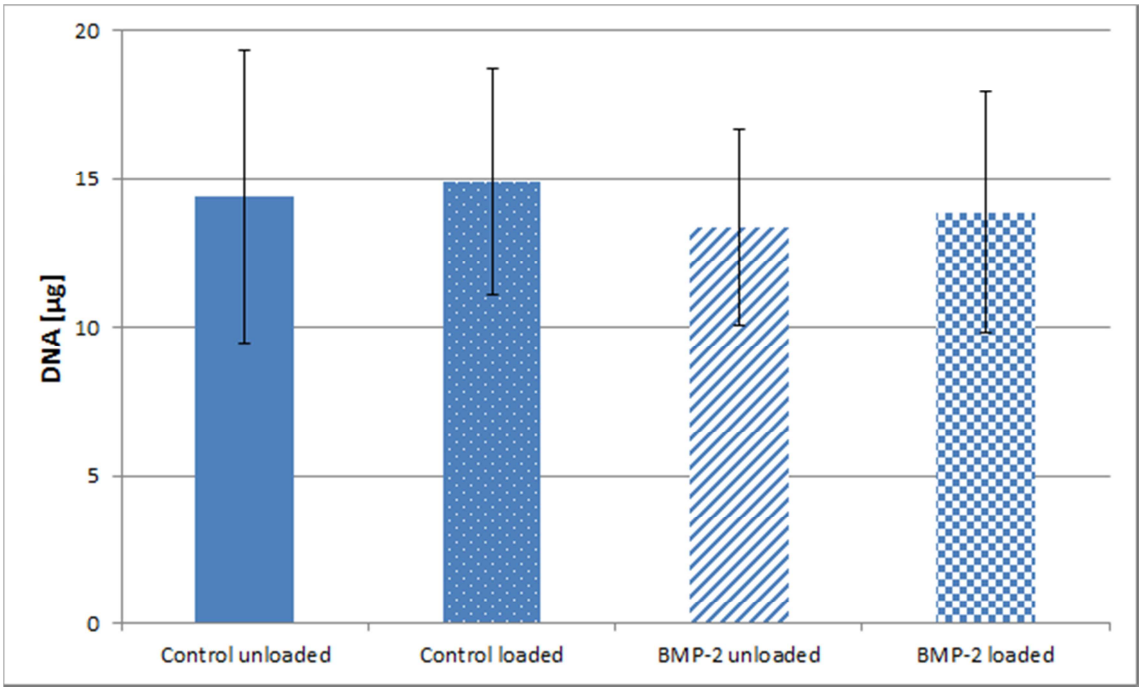

Figure 4-4: Average DNA content (calculated from day 7 and day 28 samples) of hMSCs. hMSCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). DNA content was quantified from scaffolds using the Hoechst 33258 dye assay with calf thymus DNA as standard. Further, the average between the day 7 and the day 28 samples was individually calculated for each donor and sample. Results are displayed as average ± standard deviation of triplicates from five donors.

Again, when the average DNA content (independent from the time-point) was calculated, there were no significant differences between the different groups. Total values measured were 14.41µg ± 4.79µg (control unloaded), 14.92µg ± 3.97µg (control loaded), 13.36µg ± 3.3µg (BMP-2 unloaded) and 13.88µg ± 4.05µg (BMP-2 loaded).

4.2.4 The bulk amount of synthesised GAG is released into the culture medium.

The cumulative amount of released GAG, over the course of 28 days, was quantified using the DMMB dye binding assay. Furthermore, the amount of GAG retained within the scaffolds was measured after 28 days. Thereby, matrix production of hMSCs was tracked (figure 4-5).

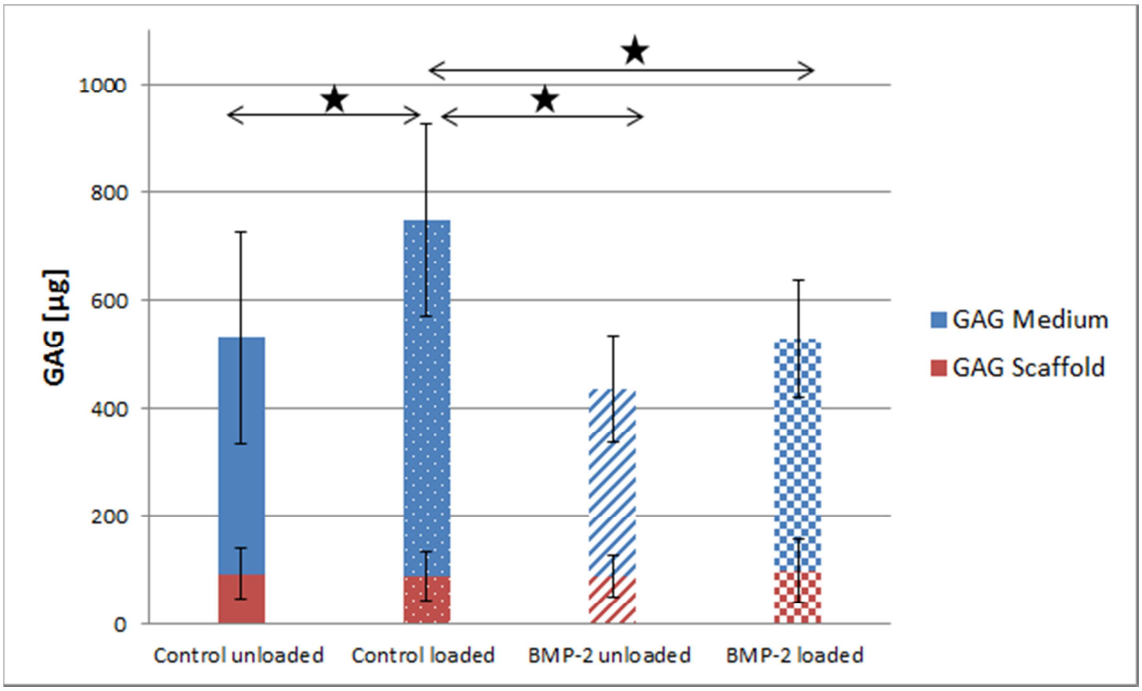

Figure 4-5: Total amount of GAG synthesised after 28 days. hMSCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Medium was changed and collected three times per week. It was pooled for weeks 1, 2, 3 and 4. Additionally, the medium of the 3 day pre-culture period was collected. GAG content was quantified (both within the scaffold and within the culture medium) using the DMMB dye binding assay with chondroitin-4-sulfate as standard. Results are displayed as average ± standard deviation of triplicates from five donors.★Significantly different from the control loaded group ($p \leq 0.05$).

A large proportion of synthesised GAG was released into the culture medium. After 28 days of culture, only 92.5µg ± 46.5µg (control unloaded), 88.6µg ± 45.2µg (control loaded), 89.1µg ± 37.6µg (BMP-2 unloaded) or 99.5µg ± 58.9µg (BMP-2 loaded) of GAG was retained within the scaffolds. On the other hand, during the same period of time, a total of 438.4µg ± 196µg (control unloaded), 660µg ± 177.7µg (control loaded), 346.5µg ± 97.2µg (BMP-2 unloaded) or 429.4µg ± 108.7µg (BMP-2 loaded) of GAG was released into the culture medium. In summary, the amount of synthesised GAG, retained within the scaffolds, was in a range between 12.1% ± 5.6% (control loaded) and 21.75% ± 3.6% (BMP-2 unloaded). A trend towards an increased GAG release in loaded vs. unloaded groups was detected. This trend reached statistical significance only in the control group and not in the BMP-2 group. Further, the total amount of GAG produced in the loaded control group was highest among all groups and significantly different from the other groups (*p=0.028* vs. control unloaded, *p=0.000* vs. BMP-2 unloaded and *p=0.011* vs. BMP-2 loaded).

4.2.5 The GAG/DNA ratio is highest in the control loaded group.

Finally, in order to normalise the amount of GAG produced to total cell number (figure 4-6), the GAG/DNA ratio was calculated. Therefore, the total amount of GAG (scaffolds + medium over 4 weeks) was divided by the average DNA content (from day 7 and day 28 samples) for each of the four groups.

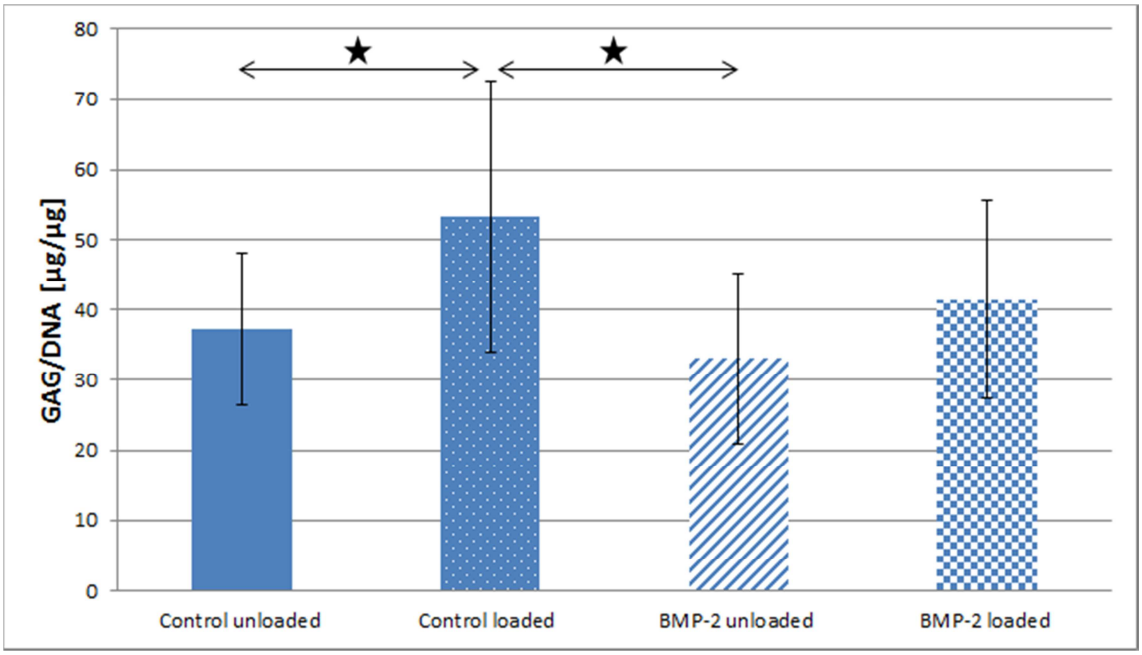

Figure 4-6: Total amount of GAG (scaffold + medium over the course of 4 weeks) normalised to average DNA content (of day 7 and day 28 samples) between the different groups after 28 days. hMSCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Total amount of GAG and amount of DNA was quantified. Subsequently, GAG production was normalised to DNA content. Results are displayed as averages ± standard deviation of triplicates from five donors.★Significantly different from the control loaded group ($p \leq 0.05$).

GAG/DNA ratios (in µg/µg) were 37.3 ± 10.8 (control unloaded), 53.3 ± 19.3 (control loaded), 33 ± 12.1 (BMP-2 unloaded) and 41.4 ± 14.1 (BMP-2 loaded). A trend towards higher GAG/DNA ratios in control vs. Ad.BMP-2 transduced and loaded vs. unloaded groups was detected. This trend reached statistical significance in the control loaded group only, where the GAG/DNA ratio was highest among all groups investigated (*p=0.048* vs. control unloaded and *p=0.014* vs. BMP-2 unloaded).

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4.3 Ad.BMP-2 and load study – Discussion

During everyday life, cartilage is subjected to mechanical forces. It is widely accepted that they play an important role in both development and maintenance of this tissue. The concept of functional TE implicates mechanical stimulation and, thereby, mimics the prevailing *in vivo* situation more precisely. The present study opted to investigate the combined effect of complex mechanical stimulation using a custom-built bioreactor system (Wimmer et al., 2004) and adenoviral-mediated over-expression of BMP-2 on chondrogenesis of monolayer-expanded hMSCs. hMSCs were encapsulated in a fibrin-PU composite scaffold system and cultured for up to 28 days. A chondro-permissive medium, which has the exact same composition as standard chondrogenic medium but lacks any exogenous growth factor, was used. The fibrin-PU system used compares favourably to pellet culture, particularly with respect to a lower expression of genes associated with endochondral ossification (Li et al., 2009a). The two stimuli were applied alone or in combination. In the fibrin-PU composite system, mechanical stimulation is known to enhance the paracrine production of TGF-β1, inducing chondrogenesis of hMSCs (Li et al., 2009b). Further, it has been demonstrated that shear plays an important role during hMSC chondrogenesis (Schatti et al., 2011). After transduction with Ad.BMP-2, hMSCs are able to endogenously produce BMP-2. This bioactive factor is widely utilised for both chondrogenic induction and enhancement of chondrogenesis in hMSCs (Majumdar et al., 2001; Schmitt et al., 2003). In the present study, BMP-2 over-production was believed to further enhance chondrogenesis. Outcome parameters measured were concentration of BMP-2 within cell culture medium, biochemical analyses (GAG, DNA and GAG/DNA) and gene expression analyses.

After Ad.BMP-2 transduction in 3D, hMSCs were able to generate high levels of transgene product over the course of 4 weeks. This expression was far more prolonged, when compared to standard 2D Ad.BMP-2 transduction (Palmer et al., 2005). Furthermore, BMP-2 concentrations of 100ng/ml or above were generated. This concentration is commonly used when BMP-2 is applied exogenously and is considered to be biologically relevant (Majumdar et al., 2001; Sailor et al., 1996; Toh et al., 2007).

It is worth mentioning that the application of mechanical stimulation led to a trend towards higher BMP-2 medium concentrations. Yet, this trend failed to reach statistical significance. The most likely explanation for this observation is that, in the unloaded group, the synthesised BMP-2 can leave the scaffold only by means of passive diffusion. In the loaded group however, application of mechanical forces will likely squeeze out most of the BMP-2 which is retained within the scaffold. Interestingly, a similar trend was seen for GAG concentration within the cell culture medium within this study and in previous work from our group (Li et al., 2009c; Schatti et al., 2011).

The DNA content did not change significantly over the time course of the experiment. Further, there was no significant change in DNA content when both stimuli were applied solely or in combination. This indicates that the total cell number stayed relatively constant and that cell numbers were not influenced by either Ad.BMP-2 transduction in 3D or mechanical stimulation. In other words, neither proliferation nor cell death dominated the cellular response towards the culture environment. These observations have already been demonstrated in different studies using the fibrin-PU composite scaffold system (Li et al., 2009b; Li et al., 2009c; Neumann et al., 2013c).

Gene expression analyses revealed that mechanical stimulation led to an up-regulation of chondrogenic marker genes (Col II and aggrecan) but also to an up-regulation of the hypertrophic marker gene Col X. This suggests that mechanical stimulation enhanced hMSCs chondrogenesis but also their progression towards hypertrophy. Interestingly, at the early time-point, this hypertrophic response might have been inhibited, or at least delayed, through adenoviral-mediated over-expression of BMP-2 (as Col X message in the control unloaded group was not different from the Ad.BMP-2 transduced and loaded group). For the gene Col II, a synergic effect was detected when both stimuli were simultaneously applied. However, this was significant only for the early time-point at day 7. After 28 days, supraphysiological doses of BMP-2 did not seem to synergistically enhance Col II gene expression, if combined with mechanical stimulation. Interestingly, for the gene aggrecan, both stimuli led to an increase in gene expression, if applied solely. However, when cells were subjected to both stimuli, the increase induced by Ad.BMP-2 transduction was not further enhanced by mechanical load. This suggests that over-expression of BMP-2 is the predominant stimulus for the gene aggrecan and that aggrecan is differently regulated, when compared to the other genes that were investigated in this study.

The same observation was made by Kupcsik et al., in 2010 (Kupcsik et al., 2010). Finally, transduction with Ad.BMP-2 led to a significant increase in SOX9 (master transcription factor for chondrogenesis) expression after 7 days. This finding indicates that Ad.BMP-2 transduction was beneficial for the initiation of chondrogenesis.

Interestingly, the gene expression results within this study conflicted with the GAG/DNA data where a trend towards a negative effect of Ad.BMP-2 transduction was detected. An up-regulation in gene expression does not always correlate to elevated protein levels. Our group and others have already described this lack of direct correlation between aggrecan gene expression and GAG synthesis for MSC (Barry et al., 2001; Kisiday et al., 2008). In previous work of our group, this lack of correlation was investigated using the DMMB assay, Safranin-O staining and immunohistochemistry for the aggrecan protein (Kupcsik et al., 2010; Schatti et al., 2011). It was also tested if the cells have the potential to produce negative regulators of GAG expression, such as interleukin-1β, but no significant changes were detected. It was proposed that some required co-factor, present in mature chondrocytes, is lacking in chondrogenically induced MSCs (Kupcsik et al., 2010). Gene expression of Col I (fibroblastic marker) and Runx2 (osteoblastic marker) was almost unaffected by both stimuli. The few detected statistically significant up-regulations in gene expression, after transduction with Ad.BMP-2, were so small that they are most likely not to be biologically relevant.

The quantification of total GAG revealed that the bulk amount of GAG was released into the culture medium and did not stay within the scaffolds. Further, a trend towards an increased GAG production and release in mechanical stimulated samples, when compared to free-swelling controls, was observed. In the control group, this trend reached statistical significance. Again, this has already been described in previous work using this culture system (Li et al., 2009c). In general, the amount of GAG that can be retained depends on the scaffold, the mechanical environment and the pericellular matrix. The observed results suggest that, under the observed conditions, the pericellular matrix was not well enough developed to retain most of the synthesised GAG. This task was further complicated by the highly porous structure, the relative large pore size of the scaffold and by the application of mechanical stimulation.

By normalising the total amount of GAG produced to the total DNA content it was demonstrated that the control loaded group had a higher GAG/DNA ratio than its free-swelling counterpart. For the Ad.BMP-2 transduced groups similar observations were made. Yet, the increase in the loaded group was not statistically significant. This observation is consistent with previously published data within our group using both hMSCs (Li et al., 2009c) and bovine chondrocytes (Salzmann et al., 2009). As synthesis of GAG is a qualitative measurement of hMSCs matrix production, this suggests that mechanical stimulation is beneficial for hMSC chondrogenesis. Comparison between Ad.BMP-2 transduced groups and untransduced controls revealed an unexpected result. The GAG/DNA ratio in the untransduced control and loaded group was highest, among all groups investigated. This was a surprising finding, as over-expression of BMP-2 was believed to enhance chondrogenesis of hMSCs, as already demonstrated by other investigators in different culture systems with cells from different species (Majumdar et al., 2001; Palmer et al., 2005; Schmitt et al., 2003; Sekiya et al., 2005). However, the reason for the conflicting results between gene expression analyses (aggrecan) and the GAG/DNA ratio will require further investigation.

This study and further experiments with the bioreactor system might help to develop a one-step protocol, which can be conducted within the operating theatre, with the aim to treat cartilage defects. Theoretically, a surgeon could harvest bone-marrow during operation, isolate the mononuclear fraction, containing hMSCs, through centrifugation, transduce these cells with an adenovirus expressing a desired transgene and implant the cells immediately into the defect. The feasibility if this approach has already been demonstrated in 2004 by Pascher et al., by transducing rabbit whole bone-marow clots intra-operatively (Pascher et al., 2004). Further, it would require the direct transduction of hMSC in 3D. This has been demonstrated by recent work within our group using monolayer-expanded cells (Neumann et al., 2013c). The suggested protocol would no longer rely on a time-consuming and money-consuming cell culture step and would remove the need for a second operation. A potential limitation to the proposed approach would involve the release of BMP-2 from modified cells within the defect into the surrounding joint compartment, which might lead to adverse effects within tissues proximal to the defect (e.g., osteophyte formation). This would have to be investigated in an *in vivo* model to determine the concentration and effect of any BMP-2 release.

In summary, the present study aimed to investigate the combined effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2 on hMSC chondrogenesis. It was demonstrated that mechanical stimulation led to an up-regulation of chondrogenic genes. Load also led to a small increase in expression of the hypertrophic marker Col X. This hypertrophic response seemed to be diminished, or at least delayed, when cells were transduced with Ad.BMP-2. Furthermore, mechanical stimulation increased the GAG/DNA ratio in this culture system which is an indicator for improved chondrogenesis. The effect of Ad.BMP-2 transduction in 3D on hMSC chondrogenesis was not as clear. On one hand, over-expression of BMP-2 increased aggrecan and SOX9 gene expression and, further, decreased Col X gene expression in the loaded group which suggests a positive effect on chondrogenesis and a negative effect on progression towards hypertrophy. Further, mechanical stimulation and transduction with Ad.BMP-2 had a synergistic effect on Col II message at the early time-point (day 7). On the other hand, transduction with Ad.BMP-2 led to a trend towards decreased GAG/DNA ratios, suggesting its use may be limited.

Chapter 5: Effect of dorsomorphin on hMSC hypertrophy.

5.1 Effect of dorsomorphin on hMSC hypertrophy – introduction.

MSCs are a commonly used cell source for cartilage TE applications. These cells can be harvested relatively easily and in a less-invasive manner, when compared to chondrocytes. MSCs can be differentiated into several cell types which belong to the mesenchymal lineage (Pittenger et al., 1999). The first study, demonstrating successful *in vitro* chondrogenic induction of MSCs, was conducted by Ashton and co-workers in 1980 (Ashton et al., 1980). In 1998, Johnstone and co-workers were able to establish a 3D micromass culture model that allows for the chondrogenic differentiation of MSCs, under defined serum-free conditions (Johnstone et al., 1998). Within this so-called pellet culture model, *in vitro* chondrogenesis of MSCs is driven by dexamethasone and TGF-β1.

One of the main drawbacks of MSCs, as a cell source for cartilage TE, is that their stable *in vitro* chondrogenic induction is still not possible. Instead, these cells advance to their terminal phenotype, the hypertrophic chondrocyte (Bosnakovski et al., 2006; Yoo et al., 1998). Additionally, after subcutaneous transplantation into SCID mice, pre-differentiated hMSC pellets underwent mineralisation (Pelttari et al., 2006). These observations resemble the sequence of events that takes place during endochondral ossification. If TE of articular cartilage using MSCs as cells source should become reality, it is absolutely necessary to block the progression towards hypertrophy. Currently, several approaches exist to prevent this hypertrophic differentiation. For instance, exogenous application of PTHrP (Kim et al., 2008; Lee and Im, 2012; Mueller et al., 2013) or co-culture of MSCs and articular chondrocytes (Fischer et al., 2010).

In recent work of Hellingman et al., a new and interesting approach was taken (Hellingman et al., 2011). The group postulated that both phosphorylated SMAD 2/3-mediated TGF-β signalling and phosphorylated SMAD 1/5/8-mediated BMP signalling are essential for early chondrogenic induction. During later stages of chondrogenesis however, the group was able to demonstrate that blocking of BMP signalling, using the small molecule dorsomorphin, led to a reduction of Col X, matrix metalloproteinase (MMP)-13 and ALP expression.

The effect of dorsomorphin on BMP-2 signalling was first described in 2008 by Yu and co-workers in a screening study which involved over 7, 500 molecules (Yu et al., 2008). The aim of the screening study was to detect substances that perturb dorsoventral axis formation in zebrafish embryos. Dorsomorphin is structurally identical to compound C, which already has been shown to antagonise the activity of adenosine monophosphate-activated kinase (Zhou et al., 2001). Yu et al., demonstrated that dorsomorphin acts by selective inhibition of the BMP type I receptors activin receptor-like kinase (ALK)2, ALK3 and ALK6 (Yu et al., 2008). Thereby, phosphorylation of SMAD 1/5/8 was inhibited and BMP signalling was selectively blocked.

As already described in section 1.5.2, BMPs and TGF-βs bind to different sub-types of type I and type II serine/threonine kinase receptors. Further, their downstream signalling is mediated by phosphorylation of different receptor-activated SMADs (SMAD 2/3 for TGF-β signalling and SMAD 1/5/8 for BMP signalling). Yet, the corresponding phosphorylated receptor-activated SMADs, of both pathways, form a complex with the same co-SMAD (SMAD 4). This SMAD complex then translocates into the nucleus and activates transcription of target genes (figure 5-1).

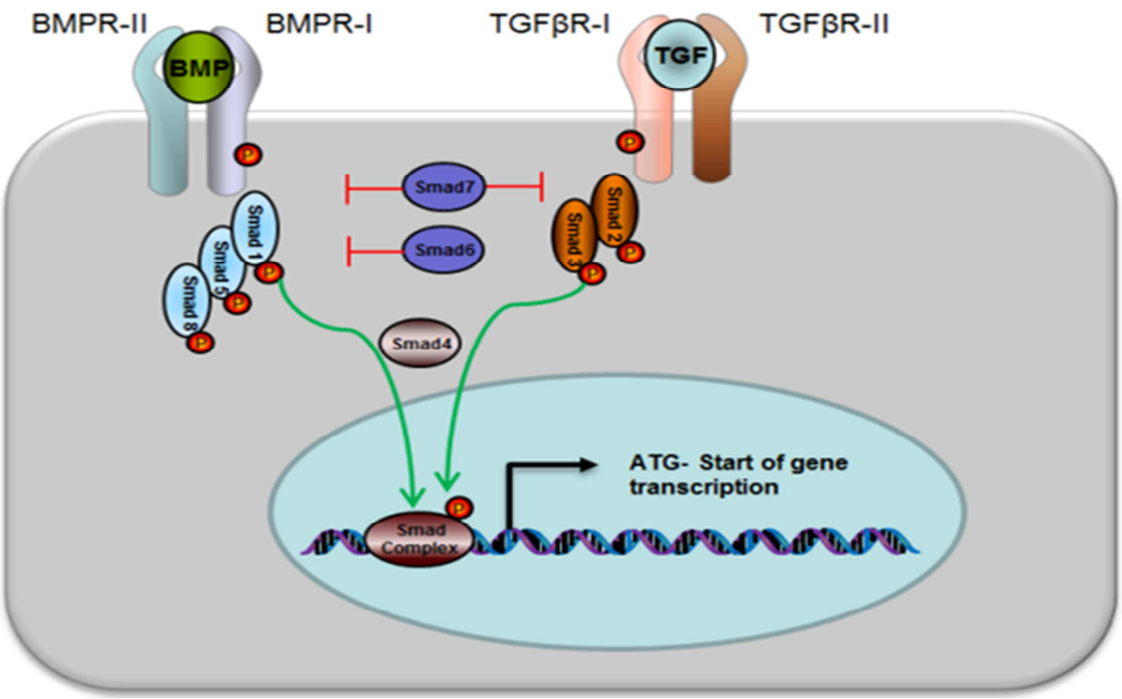

Figure 5-1: Schematic display of the BMP and the TGF-β signal transduction pathway. Both pathways use different kinds of type I and type II serine/threonine kinase receptors. Their down-stream signalling is mediated by phosphorylated SMAD 1/5/8 (BMP) or phosphorylated SMAD 2/3 (TGF-β). In both signalling pathways, the phosphorylated SMADs form a complex with SMAD 4. Afterwards, this complex translocates into the nucleus and activates target gene expression. Signal transduction can be blocked by the inhibitory SMADs 6 (BMP only) and 7 (BMP and TGF-β). Image with courtesy of Dr. Martin Stoddart (AO Research Institute Davos, AO Foundation, Davos Switzerland).

As a counter to the previous study, which investigated over-expression of BMP-2, the current study aimed to investigate the effect of dorsomorphin and thus inhibition of BMP signalling, on the chondrogenesis and progression towards hypertrophy of hMSCs. As opposed to the study of Hellingman et al., (Hellingman et al., 2011), hMSCs were cultured in fibrin-PU composite scaffolds and not as pellet cultures. First, chondrogenesis of hMSCs was induced in "classical" serum-free chondrogenic medium, containing exogenous TGF-β1. After two weeks of culture, scaffolds were divided into two groups and cultured for another two weeks. In the control group, scaffolds were further cultured under identical conditions. In the dorsomorphin group, the chondrogenic medium was supplemented with an additional 10µM of dorsomorphin. After four weeks, scaffolds were harvested. Biochemical analyses (GAG, DNA and GAG/DNA) and gene expression analyses, for genes related to chondrogenesis and progression towards hypertrophy, were conducted. Further, scaffolds were subjected to histological (Safranin-O/Fast Green staining) and immunohistochemical (for the matrix proteins aggrecan, Col II and Col X) analysis, in order to track chondrogenic and hypertrophic matrix protein production.

5.2 Effect of dorsomorphin on hMSC hypertrophy – results.

In this experiment, triplicate samples were carried out for each group and each outcome measurement (biochemical analyses, histology and gene expression analyses). The experiment was independently repeated with cells from three donors (♀ age 49, ♂ age 24 and ♀ age 20).

5.2.1 Exposure to 10µM of dorsomorphin significantly reduces DNA content.

First, it was tested if exposure to 10µM of dorsomorphin influences the DNA content (indicator for total cell number) of hMSCs seeded into fibrin-PU scaffolds and cultured in chondrogenic medium. Therefore, total DNA content was determined, from PK-digested scaffolds, using the Hoechst 33258 dye binding assay (figure 5-2). The control group (cultured for 4 weeks in standard chondrogenic medium) was compared to the dorsomorphin group (cultured for 2 weeks in standard chondrogenic medium and then for another 2 weeks in standard chondrogenic medium supplemented with an additional 10µM of dorsomorphin).

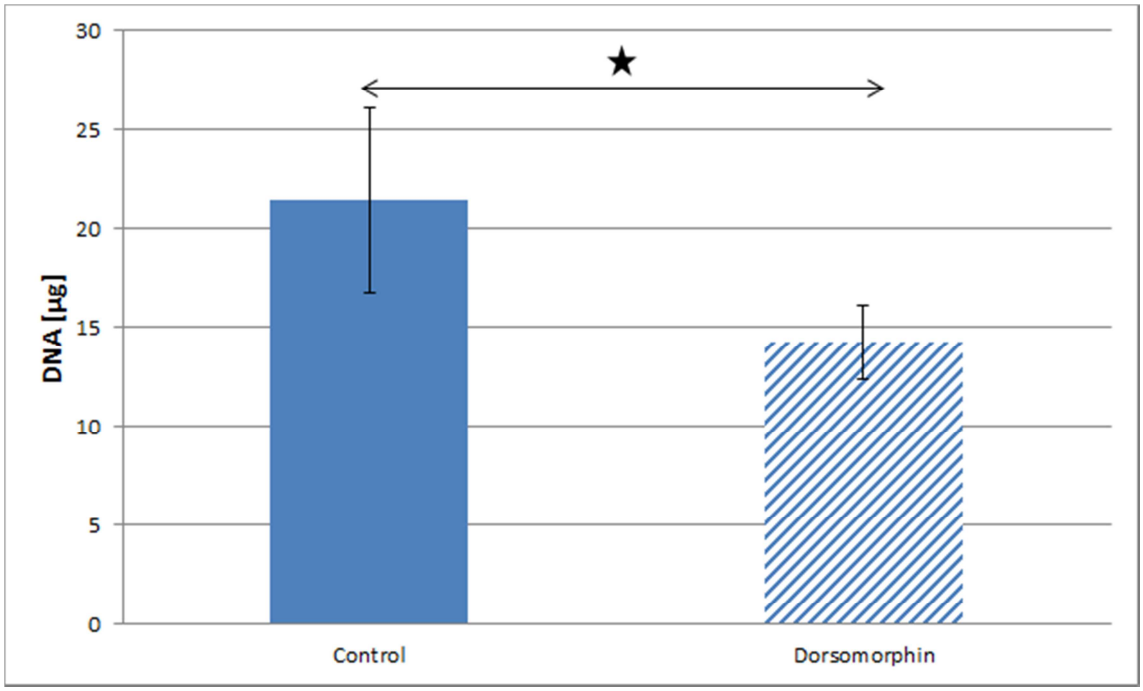

Figure 5-2: Total DNA content of hMSCs after 4 weeks of culture. hMSCs were seeded into fibrin-PU composite scaffolds. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). DNA content was quantified from scaffolds using the Hoechst 33258 dye assay with calf thymus DNA as standard. Results are displayed as average ± standard deviation of triplicates from three donors.★Significant difference between the control group and the dorsomorphin group (*p = 0.001*).

The total DNA content in the dorsomorphin group was significantly lower, when compared to the control group (*p=0.001*). Absolute values measured were 21.44µg ± 4.67µg (control) and 14.22µg ± 1.87µg (dorsomorphin).

5.2.2 The bulk amount of synthesised GAG is released into the culture medium. Significantly more GAG is produced in the control group, when compared to the dorsomorphin group.

Next, the total amount of GAG (culture medium + amount retained within the scaffolds) was quantified using the DMMB dye binding assay (figure 5-3). Thereby, matrix production in the two experimental groups was compared. Additionally, to gain more information about how matrix production changes during the individual time-points (week 1, week 2, week 3 and week 4), figure 5-4 displays the amount of GAG released into the culture medium from both groups, over the course of 4 weeks.

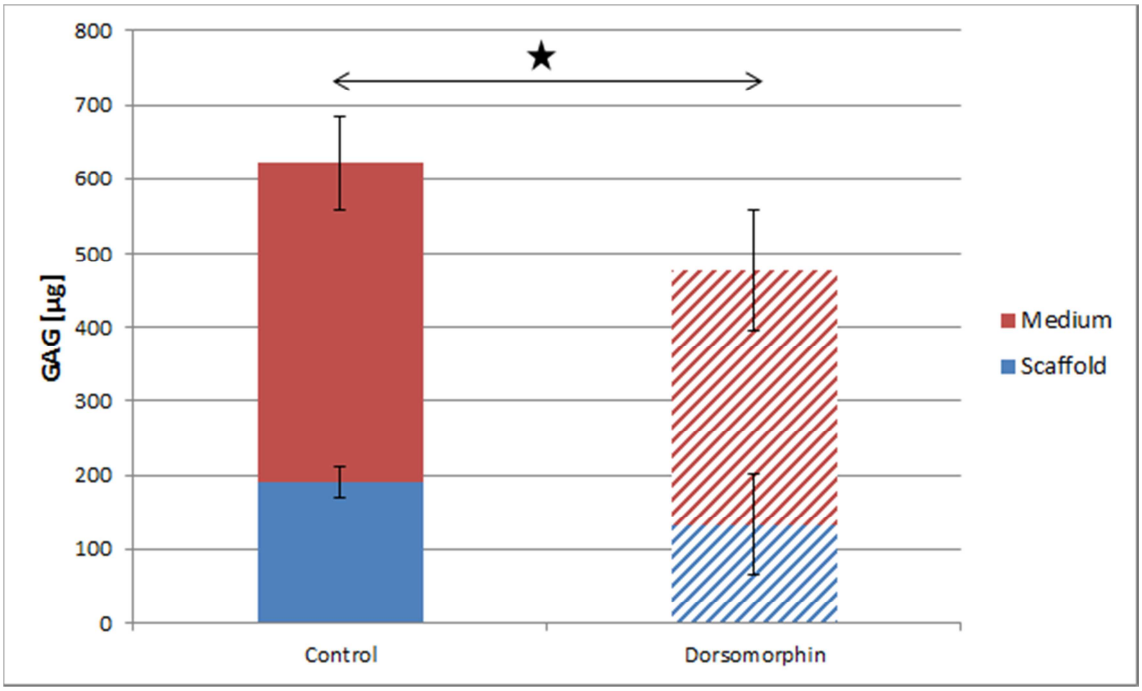

Figure 5-3: Total amount of GAG synthesised after 28 days. hMSCs were seeded into fibrin-PU composite scaffolds. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). Medium was changed and collected three times per week. It was pooled for weeks 1, 2, 3 and 4. The GAG content was quantified (both within the scaffold and within the culture medium) using the DMMB dye binding assay with chondroitin-4-sulfate as standard. Results are displayed as average ± standard deviation of triplicates from three donors. ★ Significant differences between the control group and the dorsomorphin group (*p = 0.006*).

Cells in the control group produced significantly more GAG, when compared to cells in the dorsomorphin group (*p=0.006*). The Total amount of GAG produced (medium + scaffolds) was 621.9µg ± 63.8µg (control) and 476.4µg ± 122.2µg (dorsomorphin).

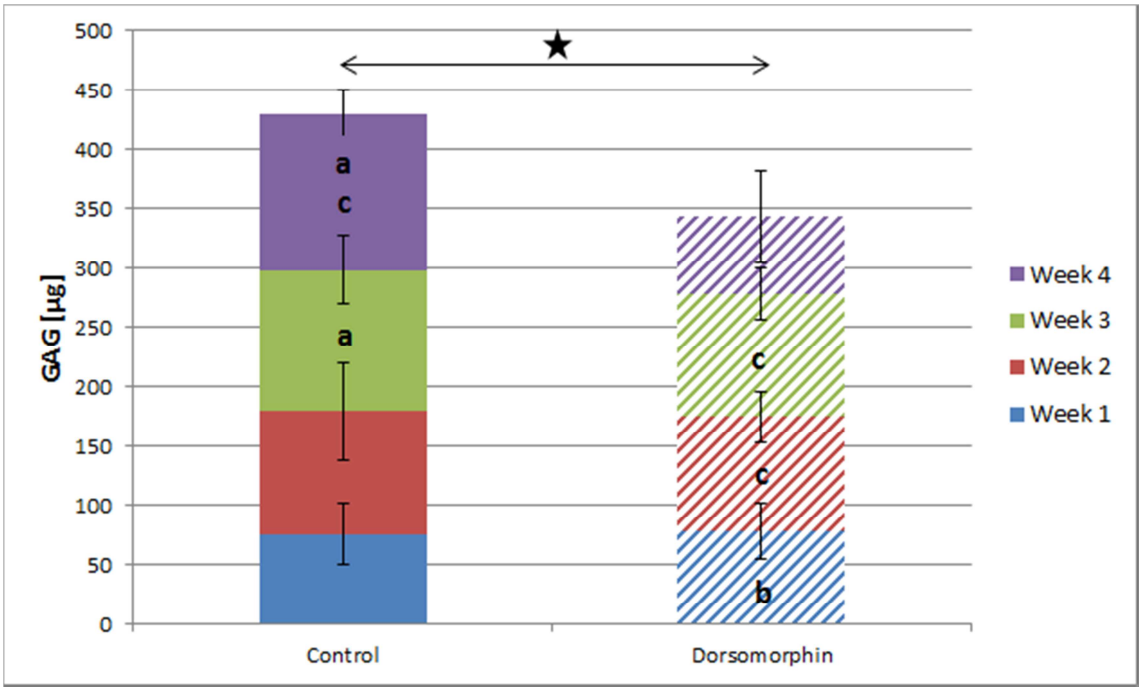

Figure 5-4: Total amount of GAG, released into the culture medium after 28 days. hMSCs were seeded into fibrin-PU composite scaffolds. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). Medium was changed and collected three times per week. It was pooled for weeks 1, 2, 3 and 4. The GAG content was quantified (from the culture medium) using the DMMB dye binding assay with chondroitin-4-sulfate as standard. Results are displayed as average ± standard deviation of triplicates from three donors.★ Significant difference in total GAG content after 4 weeks of culture ($p \leq 0.05$), [a] significantly different from the control week 1 group ($p \leq 0.05$), [b] significantly different from the dorsomorphin week 3 group ($p \leq 0.05$), [c] significantly different from the dorsomorphin week 4 group ($p \leq 0.05$).

During the first two weeks of culture, no significant differences were detected. In the control group, the GAG release increased with time and was significantly higher during week 3 ($p=0.04$) and week 4 ($p=0.000$), when compared to week 1. For the dorsomorphin group, the GAG release was highest at week 3 (significantly higher, when compared to week 1 ($p=0.031$) and week 4 $p=(0.017)$) and lowest at week 4 (significantly lower, when compared to week 2 ($p=0.045$) and week 3 ($p=0.017$)). During the fourth week of culture, the GAG production in the control group was significantly higher, when compared to the dorsomorphin group ($p=0.001$). After 4 weeks of culture, the cumulative amount of GAG released was significantly higher in the control group, when compared to the dorsomorphin group ($p= 0.008$). In the control group, the total amount of GAG, released into the culture medium, was 75.69µg ± 25.62µg (week 1), 103.89µg ± 41.03µg (week 2), 11851µg ± 28.84µg (week 3) and 132.46µg ± 20.47µg (week 4).

In the dorsomorphin group, the total amount of GAG, released into the culture medium, was 78.45µg ± 23.67µg (week 1), 96.28µg ± 21.23µg (week 2), 104.06µg ± 22.21µg (week 3) and 64.23µg ± 38.9µg (week 4).

5.2.3 The GAG/DNA ratio is not significantly different between the control and the dorsomorphin group.

In order to finalise biochemical analyses, the GAG/DNA ratio for both groups was calculated (figure 5-5). This procedure normalises GAG content (matrix production) to DNA content (indicator for total cell number) and, thereby, allows for a better comparison of matrix production between groups. For both groups, the total amount of synthesised GAG (medium + scaffolds) was divided by the total DNA content after 4 weeks of culture.

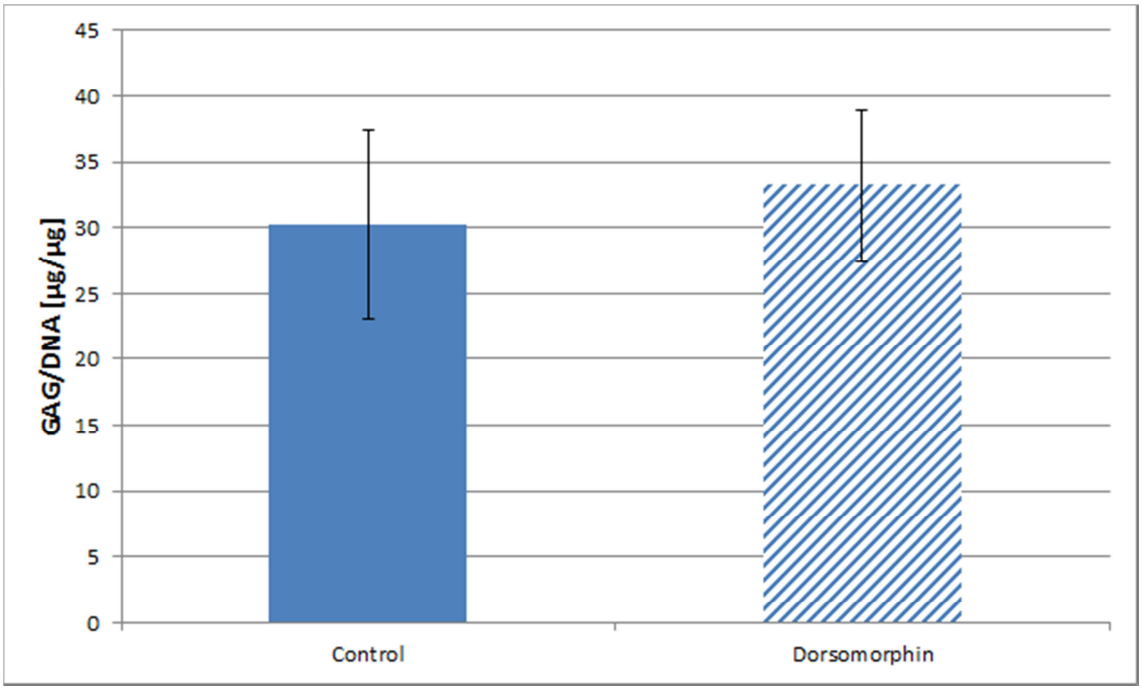

Figure 5-5: Total amount of GAG (scaffold + medium over the course of 4 weeks) normalised to DNA content. hMSCs were seeded into fibrin-PU composite scaffolds. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). The total amount of GAG and the amount of DNA was quantified. Next, GAG production was normalised to DNA content. Results are displayed as averages ± standard deviation of triplicates from three donors.

When matrix production was normalised to DNA content, no significant difference between the groups was detected. The GAG/DNA ratio (in µg/µg) was 30.19 ± 7.17 (control) and 33.22 ± 5.75 (dorsomorphin).

5.2.4 Exposure to 10µM dorsomorphin significantly reduces the expression of genes collagen I, collagen II, collagen X and aggrecan, when compared to the control group.

Subsequently, the effect of exposure to 10µM of dorsomorphin, on the gene expression profile of hMSCs, was investigated. Gene expression analyses were conducted using the comparative $\Delta\Delta C_T$ method with 18s rRNA as internal control. The control group was used as normaliser and, therefore, set to 1. The following genes were tested; Col II, aggrecan, SOX9 and BMP-2 (figure 5-6) and Col I, Col X and Runx2 (figure 5-7).

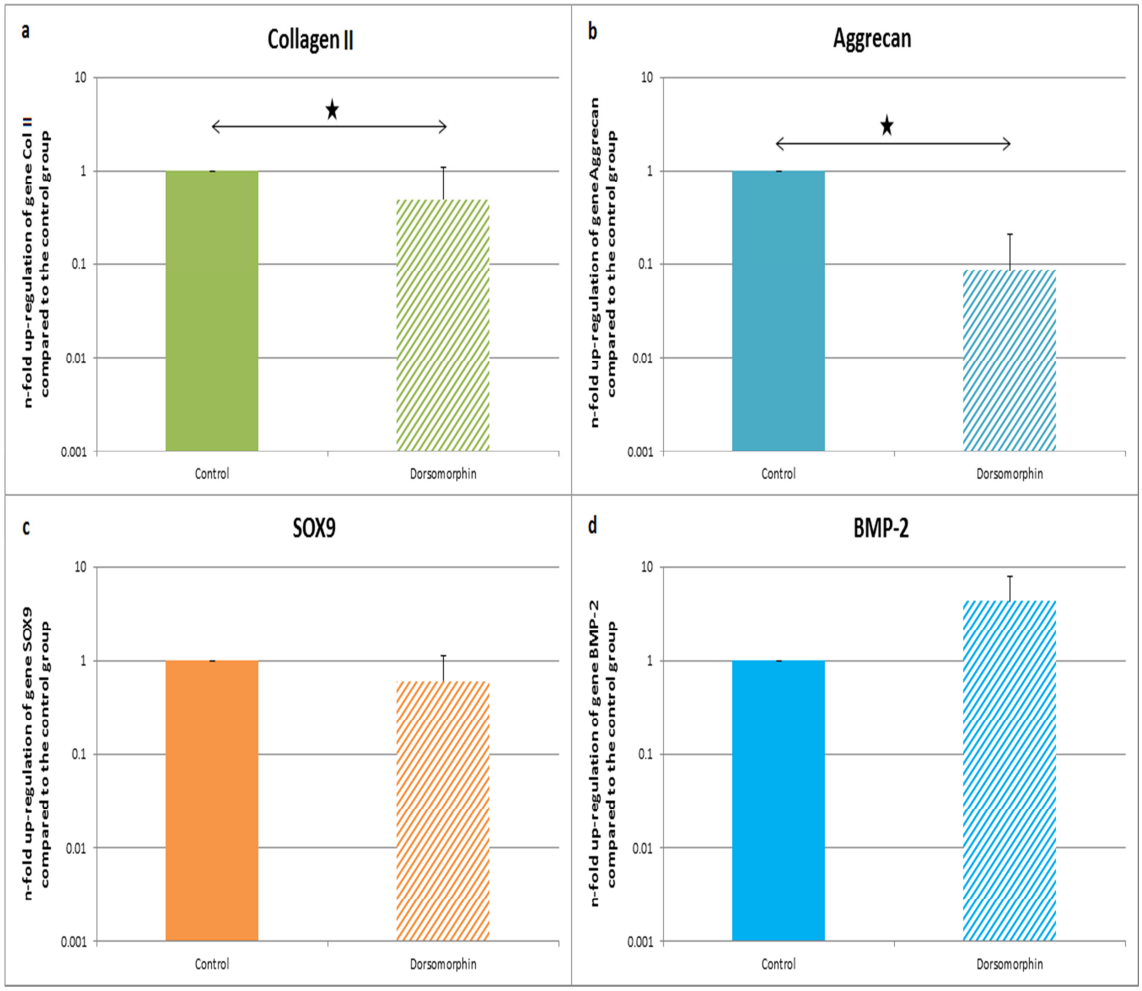

Figure 5-6: Relative gene expression of hMSCs. Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Col II (a), aggrecan (b), SOX9 (c) and BMP-2 (d). 18s rRNA was used as internal control. The control group was used as calibrator and, therefore, set to 1. Results are displayed as average + standard deviation of triplicates from three donors.★Significant differences between the control group and the dorsomorphin group ($p \leq 0.05$).

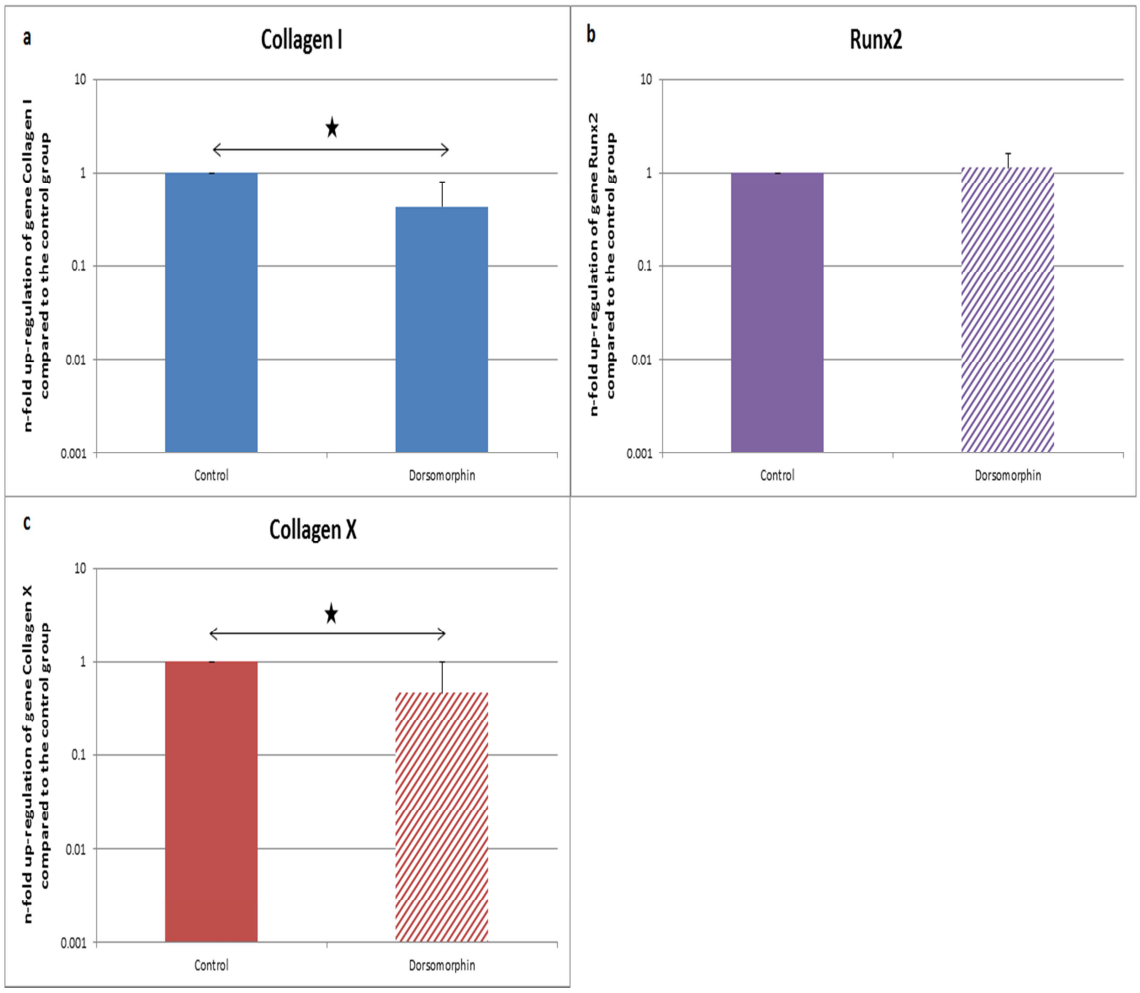

Figure 5-7: Relative gene expression of hMSCs Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (dorsomorphin). Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Col I (a), Runx2 (b) and Col X (c). 18s rRNA was used as internal control. The control group was used as calibrator and, therefore, set to 1. Results are displayed as average + standard deviation of triplicates from three donors.★ Significant differences between the control group and the dorsomorphin group ($p \leq 0.05$).

Generally, the expression of most genes investigated was down-regulated in cells exposed to 10µM of dorsomorphin, when compared to cells in the control group. Exceptions were the genes Runx2, SOX9 and BMP-2. Runx2 and SOX9 message were comparable between the control and the dorsomorphin group. For the gene BMP-2, there was no significant difference between the control and the dorsomorphin group. Yet, a trend towards an up-regulation in the dorsomorphin group (4.34-fold ± 3.68-fold elevated, when compared to the control group) was detected.

The other four genes (Col I, Col II, Col X and aggrecan) investigated were significantly down-regulated in the dorsomorphin group, when compared to the control group. There gene expression reached only 48.3% ± 59.3% (Col II, *p=0.019*), 46.1% ± 52.1% (Col X, *p=0.007*), 43.1% ± 35.3% (Col I, *p=0.001*) and 8.6% ± 12.5% (aggrecan, *p=0.000*) of the control group.

5.2.5 On the protein level, exposure to 10µM of dorsomorphin leads to a reduction in chondrogenic and hypertrophic matrix protein production.

In order to finalise the evaluation for this experiment, scaffolds were cryo-sectioned and histological and immunohistochemical analyses were conducted. This was done to track and visualise differences in matrix production between the groups on the protein level. The sections were stained with Safranin-O/Fast Green to detect negatively charged proteoglycans (figure 5-8). Further, immunohistochemical detection of the chondrogenic matrix proteins aggrecan and Col II and the hypertrophic matrix protein Col X were conducted. The experiment was independently repeated with cells from three donors. Negative controls were similar between experiments and representative sections are shown. All three donors displayed the same trend towards *in vitro* chondrogenesis (induced by 10ng/ml of exogenous TGF-β1) and towards exposure to 10µm of dorsomorphin. There were only slight variations in Col X protein labelling. Yet, the magnitude of their response was different. Cells from one donor (Pat 52, ♂ age 24) hardly responded towards the chondrogenic stimulus. One donor (Pat 51, ♀ age 49) showed a "normal" (moderate) chondrogenic response. The third donor (Pat 53, ♀ 20) underwent massive *in vitro* chondrogenesis. For immunohistochemical detection of the matrix proteins aggrecan (figure 5-9), Col II (figure 5-10) and Col X (figure 5-11), only the images of Pat 51 were shown in the results section. For this donor ("normal responder") it was easiest to detect differences in DAB labelling between the groups. The results of the remaining two donors are shown in the Appendix (section A 5).

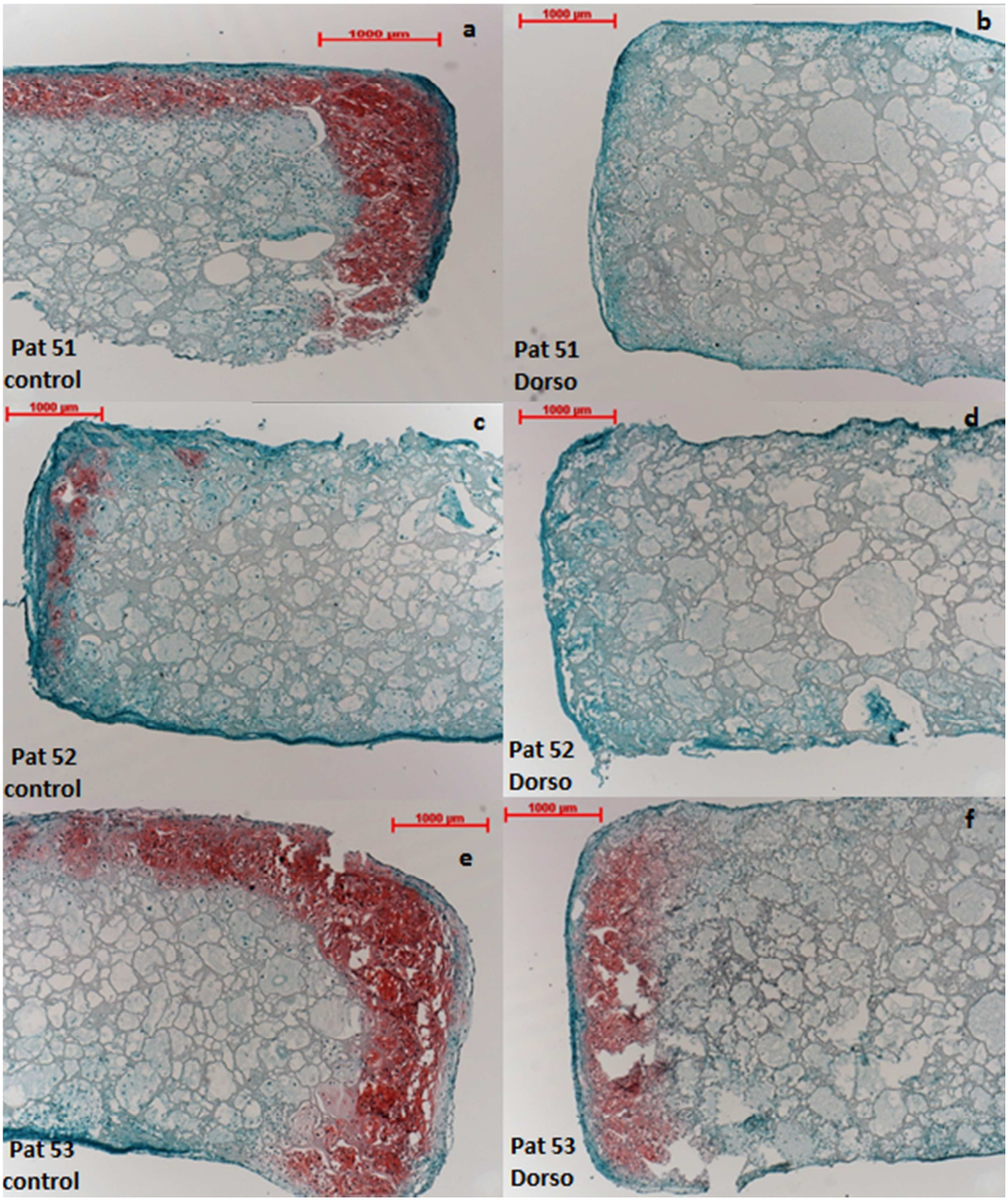

Figure 5-8: Safranin-O/Fast Green staining of hMSCs. Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Negatively charged proteoglycans were stained red with Safranin-O. Fast Green was used as counterstain to stain proteins green. Representative images for donor Pat 51 (♀ age 49, control [a], Dorso[b]), donor Pat 52 (♂ age 24, control [c], Dorso [d]) and donor Pat 53 (♀ 20, control [e], Dorso [f]) are shown.

In the control group (10ng/ml of exogenous TGF-β1), production and deposition of negatively charged proteoglycans into the ECM (on top and on the side of the PU scaffolds) was evidenced by Safranin-O staining. Pat 51 and Pat 53 showed an abundant proteoglycan production and deposition. Pat 52, however, hardly produced and deposited any proteoglycans. In the dorsomorphin group (additional application of 10µM of dorsomorphin during week 3 and week 4 of culture), proteoglycan production and deposition was completely blocked (Pat 51 and Pat 52) or significantly diminished (Pat 53).

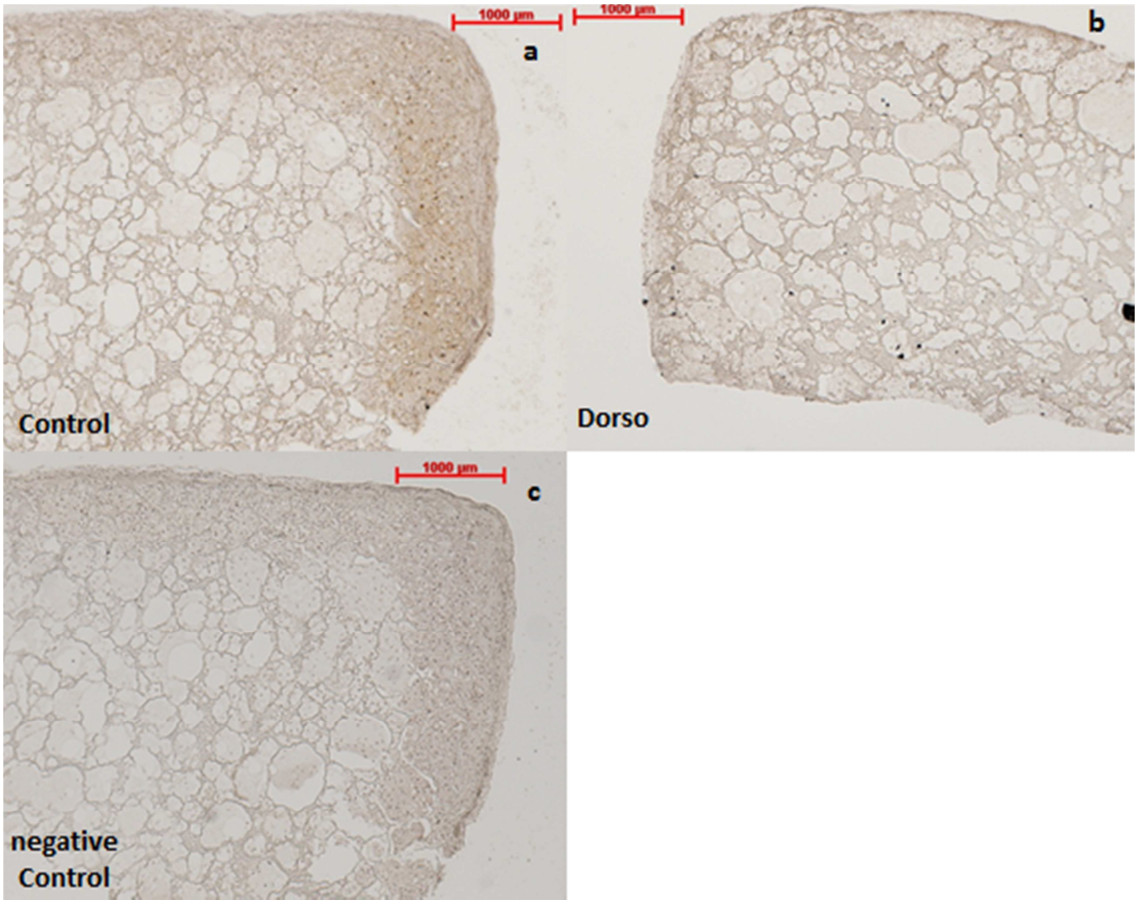

Figure 5-9: Aggrecan immunohistochemistry of hMSCs from donor Pat 51 (♀ age 49). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Aggrecan immunohistochemistry was conducted using the 1-C-6 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control [a], Dorso [b] and negative control [c]) are shown.

The result of the immunohistochemistry for the aggrecan protein confirmed the results of the Safranin-O/Fast Green staining (figure 5-8 a and figure 5-8 b). Yet, the labelling of the immunohistochemistry was not as intense as the Safranin-O/Fast Green staining. In the control group (figure 5-9 a), labelling for the aggrecan protein was detected at the same locations (on top and especially on the side of the PU scaffolds) within the freshly synthesised ECM. In the dorsomorphin group (figure 5-9 b), labelling for the aggrecan protein was no longer detectable. The negative control section (figure 5-9 c) demonstrated no unspecific binding of the secondary antibody.

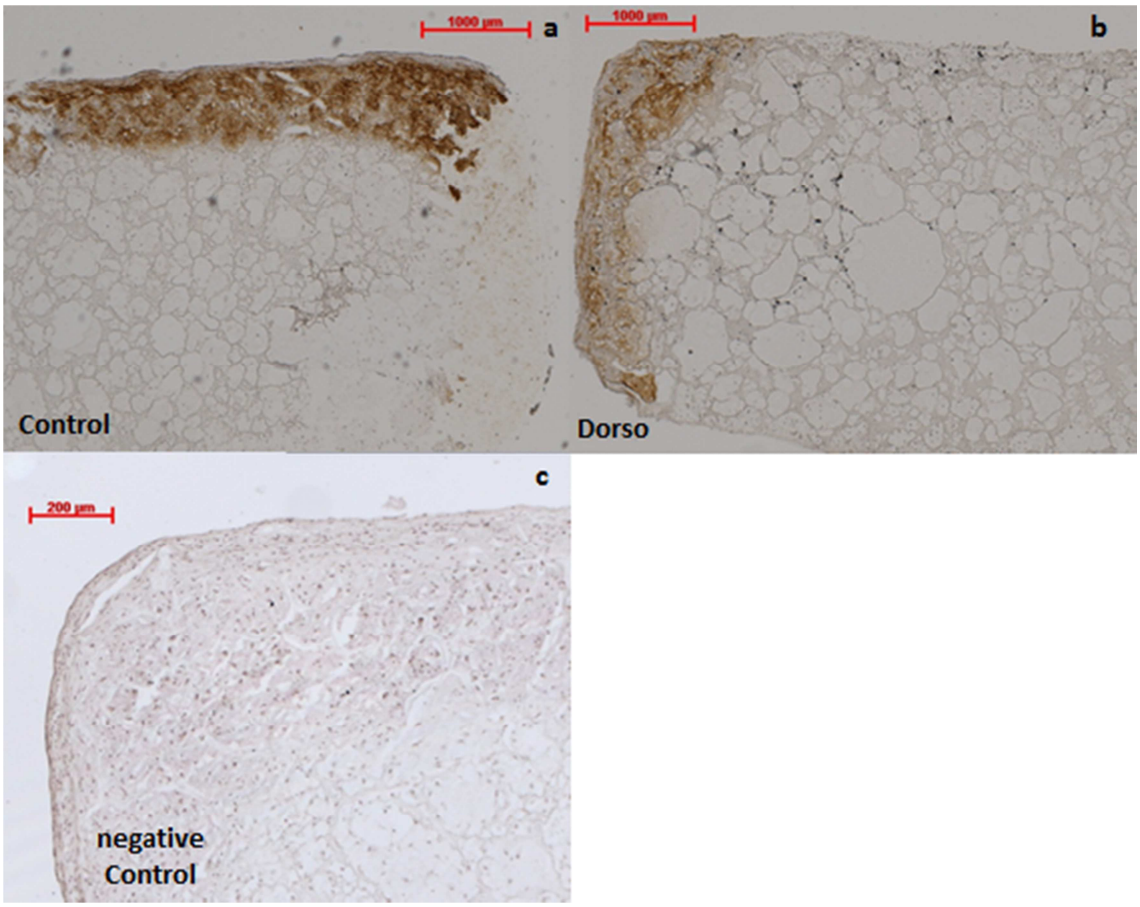

Figure 5-10: Col II immunohistochemistry of hMSCs from donor Pat 51 (♀ age 49). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col II immunohistochemistry was conducted using the CIICI antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control [a], Dorso [b] and negative control [c]) are shown.

In the control group, the Col II protein was mostly deposited in the ECM on top of the fibrin-PU composite scaffold (figure 5-10 a). At the side however, labelling for the Col II protein was not as strong. In the dorsomorphin group, the Col II labelling was less intense, when compared to the control group (figure 5-10 b). Additionally, most of the labelling was located in the ECM at the side of the scaffolds and not so much on top of the scaffold. The negative control section (figure 5-10 c) demonstrated no unspecific binding of the secondary antibody.

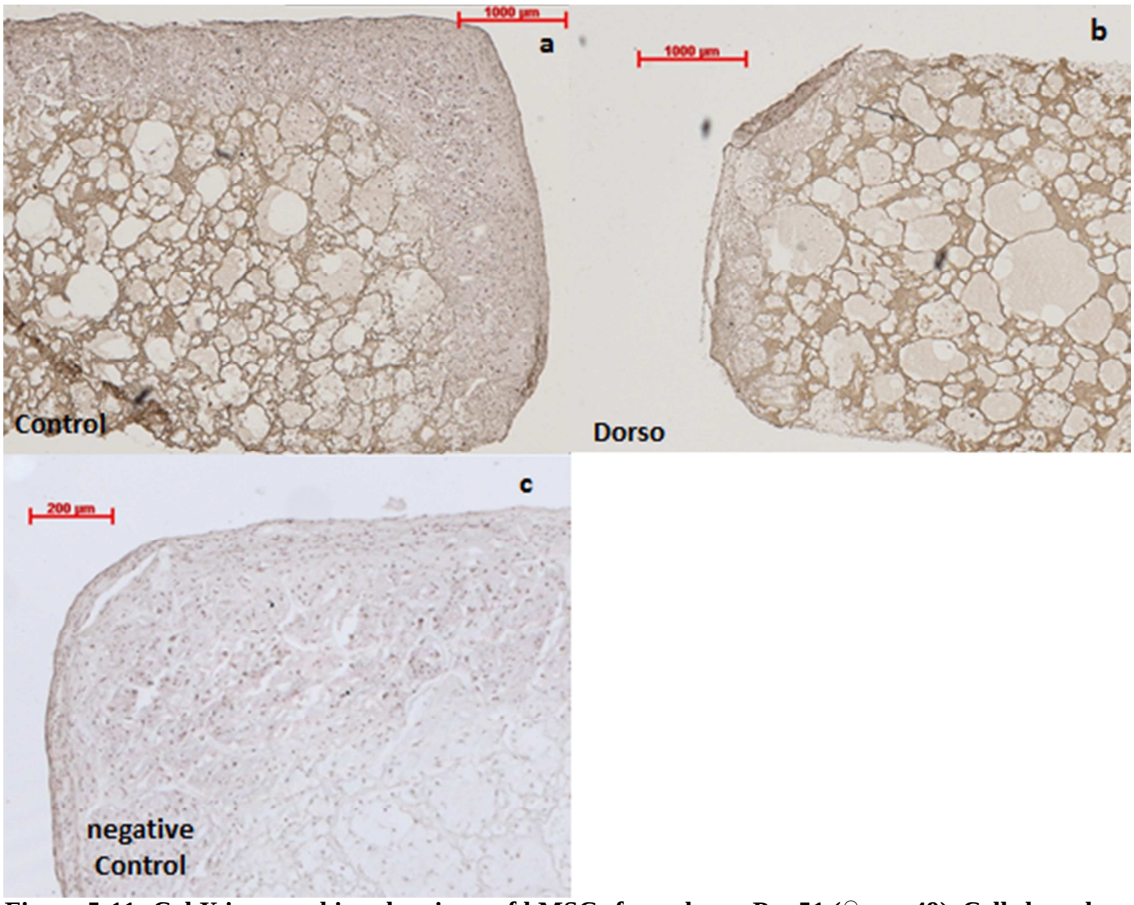

Figure 5-11: Col X immunohistochemistry of hMSCs from donor Pat 51 (♀ age 49). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col X immunohistochemistry was conducted using the Col-10 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control [a], Dorso [b] and negative control [c]) are shown. Images of the negative control sections were taken with a higher magnification.

Unspecific binding of the Col-10 antibody to the PU scaffolds was detected. This was evident as in the control and in the dorsomorphin group (figure 5-11 a and b), the PU scaffolds were strongly labelled, whereas in the negative control group (figure 5-11 c), which was not treated with the Col-10 antibody, the PU scaffold was not labelled. Yet, the unspecific labelling of the PU scaffold was clearly distinguishable from specific labelling for the Col X protein within the freshly synthesised matrix. In the control group (figure 5-11 a), labelling for the Col X protein was detectable within the ECM. This labelling was reduced in the dorsomorphin group (figure 5-11 b). The negative control section (figure 5-11 c) demonstrated no unspecific binding of the secondary antibody.

5.3 Effect of dorsomorphin on hMSC hypertrophy – discussion.

The fact that a stable *in vitro* chondrogenic differentiation of MSCs is currently not yet possible represents a major limitation for their use as cell source in cartilage TE. Instead, these cells tend to undergo terminal differentiation into hypertrophic chondrocytes (Bosnakovski et al., 2006; Pelttari et al., 2006; Yoo et al., 1998). There exist several approaches to prevent this hypertrophic differentiation, such as application of PTHrP or co-culture with mature articular chondrocytes (Fischer et al., 2010; Kim et al., 2008; Lee and Im, 2012; Mueller et al., 2013). Recently, a different approach was taken by Hellingman and co-workers. The group was able to demonstrate that specific blocking of phosphorylated SMAD 1/5/8 signalling, after the onset of chondrogenic differentiation, reduced expression of MMP 13, Col X and ALP (Hellingman et al., 2011). The group used the pellet culture model and dorsomorphin as agent for blocking of phosphorylated SMAD 1/5/8 (BMP) signalling. Based on these promising results, it was of interested to determine the effect of dorsomorphin on hMSCs chondrogenesis and progression towards hypertrophy, within the fibrin-PU scaffold system. Previously, it already has been demonstrated that this scaffold system compares favourably to the classic pellet culture model, in terms of gene expression related to endochondral ossification (Li et al., 2009a). It was hypothesised that, by administration of dorsomorphin after 2 weeks of culture, hypertrophic differentiation of hMSCs can be reduced or supressed, without affecting chondrogenesis. hMSCs were encapsulated in fibrin and seeded into 2 x 8 mm PU scaffolds. A total of 2.5 x 10^6 cells were seeded per scaffold. Scaffolds were cultured in 12-well plates. During the first two weeks of culture, cells were cultured in classical serum-free chondrogenic medium with 10ng/ml of exogenous TGF-β1. The medium was changed three times per week and pooled and collected for week 1, week 2, week 3 and week 4. After 2 weeks, cells were divided into two groups. The first group (control) was cultured under identical conditions. In the second group (dorsomorphin), the culture medium was additionally supplemented with 10µM of dorsomorphin. After 4 weeks of culture, samples were harvested. Biochemical analyses (GAG, DNA and GAG/DNA) and gene expression analyses (Col I, Col II, Col X, aggrecan, BMP-2, SOX9 and Runx2) were conducted. Additionally, the presence and distribution of matrix proteins was analysed using histology (Safranin-O/Fast Green) and immunohistochemistry (for the matrix proteins aggrecan, Col II and Col X).

Quantification of the total DNA content, after 4 weeks of culture, demonstrated that exposure to 10 µM of dorsomorphin significantly diminished the total DNA content. The DNA content (indicator for total cell number) was significantly lower in the dorsomorphin group, when compared to the control group. This result indicates that exposure to dorsomorphin led to an increase in cellular death and/or a reduction in proliferation of hMSCs, when compared to cells cultured under control conditions. This finding had major implications for the discussion of the remaining data. The reduced chondrogenic differentiation potential was likely a direct consequence of the cytotoxic effect that was observed, when hMSCs were exposed to 10 µM of dorsomorphin in the fibrin-PU composite scaffold system.

The total amount of GAG, secreted into the culture medium was significantly lower in the dorsomorphin group, when compared to the control group. In order to gain a more detailed insight into GAG synthesis, the amount of GAG, secreted into the culture medium, was compared between the individual weeks. Both differences between different time-points within the same group and differences between the same time-point in the different groups were investigated. During the first two weeks of culture, no significant differences were detected. In the control group, GAG synthesis increased with time. This led to a significantly higher GAG release during both week 3 and week 4 of culture, when compared to the first week of culture. In the dorsomorphin group, similar observations were made during the first three weeks of culture. Again, significantly more GAG was produced during week 3 of culture, when compared to week 1 of culture. Yet, during the fourth week of culture, GAG production was significantly diminished in the dorsomorphin group. The total amount of GAG, secreted into the culture medium, was significantly lower, when compared to the control group at week 4 and the dorsomorphin group at week 2 and week 3. These data demonstrated that the significant decrease in total GAG content, between both groups, originated mainly from the differences that were present during the fourth week of culture. The cells either reduced their GAG production starting from the second week after exposure to dorsomorphin or they already started to reduce the GAG production immediately after exposure to dorsomorphin and also started to degrade their ECM at the same time. The GAGs from the degraded matrix would then be released into the culture medium and, if still sulphated, would be detected by the DMMB assay. This hypothetical release could compensate for the reduced GAG production, after exposure to dorsomorphin.

This hypothesis would explain why the amount of GAG, released into the culture medium, was not different between the control and the dorsomorphin group during the third week of culture (reduced production and partial compensation by release of degraded ECM).

When the total GAG production was normalised to the DNA content, no significant differences between the control and the dorsomorphin group were detected. Both the total amount of GAG produced and the DNA content were reduced by approximately one third in the dorsomorphin group, when compared to the control group. This result indicated that the matrix production, on the single cell basis, was not different between the dorsomorphin and the control group. Yet, this result will have to be interpreted with caution. It can be assumed that the DNA content, during the first two weeks of culture, was not different between the control group and the dorsomorphin group, as both groups were cultured under identical conditions. Then, after exposure to dorsomorphin, cells started to die and/or stopped proliferating within the dorsomorphin group, which resulted in the observed difference in total DNA content between the groups. However, for the calculation of the GAG/DNA ratio, the total amount of GAG and the DNA content, after 4 weeks of culture, were used. This might have lead to a false high GAG/DNA ratio within the dorsomorphin group. During the first two week of culture, more cells were actually producing the GAG. Still the lower DNA content, after 4 weeks of culture, had to be used for the calculation of the GAG/DNA ratio. In retrospect, it would have been ideal to additionally harvest some samples after 2 weeks and 3 weeks of culture, quantify their DNA content and use these values for the calculation of the GAG/DNA ratio during the first two weeks of culture respectively the third week of culture. Thereby, it could have definitely been determined if exposure to dorsomorphin leads to fewer cells that produce the same amount of matrix or to fewer cells that additionally produce less matrix. Unfortunately, during the initial study design, these considerations were not taken into account. It was not expected that the exposure of hMSCs, seeded into fibrin-PU composite scaffolds, to 10µM of dorsomorphin leads to a cytotoxic effect.

Gene expression analyses of fibroblastic (Col I), chondrogenic (SOX9, aggrecan, Col II) and hypertrophic (Col X) markers showed a general trend towards a decrease in gene expression within the dorsomorphin group. This trend reached significance for the genes aggrecan, Col II and Col X and indicated a decreased chondrogenic and hypertrophic response of these cells, when compared to control cells. SOX9 (chondrogenic transcription factor) and Runx2 (osteogenic transcription factor) expression levels were not significantly influenced. This observation indicates that the decrease of aggrecan message was mediated through a mechanism independent of SOX9 and that the decrease of Col X message was mediated through a mechanism independent of Runx2.

Histological and immunohistochemical evaluation of matrix proteins demonstrated that hMSCs had a reduced chondrogenic and hypertrophic differentiation potential, after exposure to 10 µM of dorsomorphin. As already mentioned, the reduced differentiation potential was likely a direct consequence of the cytotoxic effect that was observed, when hMSCs were exposed to 10 µM of dorsomorphin in the fibrin-PU composite system. In two out of three donors, Safranin-O staining was completely absent in the dorsomorphin group. This result was confirmed using immunohistochemistry for the aggrecan protein. In the third donor, exposure to dorsomorphin clearly reduced both Safranin-O staining and labelling for the aggrecan protein. Immunohistochemistry for the protein Col II confirmed this negative influence of dorsomorphin. In two out of three donors, immunolabelling for the Col II protein was markedly reduced in the dorsomorphin group, when compared to the control group. In the donor with the lowest chondrogenic response, the weak immunolabelling for the Col II protein, which was detected within the control group, was absent after exposure to dorsomorphin. Immunolabelling for the hypertrophic matrix protein Col X showed similar results. Again, exposure to dorsomorphin led to a reduction of Col X protein labelling (in two out of three donors) or to the complete absence of Col X protein labelling (in the donor with the weakest chondrogenic response). It must be noted that the results of the Safranin-O staining and the immunohistochemistry for the aggrecan protein seemed to conflict with the biochemical analyses. The GAG/DNA ratio was not different between the control and the dorsomorphin group. Yet, as already discussed, the results of the GAG/DNA ratio have to be interpreted with caution.

Further, even if the GAG to DNA ratio was comparable between the groups, the exposure to 10 µM of dorsomorphin led to a significant reduction in cell number. Therefore, fewer cells were present which produced less total GAG, as reflected by histology and immunohistochemistry. On top of that, only the GAG that was secreted into the culture medium was measured. On the other hand, histology and immunohistochemistry detect the proteins within the ECM or within the scaffold. A possible explanation might be that exposure to dorsomorphin induced matrix degradation. Thereby, the matrix that was already built, during the first two weeks of culture, would be degraded and released into the culture medium. If the GAG fragments were still sulphated, they would, nevertheless, be detected by the DMMB assay. Further, it might be that two weeks of culture are not sufficient to build a cartilaginous ECM. After exposure to dorsomorphin, Col II and aggrecan gene expression were significantly down-regulated. Therefore, the matrix proteins which were detected within the dorsomorphin group might only be the result of matrix production within the first two weeks of culture. In the control group however, the cells would have another two weeks to build their ECM. Given that exposure to dorsomorphin significantly reduced the total cell number, this explanation is not unlikely. Cells that are stressed and start to undergo cell death stop to produce matrix. Hellingman et al., made similar observations in their pellet culture study (Hellingman et al., 2011). Again, they detected differences in chondrogenic response, depending on the particular donor. If labelling for the Col II protein was not evident after 14 days of culture, exposure to dorsomorphin diminished and almost completely blocked Col II protein incorporation. Only very little Col II protein was then evident in the pellets after 5 weeks of culture. However, if the matrix was already well developed after 2 weeks of culture, dorsomorphin had no negative effect on further Col II protein incorporation. Consistently, when the fibrin-PU composite scaffold was established and compared to the pellet culture model, labelling for the matrix proteins Col II and aggrecan was only weakly or moderately evidenced after two weeks of culture (Li et al., 2009a).

In summary, it was demonstrated that exposure to 10µM of dorsomorphin led to a cytotoxic effect, on hMSCs seeded into fibrin-PU composite scaffolds. This cytotoxic effect is most likely the cause for the reduced differentiation potential, which was observed, in the dorsomorphin group.

The total GAG production was reduced, when compared to the control group. The GAG/DNA ratio was not significantly different between the two groups. Still, as already discussed, this result has to be interpreted with caution. Gene expression analyses revealed that the dorsomorphin group displayed a significant reduction in Col I, Col II, aggrecan and Col X expression, when compared to the control group. Histology and immunohistochemistry demonstrated that, depending on the donor, exposure to 10 µM of dorsomorphin led to a matrix production (both chondrogenic and hypertrophic) that was either decreased or completely abolished. Possible explanations might be matrix degradation or a decrease in matrix protein production and incorporation. Both effects would likely be a direct consequence of to the cytotoxic effect that was observed. It must be noted that these results conflict with the study of Hellingman et al., (Hellingman et al., 2011). This group could demonstrate that, at least for some of the investigated donors, exposure to dorsomorphin led to a decreased hypertrophic differentiation without negatively affecting the chondrogenic response. In the current study, exposure to dorsomorphin led to an increase in cell death and/or a reduced proliferation potential of hMSCs, which then likely led to the observed general reduction in differentiation potential. Other reasons for these observations, which are far less likely, might be differences in culture time (4 weeks in this study, 5 weeks in the Hellingman study) or chondrogenic inducer (TGF-β1 in this study, TGF-β2 in the Hellingman study). In 2001, Barry et. al., were able to show that TGF-β2 is a more potent chondrogenic inducer (using hMSCs in the pellet culture model), when compared to TGF-β1 (Barry et al., 2001). Summarised, the dorsomorphin concentration has been chosen according to the study of Hellingman et al., (Hellingman et al., 2011), which used the pellet culture model where cells are densely packed. In contrary, the fibrin-PU composite system allows for better medium penetration, when compared to the densely packed pellets. This difference probably led to a higher actual dorsomorphin concentration within the fibrin-PU composite system, which then likely led to the observed negative effect on total cell number. It would be necessary to conduct a dose-response test and determine a concentration of dorsomorphin, which does not longer display a negative effect on total cell number. Then, this concentration could be used to analyse the effect of dorsomorphin on hMSC chondrogenesis and their progression towards hypertrophy in the fibrin-PU composite system.

Chapter 6: Co-culture of hMSCs and hACPCs.

6.1 Co-culture of hMSCs and hACPCs – introduction.

Most studies in cartilage TE focus on the application of a single cell type, such as chondrocytes, MSCs or ACPCs. Currently, a large amount of work is carried out in the field with the aim to detect the best possible starting cell source for cartilage TE or with the aim to improve the chondrogenic differentiation of a specific cell source. In the last couple of years, co-cultures of more than one cell type are starting to gain an increasing amount of attention (reviewed for example in: (Leijten et al., 2012)). It is believed that the different cell types can (beneficially) influence each other during differentiation.

The following section briefly highlights a few examples for different co-culture models in cartilage TE. These co-cultures can be derived from the same cell type in a different phenotypic state. For example, primary and passaged chondrocytes can be co-cultured with the aim to aid re-differentiation of de-differentiated (passaged) chondrocytes (Gan and Kandel, 2007). Yet, usually, these co-culture models consist of two different cell types, most often articular chondrocytes and MSCs. For example, it has been shown that chondrocytes can create a chondrogenic niche in a subcutaneous environment. This feature then directs chondrogenic induction of MSCs (Liu et al., 2010). Further, human articular chondrocytes have been shown to suppress hypertrophy of hMSCs in a pellet co-culture model (Fischer et al., 2010).

The discovery of ACPCs that can be derived from bovine (Dowthwaite et al., 2004), equine (McCarthy et al., 2011) and human (Williams et al., 2010) articular cartilage introduces a new, potentially powerful, cell source for cartilage TE applications. It already has been demonstrated that equine ACPCs compare favourably to equine MSCs, during TGF-β1-driven *in vitro* chondrogenic differentiation in the pellet culture model. MSCs underwent hypertrophy. In contrary, ACPCs underwent stable chondrogenesis with no signs of progression towards hypertrophy (McCarthy et al., 2011). Unfortunately, it is not guaranteed that these exciting results directly translate from equine to human. Therefore, the possibility that hACPCs behave differently cannot be excluded.

This led to the development of the following study which had two main aims. First, it was investigated if the positive results, obtained using equine ACPCs, can be translated to hACPCs. Therefore, hMSCs and hACPCs were individually cultured in the pellet culture model. Chondrogenesis was induced using the "classic", serum-free chondrogenic medium containing exogenous TGF-β1. Second, it was of interest how co-cultures of these cells behave. The hypothesis was that the cell populations might influence each other during chondrogenic differentiation within co-culture. In order to test this hypothesis, hMSCs and hACPCs were mixed in varying ratios and cultured as pellets. Again, chondrogenic induction was performed using the "classic", serum-free chondrogenic medium containing exogenous TGF-β1. Cells were cultured for three weeks as pellets containing 250, 000 cells. Ninety-six-well V-bottom plates were used for culture. In one repeat of the experiment, cells were labelled with fluorescent dyes in order to track if the ratio between the two cell populations remains constant during co-culture. The fluorescently labelled cell pellets were harvested after 7 and 21 days. Additionally, after 3 weeks of culture, pellets were harvested and chondrogenesis and progression towards hypertrophy were monitored using several assays. Biochemical analyses (GAG, DNA and GAG/DNA) were conducted in order to track matrix production. Further, the expression of chondrogenic and hypertrophic marker genes was evaluated. Additionally, the matrix protein production of pellets was evaluated histologically (Safranin-O/Fast Green staining) and immunohistochemically (for the proteins aggrecan, Col II and Col X). Finally, the secretome of hMSCs and hACPCs was analysed in order to track differences in their cytokine release profile.

6.2 Co-culture of hMSCs and hACPCs – results.

For this experiment, cells were seeded into 96-well V-bottom plates (Corning Incorporated, Corning, NY) according to table 6-1. Seven different groups were examined. In run I, triplicate samples were carried out per group (for GAG quantification from medium and gene expression analyses). In run II, six samples were carried out per group (triplicates for biochemical analyses and gene expression analyses). In run III, nine samples were carried out per group (triplicates for biochemical analyses, gene expression analyses and histology). In run IV, triplicate samples were carried out per group for biochemical analyses. The following donors were used for the different runs (run 1: ♀ age 55 for hMSCs and ♂ age 75 for hACPCs, run 2: ♀ age 20 for hMSCs and ♂ age 33 for hACPCs, run 3: ♀ age 86 for hMSCs and ♂ age 56 for hACPCs, run 4: ♂ age 24 for hMSCs and ♂ age 56 for hACPCs). In the fourth run, hACPCs (♂ age 56) and hMSCs (♂ age 24) were additionally labelled with fluorescent dyes. It would have been ideal to us hACPCs and hMSCs from the same donor, Unfortunately, that was not possible (hACPCs were kindly provided by Prof. Archers group at Cardiff University. hMSCs were used from the common stock at the AO Research Institute Davos, Davos, Switzerland).

Group	hMSCs [x 1 000 cells]	hACPCs [x 1 000 cells]
100% hMSCs	250	0
90% hMSCs + 10% hACPCs	225	25
75% hMSCs + 25% hACPCs	187.5	62.5
50% hMSCs + 50% hACPCs	125	125
25% hMSCs + 75% hACPCs	62.5	187.5
10% hMSCs + 90% hACPCs	25	225
100% hACPCs	0	250

Table 6-1: Seeding scheme for the seven different groups, which were investigated during the co-culture of hMSCs and hACPCs study.

The secretome analysis of hMSCs and hACPCs was independently repeated with triplicates samples for each time-point and cells from two donors (♀ age 40 and ♀ age 49 for hMSCs, ♂ age 75 and ♀ age 30 for hACPCs). Population doublings were not tracked for the secretome analysis.

6.2.1 The DNA content does not differ significantly between the different groups.

First, it was of interest to investigate if co-culture of hMSCs and hACPCs affect the total DNA content (indicator for total cell number). Thus, after 3 weeks of culture, the total DNA content was quantified, from PK-digested pellets, using the Hoechst 33258 dye assay. Figure 6-1 displays the DNA content for each of the seven experimental groups.

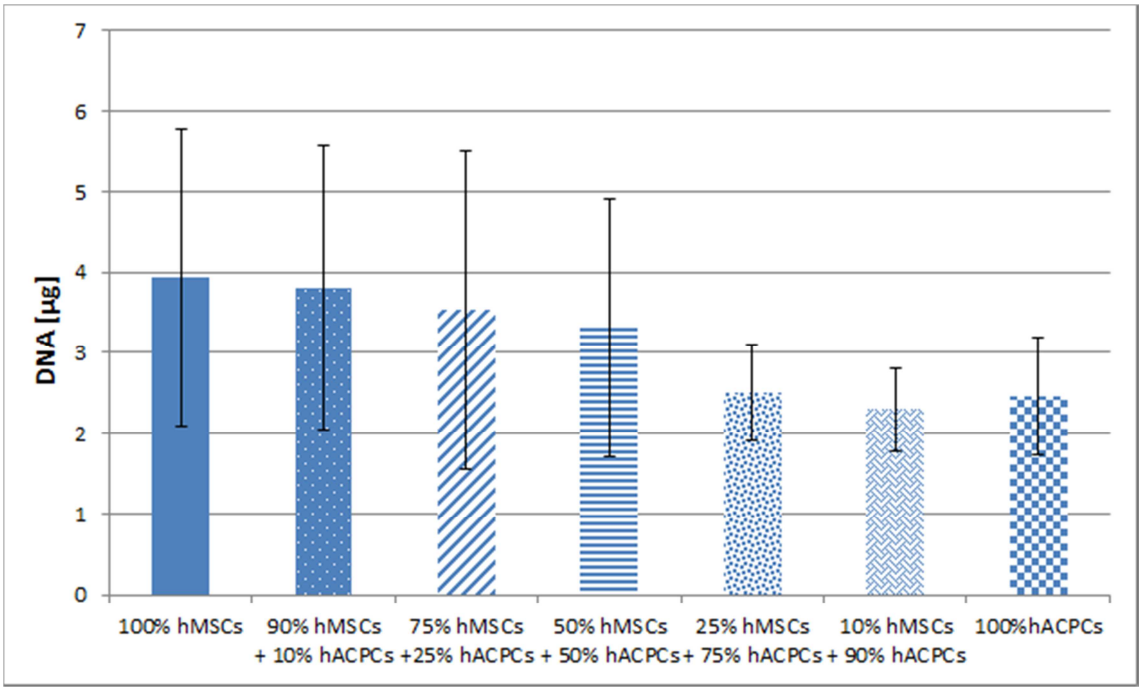

Figure 6-1: Total DNA content after 21 days. hMSCs and hACPCs were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. DNA content was quantified, from PK-digested pellets, using the Hoechst 33258 dye assay with calf thymus DNA as standard. Results are displayed as average ± standard deviation of triplicates from three experiments.

There was a general trend towards a decrease in total DNA content with increasing percentage of hACPCs within the co-culture pellets. Yet, this trend did not reach statistical significance. Total values measured were 3.94µg ± 1.83µg (100% hMSCs), 3.81µg ± 1.76µg (90% hMSCs + 10% hACPCs), 3.54µg ±1.98µg (75% hMSCs + 25% hACPCs), 3.31µg ± 1.6µg (50% hMSCs + 50% hACPCs), 2.51µg ± 0.59µg (25% hMSCs + 75% hACPCs), 2.3µg ± 0.5µg (10% hMSCs + 90% hACPCs) and 2.46µg ± 0.72µg (100% hACPCs).

6.2.2 The total amount of GAG, secreted into the culture medium, decreases with increasing percentage of hACPCs within the co-culture pellets.

Next, the amount of GAG, as an indicator for matrix production, was determined using the DMMB dye assay. Total amount of GAG, synthesised and secreted into the culture medium, was quantified for each of the seven experimental groups (figure 6-2). Significant differences between groups were tracked for the combined release of GAG, over the course of three weeks.

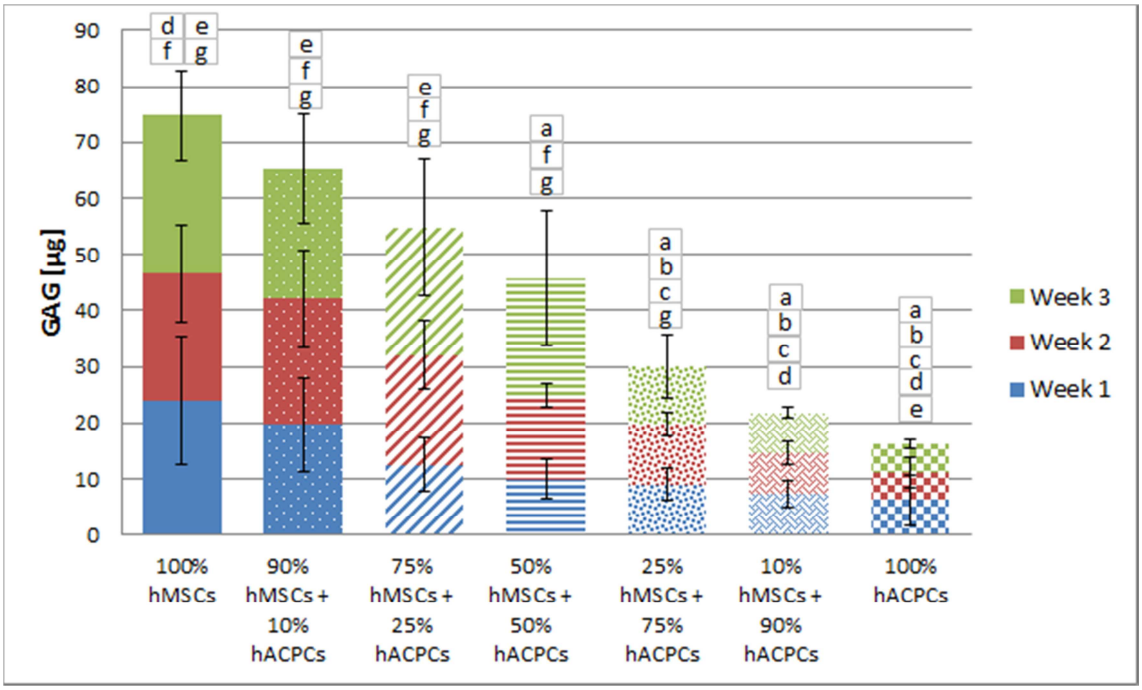

Figure 6-2: Total amount of GAG, released into the culture medium, after 21 days. hMSCs and hACPCs were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. The total amount of GAG was quantified using the DMMB dye binding assay with chondroitin-4-sulfate as standard. Results are displayed as average ± standard deviation of triplicates from three experiments. [a] significantly different from the 100% hMSCs group ($p \leq 0.05$), [b] significantly different from the 90% hMSCs + 10% hACPCs group ($p \leq 0.05$), [c] significantly different from the 75% hMSCs + 25% hACPCs group ($p \leq 0.05$), [d] significantly different from the 50% hMSCs + 50% hACPCs group ($p \leq 0.05$), [e] significantly different from the 25% hMSCs + 75% hACPCs group ($p \leq 0.05$), [f] significantly different from the 10% hMSCs + 90% hACPCs group ($p \leq 0.05$), [g] significantly different from the 100% hACPCs group ($p \leq 0.05$).

The total amount of GAG, secreted into the culture medium, decreased with increasing percentage of hACPCs within the co-culture pellets. For an overview about significant differences between groups see also table 6.2. The 100% hMSCs group had the highest GAG content and the 100% hACPCs group had the lowest GAG content, among all groups investigated. The following statistically significant differences in GAG content were observed; the 100% hMSCs group was higher than the 50% hMSCs + 50% hACPCs group (*p=0.001*), the 25% hMSCs + 75% hACPCs group (*p=0.000*), the 10% hMSCs + 90% hACPCs group (*p=0.000*) and the 100% hACPCs group (*p=0.000*). The 90% hMSCs + 10% ACPCs group was higher than the 25% hMSCs + 75% hACPCs group (*p=0.007*), the 10% hMSCs + 90% hACPCs group (*p=0.002*) and the 100% hACPCs group (*p=0.001*). The 75% hMSCs + 25% hACPCs group was higher than the 25% hMSCs + 75% hACPCs group (*p=0.015*), the 10% hMSCs + 90% hACPCs group (*p=0.002*) and the 100% hACPCs group (*p=0.000*). The 50% hMSCs + 50% hACPCs group was higher than the 10% hMSCs + 90% hACPCs group (*p=0.004*) and the 100% hACPCs group (*p=0.001*) but lower than the 100% hMSCs group (*p=0.001*). The 25% hMSCs + 75% hACPCs group was higher than the 100% hACPCs group (*p=0.020*) but lower than the 100% hMSCs group (*p=0.000*), the 90% hMSCs + 10% hACPCs group (*p=0.007*) and the 75% hMSCs + 25% hACPCs group (*p=0.015*). The 10% hMSCs + 90% hACPCs group was lower than the 100% hMSCs group (*p=0.000*), the 90% hMSCs + 10% hACPCs group (*p=0.002*), the 75% hMSCs + 25% hACPCs group (*p=0.002*) and the 50% hMSCs + 50% hACPCs group (*p=0.004*). Finally, the 100% hACPCs group was lower than the 100% hMSCs group (*p=0.000*), the 90% hMSCs + 10% hACPCs group (*p=0.001*), the 75% hMSCs + 25% hACPCs group (*p=0.000*), the 50% hMSCs + 50% hACPCs group (*p=0.001*) and the 25% hMSCs + 75% hACPCs group (*p=0.020*). The total amount of GAG, released into the culture medium, over the course of three weeks, was 74.76µg ± 10.71µg (100% hMSCs), 65.19µg ± 18.15µg (90% hMSCs + 10% hACPCs), 54.78µg ± 15.63µg (75% hMSCs + 25% hACPCs), 45.61µg ± 12.55µg (50% hMSCs + 50% hACPCs), 30.13µg± 7.79µg (25% hMSCs + 75% hACPCs), 21.72µg ± 3.58µg (10% hMSCs + 90% hACPCs) and 16.33µg ± 7.5µg (100% hACPCs).

Group	100% hMSCs	90% hMSCs + 10% hACPCs	75% hMSCs + 25% hACPCs	50% hMSCs + 50% hACPCs	25% hMSCs + 75% hACPCs	10% hMSCs + 90% hACPCs	100% hACPCs
100% hMSCs	-	-	-	**Higher**	**Higher**	**Higher**	**Higher**
90% hMSCs + 10% hACPCs	-	-	-	-	**Higher**	**Higher**	**Higher**
75% hMSCs + 25% hACPCs	-	-	-	-	**Higher**	**Higher**	**Higher**
50% hMSCs + 50% hACPCs	**Lower**	-	-	-	-	**Higher**	**Higher**
25% hMSCs + 75% hACPCs	**Lower**	**Lower**	**Lower**	-	-	-	**Higher**
90% hMSCs + 10% hACPCs	**Lower**	**Lower**	**Lower**	**Lower**	-	-	-
100% hACPCs	**Lower**	**Lower**	**Lower**	**Lower**	**Lower**	-	-

Table 6-2: Overview of the significant differences, regarding GAG release into the culture medium, in the co-culture of hMSCs and hACPCs study. The differences are indicated between the group on the left (highlighted in green) and the group on the top (highlighted in orange). Lower indicates significantly lower in the left group, when compared to the top group, higher indicates significantly higher in the left group, when compared to the top group and – indicates no significant difference between the left and the top group.

6.2.3 The 100% hMSCs group and the 90% hMSCs + 10% hACPCs group have the highest GAG/DNA ratio, among all groups investigated.

In order to exclude that differences in the amount of GAG, released into the culture medium, originate from varying cell numbers between the groups, GAG content was normalised to DNA content (indicator for total cell number). For each group, the total amount of GAG released, over the course of three weeks, was divided by total DNA content (figure 6-3).

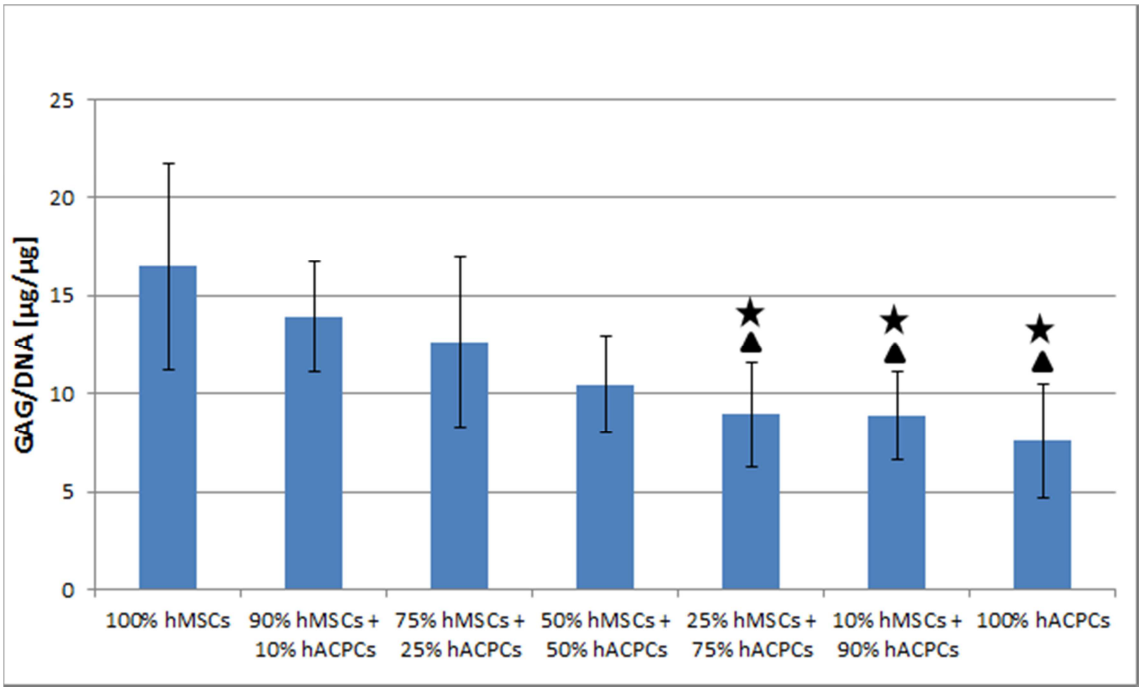

Figure 6-3: Total amount of GAG, released into the culture medium over the course of three weeks, normalised to total DNA content. hMSCs and hACPCs were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Total amount of GAG and amount of DNA was quantified. Next, GAG production was normalised to DNA content. Results are displayed as average ± standard deviation of triplicates from three experiments.★ significantly different from the 100% hMSCs group ($p \leq 0.05$),▲significantly different from the 90% hMSCs + 10% hACPCs group ($p \leq 0.05$).

A trend towards a decrease in GAG/DNA ratio, with increasing percentage of hACPCs within the co-culture pellets, was detected. This trend reached significance for the 100% hMSCs and the 90% hMSCs + 10% hACPCs group. The GAG/DNA ratio, in both groups, was significantly higher, when compared to the 25% hMSCs + 75% hACPCs group (*p=0.030* for the 100% hMSCs group and *p=0.017* for the 90% hMSCs + 10% hACPCs group), the 10% hMSCs + 90% hACPCs group (*p=0.027* for the 100% hMSCs group and *p=0.012* for the 90% hMSCs + 10% hACPCs group) and the 100% hACPCs group (*p=0.011* for the 100% hMSCs group and *p=0.005* for the 90% hMSCs + 10% hACPCs group. The GAG/DNA ratio, in µg/µg, was 16.47 ± 5.29 (100% hMSCs), 13.96 ± 2.8 (90% hMSCs + 10% hACPCs), 12.67 ± 4.35 (75% hMSCs + 25% hACPCs), 10.46 ± 2.47 (50% hMSCs + 50% hACPCs), 8.92 ± 2.67 (25% hMSCs + 75% hACPCs), 8.86 ± 2.28 (10% hMSCs + 90% hACPCs) and 7.57 ± 2.91 (100% hACPCs).

6.2.4 Co-culture of hMSCs and hACPCs does not significantly change the expression of most genes that were investigated. Significant differences, compared to the 100% hMSCs group, were only detectable for the genes collagen I and collagen II in some of the groups.

Further, the effect of co-culture of hMSCs and hACPCs on their gene expression profile was investigated. Gene expression analyses were conducted using the comparative $\Delta\Delta C_T$ method with 18s rRNA as internal control. The 100% hMSCs group was used as normaliser and, therefore, set to 1. The following genes were tested; Col I, Col II, Collagen X and aggrecan (figure 6-4).

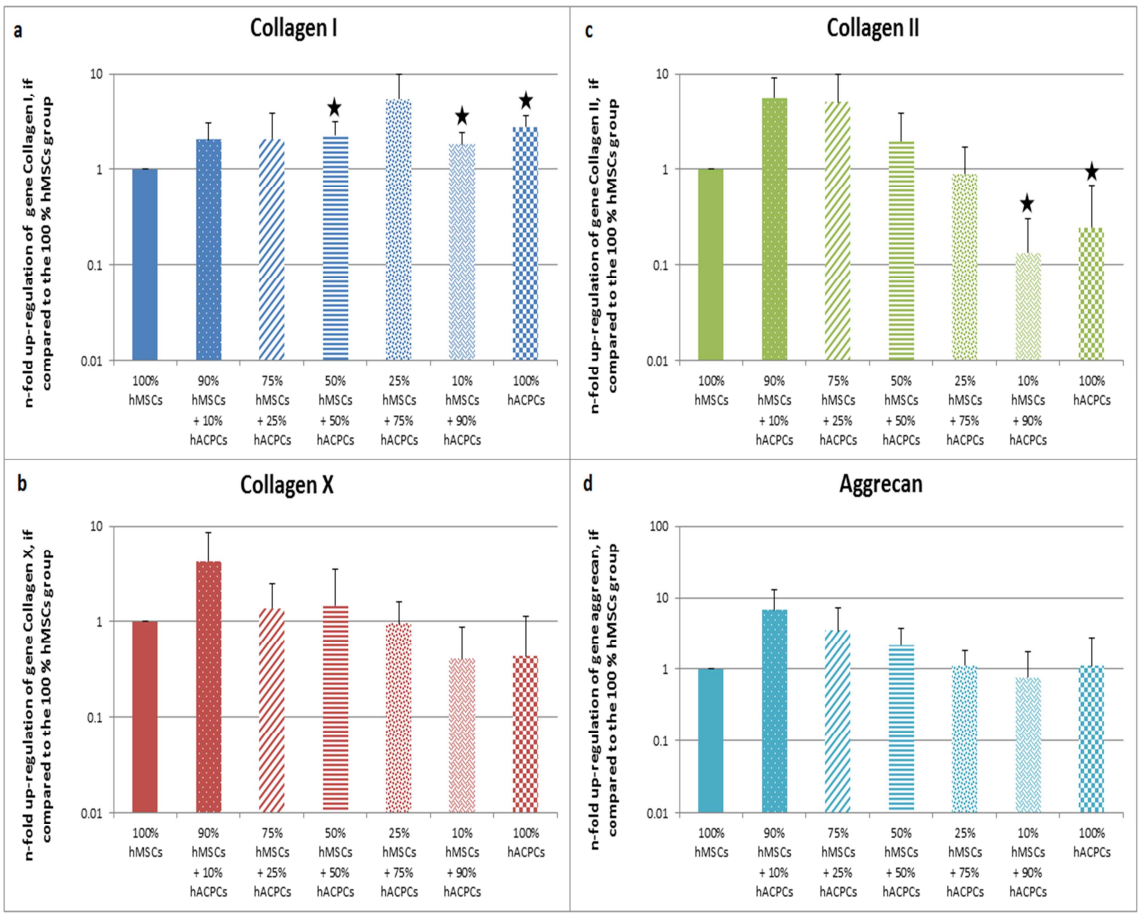

Figure 6-4: Relative gene expression of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Col I (a), Col X (b), Col II (c) and aggrecan (d). 18s rRNA was used as internal control. The 100% hMSCs group was used as calibrator and therefore set to 1. Results are displayed as average + standard deviation of triplicates from three experiments★Significantly different from the 100% hMSCs group ($p \leq 0.05$).

The gene Col II was down-regulated in the 10% hMSCs + 90% hACPCs group (*p=0.000*) and the 100% hACPCs group (*p=0.035*), when compared to the 100% hMSCs group. For the gene Col I, gene expression was lowest in the 100% hMSCs group. It was significantly lower, when compared to the 50% hMSCs + 50% hACPCs group (*p=0.026*), the 10% hMSCs + 90% hACPCs group (*p=0.050*) and the 100% hACPCs group (*p=0.006*).

6.2.5 Fluorescent labelling of hMSCs and hACPCs indicates a loss of hMSCs with time, if the percentage of hACPCs in co-culture pellets is 25% or higher.

In order to ascertain if the ratio between the different cell populations in the co-culture pellets stayed constant, hMSCs (PKH67, green) and hACPCs (PKH26, red) were labelled with fluorescent dyes. After 7 and 21 days, pellets were harvested and cryo-sectioned, cell nuclei were counterstained with DAPI (blue) and sections were viewed using fluorescence microscopy (figure 6-5, figure 6-6 and figure 6-7).

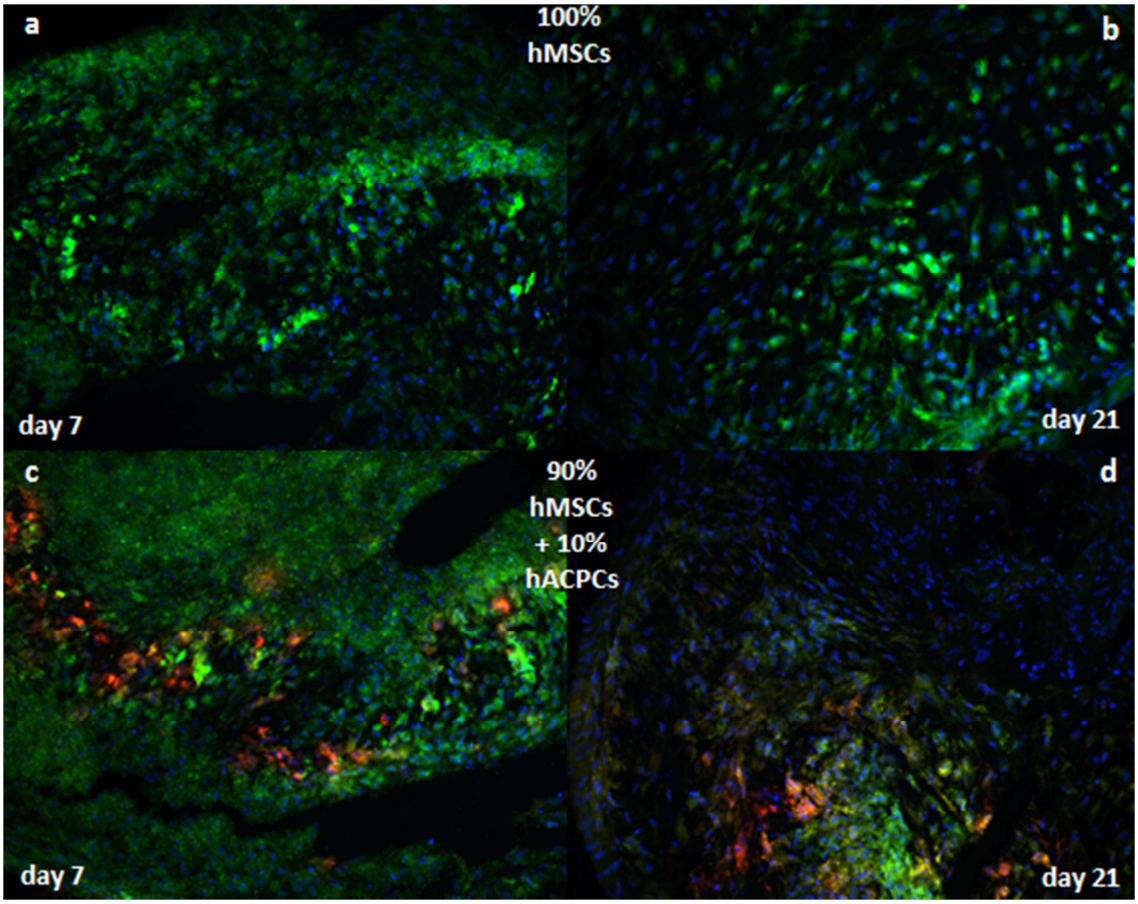

Figure 6-5: Fluorescent images of PKH67-labelled hMSCs (green) and PKH26-labelled hACPCs (red). Labelled cells were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. After 7 and 21 days, pellets were harvested and cryo-sectioned. Before picture acquisition, cell nuclei were counterstained with ProLong® antifade reagent with DAPI (blue) and sections were cover-slipped. The following channels were used; the DAPI channel for DAPI, the FITC channel for PKH67-labelled hMSCs and the Cy3 channel for PKH26-labelled hACPCs. The picture is a merge of three images which were individually taken with the different channels. The 100% hMSCs (a and b) and the 90% hMSCs + 10% hACPCs (c and d) pellets after 7 (a and c) and 21 (b and d) days are shown. The images were taken with a total magnification of 12.6.

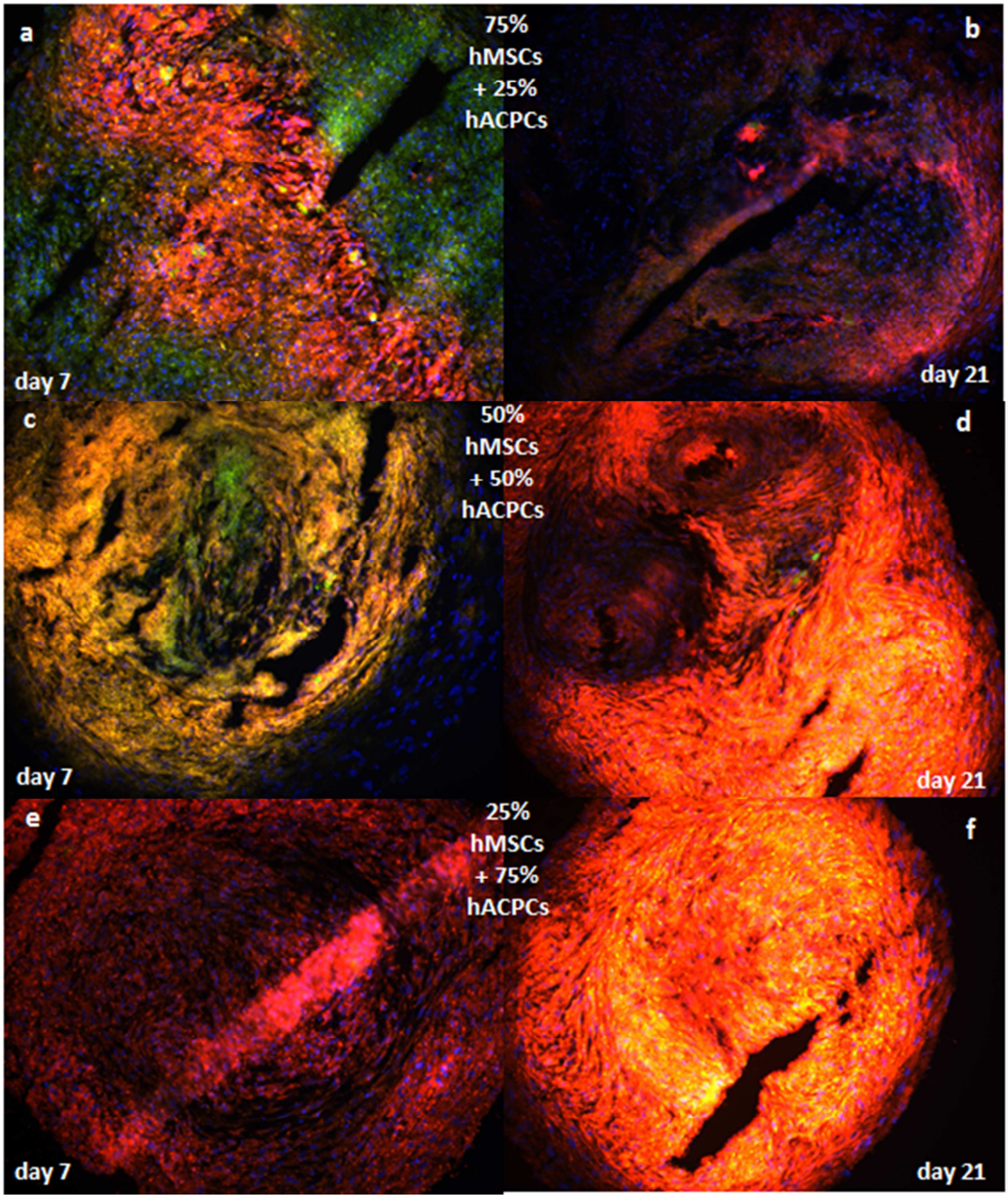

Figure 6-6: Fluorescent images of PKH67-labelled hMSCs (green) and PKH26-labelled hACPCs (red). Labelled cells were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. After 7 or 21 days, pellets were harvested and cryo-sectioned. Before picture acquisition, cell nuclei were counterstained with ProLong® antifade reagent with DAPI (blue) and sections were cover-slipped. The following channels were used; the DAPI channel for DAPI, the FITC channel for PKH67-labelled hMSCs and the Cy3 channel for PKH26-labelled hACPCs. The picture is a merge of three images which were individually taken with the different channels. The 75% hMSCs + 25% hACPCs (a and b), the 50% hMSCs + 50% hACPCs (c and d) and the 25% hMSCs + 75% hACPCs (e and f) pellets after 7 (a, c and e) and 21 (b, d and f) days are shown. Differences in fluorescence intensity between the images originate from varying exposure times. The images were taken with a total magnification of 12.6.

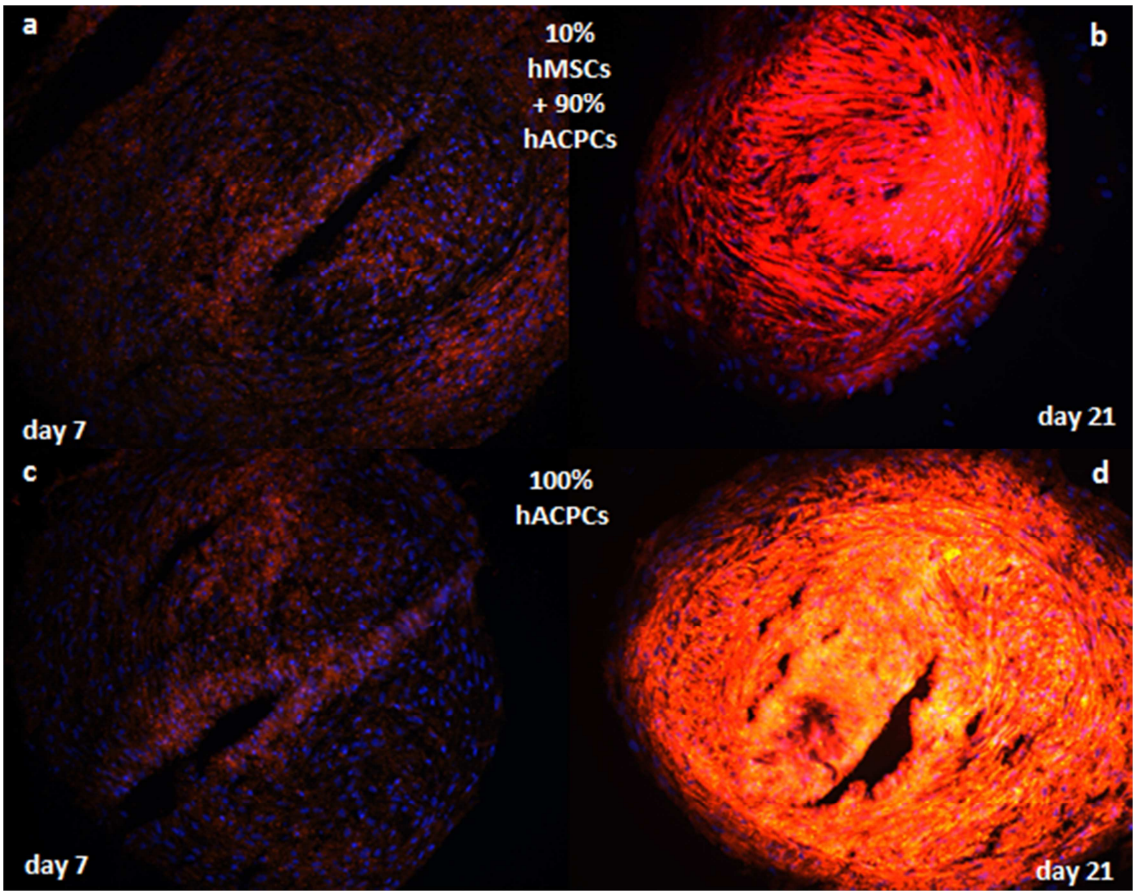

Figure 6-7: Fluorescent images of PKH67-labelled hMSCs (green) and PKH26-labelled hACPCs (red). Labelled cells were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. After 7 or 21 days, pellets were harvested and cryo-sectioned. Before picture acquisition, cell nuclei were counterstained with ProLong® antifade reagent with DAPI (blue) and sections were cover-slipped. The following channels were used; the DAPI channel for DAPI, the FITC channel for PKH67-labelled hMSCs and the Cy3 channel for PKH26-labelled hACPCs. The picture is a merge of three images which were taken with the different channels. The 10% hMSCs + 90% hACPCs (a and b) and the 100% hACPCs (c and d) pellets after 7 (a and c) and 21 (b and d) days are shown. Differences in fluorescence intensity between the images originate from varying exposure times. The images were taken with a total magnification of 12.6.

After 7 days, the 100% hMSCs (figure 6-5 a), the 90% hMSCs + 10% hACPCs (figure 6-5 c), the 75% hMSCs + 25% hACPCs (figure 6-6 a) and the 50% hMSCs + 50% hACPCs (figure 6-6 c) groups showed the expected ratio between PKH67-labelled hMSCs (green) and PKH26-labelled hACPCs (red). Interestingly, if the percentage of hACPCs was 75% or higher (figures 6-6 e, 6-7 a and 6-7 c), hMSCs were no longer visible. After 21 days, the ratio within the co-culture pellets was shifted in favour of PKH26-labelled hACPCs. An exceptions was the 90% hMSCs + 10% hACPCs group (figure 6-5 d), where the ratio stayed constant. In the 75% hMSCs + 25% hACPCs group (figure 6-6 b) and the 50% hMSCs + 50% hACPCs group (figure 6-6 d), the ratio was significantly shifted to hACPCs. In the remaining groups (figure 6-6 f, figure 6-7 b and figure 6-7 d), hMSCs were not detectable (consistent with the results from day 7).

6.2.6 On the protein level, *in vitro* chondrogenesis is only detectable in the 100% hMSCs and the 90% hMSCs + 10% hACPCs group.

Subsequently, co-culture pellets were cryo-sectioned and histological and immunohistochemical analyses were conducted. Thereby, distribution of matrix proteins within the pellets was monitored. The presence of negatively charged proteoglycans was detected by Safranin-O/Fast Green staining (figure 6-8 and figure 6-9). Further, immunohistochemical detection for the chondrogenic matrix proteins aggrecan (figure 6-10 and figure 6-11) and Col II (figure 6-12 and figure 6-13) and the hypertrophic matrix protein Col X (figure 6-14 and figure 6-15) was conducted.

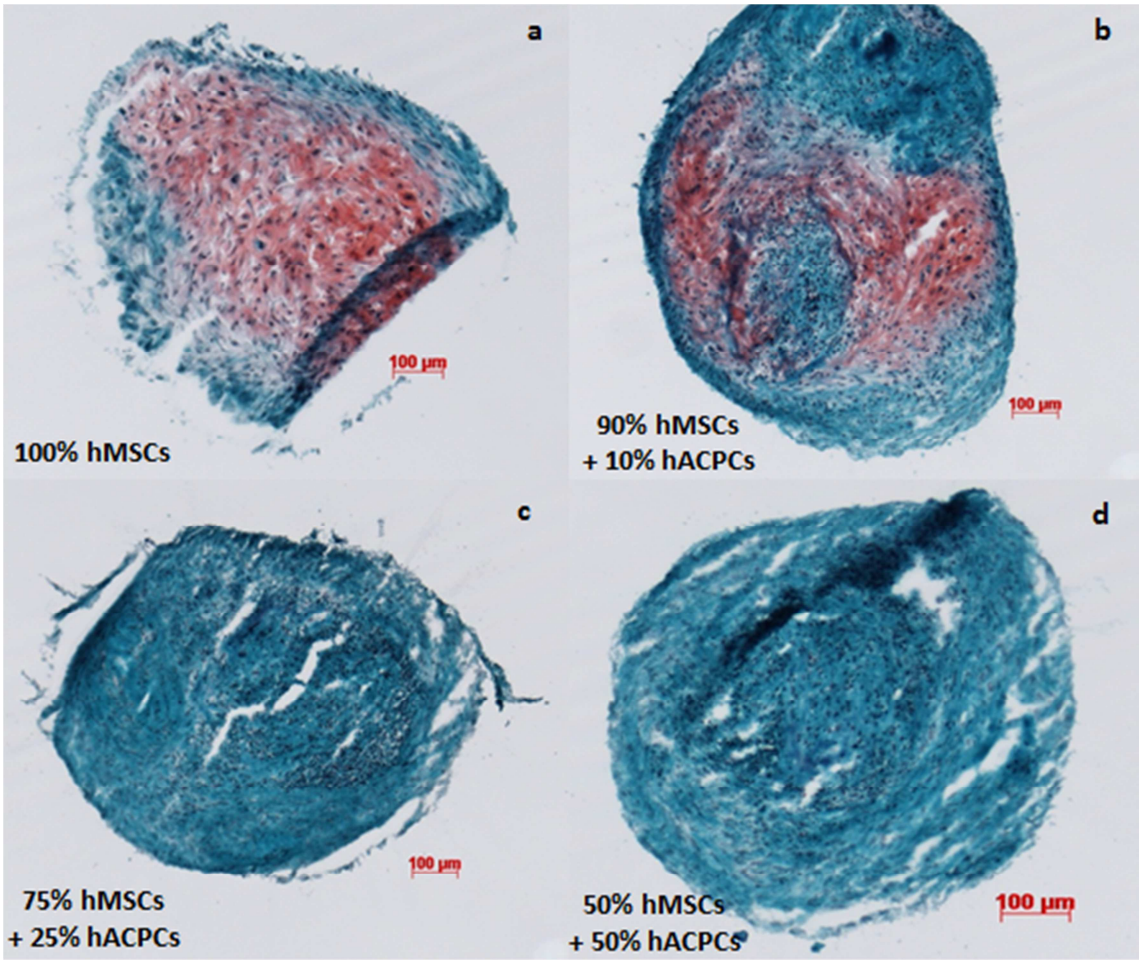

Figure 6-8: Safranin-O/Fast Green staining of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and negatively charged proteoglycans were stained red with Safranin-O. Fast Green was used as counterstain to stain proteins green. Representative images of the 100% hMSCs (a), the 90% hMSCs + 10% hACPCs (b), the 75% hMSCs + 25% hACPCs (c) and the 50% hMSCs + 50% hACPCs (d) group are shown.

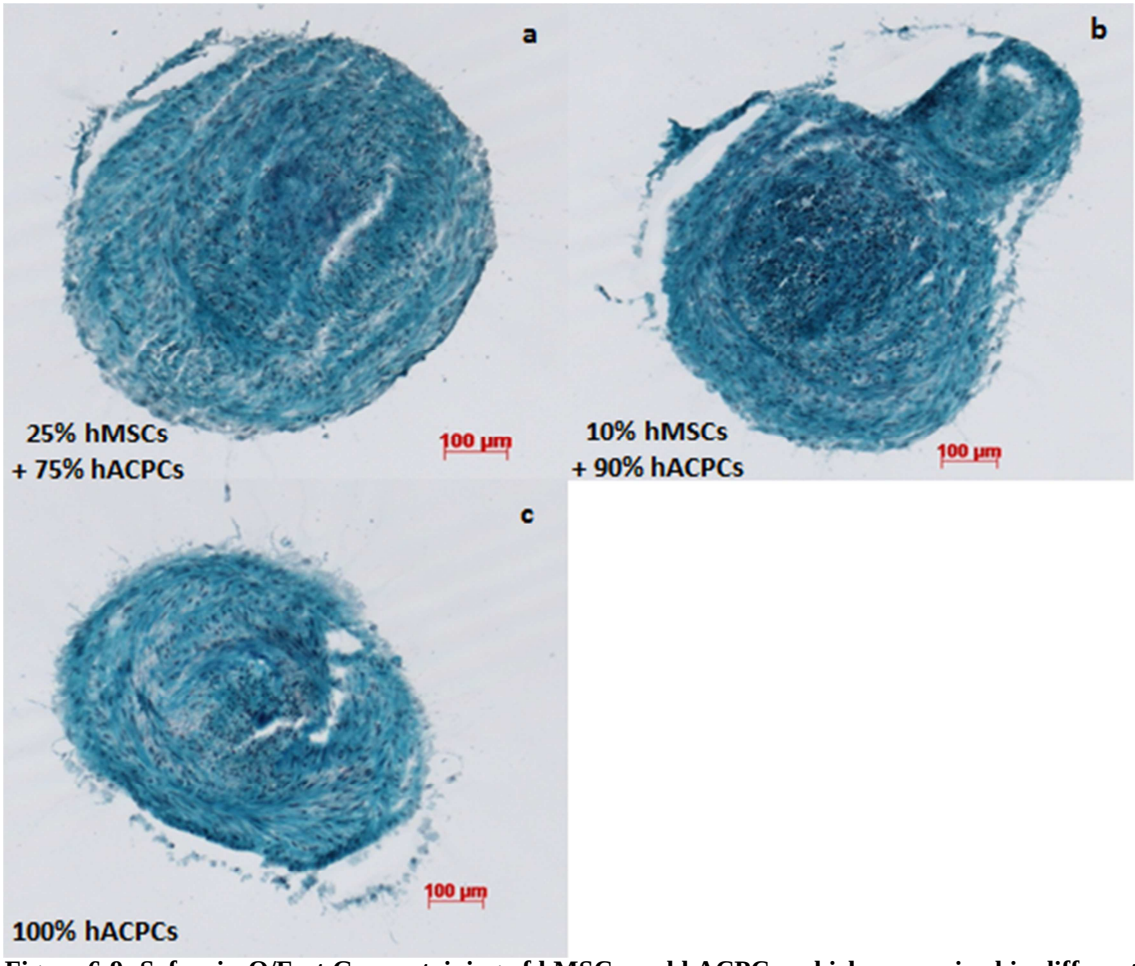

Figure 6-9: Safranin-O/Fast Green staining of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and negatively charged proteoglycans were stained red with Safranin-O. Fast Green was used as counterstain to stain proteins green. Representative images of the 25% hMSCs + 75% hACPCs (a), the 10% hMSCs + 90% hACPCs (b) and the 100% hACPCs (c) group are shown.

The presence of negatively charged proteoglycans, within the ECM of co-culture pellets, was only detected in the 100% hMSCs and the 90% hMSCs + 10% hACPCs group. In the 100% hMSCs group, the Safranin-O staining was more abundant, when compared to the 90% hMSCs + 10% hACPCs group. In the remaining five groups, Safranin-O staining was not detected.

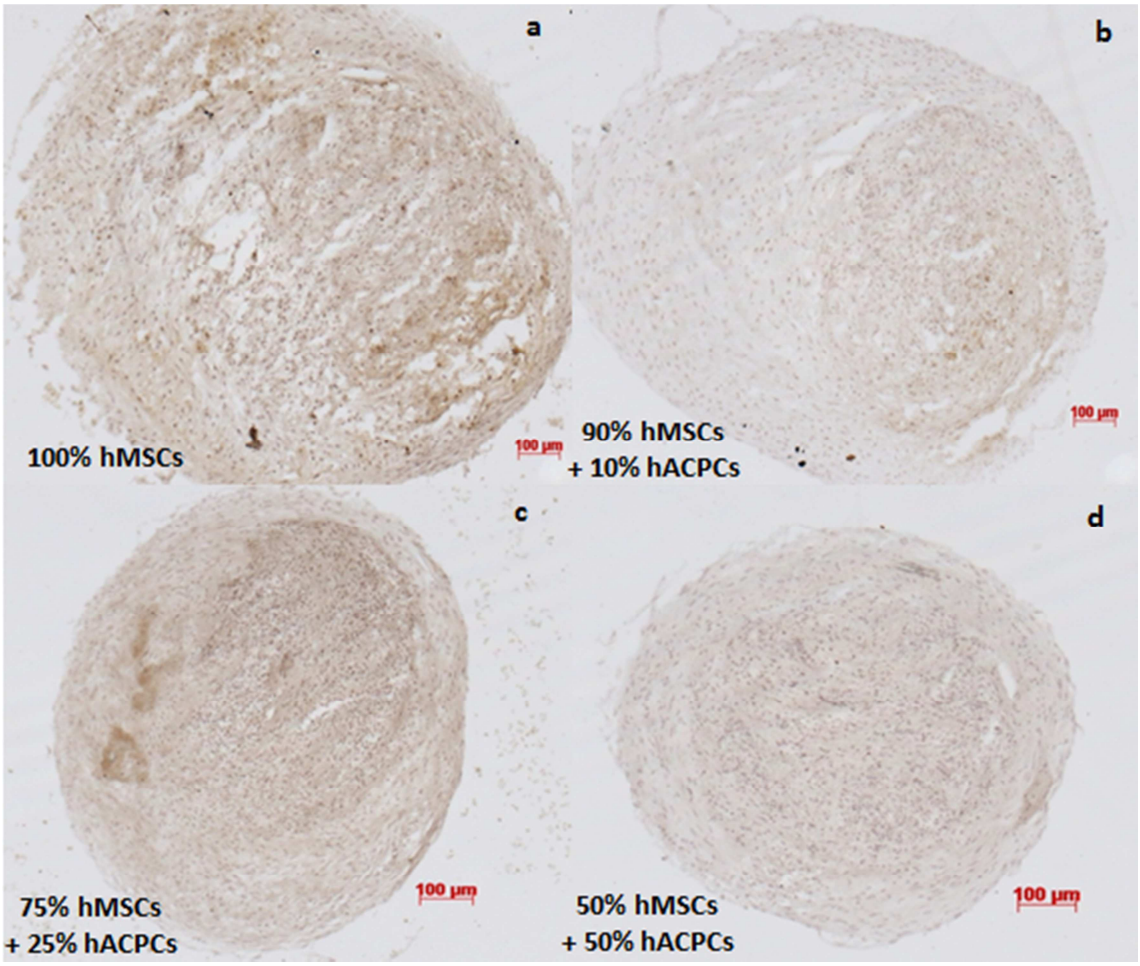

Figure 6-10: Aggrecan immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and aggrecan immunohistochemistry was conducted using the 1-C-6 antibody as primary antibody. The cell nuclei were counter-stained with haematoxyline. Representative images of the 100% hMSCs (a), the 90% hMSCs + 10% hACPCs (b), the 75% hMSCs + 25% hACPCs (c) and the 50% hMSCs + 50% hACPCs (d) group are shown.

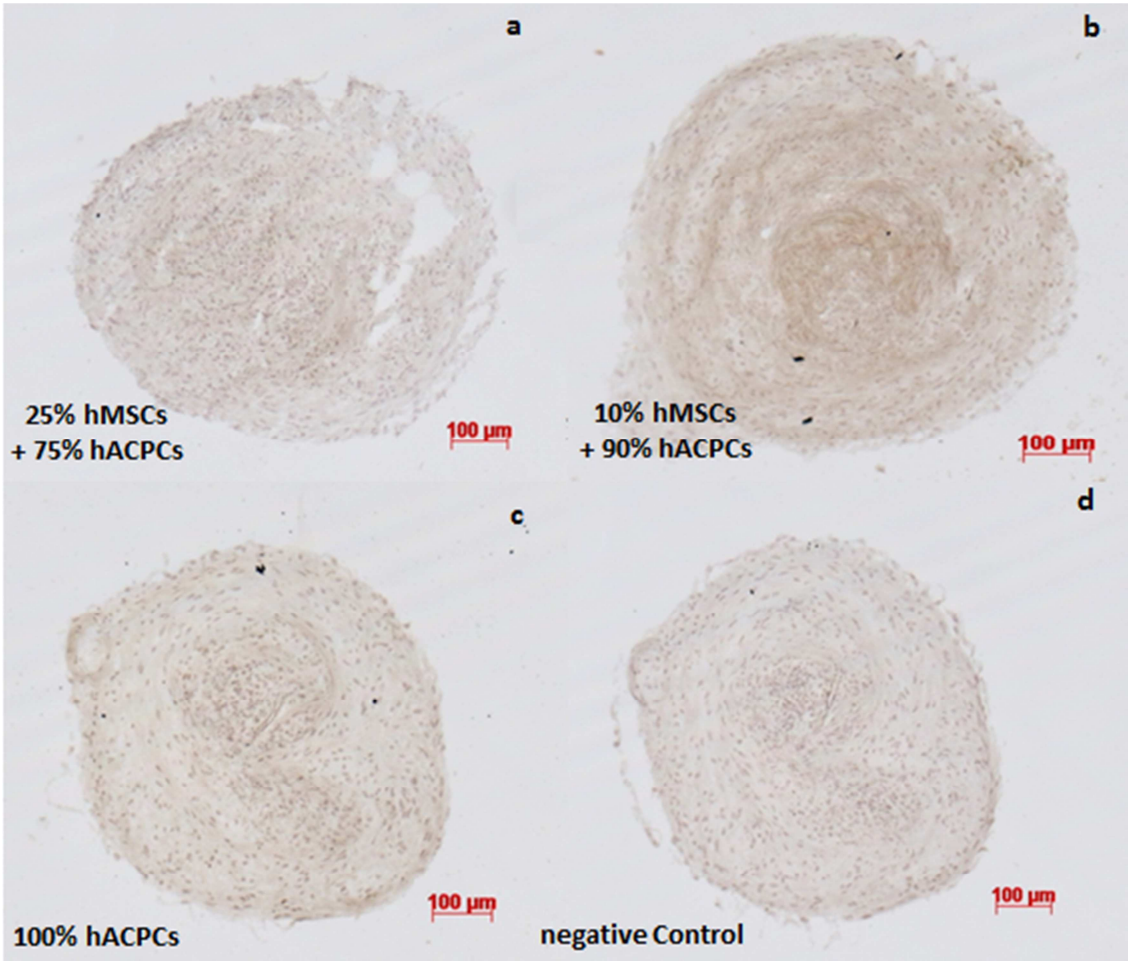

Figure 6-11: Aggrecan immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and aggrecan immunohistochemistry was conducted using the 1-C-6 antibody as primary antibody. Negative control sections were treated with PBS-T instead. The cell nuclei were counter-stained with haematoxyline. Representative images of the 25% hMSCs + 75% hACPCs (a), the 10% hMSCs + 90% hACPCs (b), the 100% hACPCs (c) and the negative control group (d) are shown.

Generally, it was difficult to distinguish between the specific labelling for the aggrecan protein and the brown basic colouration of the pellets (see negative control figure 6-11 d). In some cases, this task was further complicated by the black stain of the cell nuclei. Distinct immunolabelling for the aggrecan protein was detected in the 100% hMSCs group (figure 6-10 a). In the 90% hMSCs + 10% hACPCs group (figure 6-10 b), weaker labelling was detected. In the 75% hMSCs + 25% hACPCs group (figure 6-10 c), the brown basic colouration was quite dark and the cells were densely packed. Yet, some immunolabelling for the aggrecan protein was detected at the left side of the pellet. In the other groups, no clear immunolabelling for the aggrecan protein was evident.

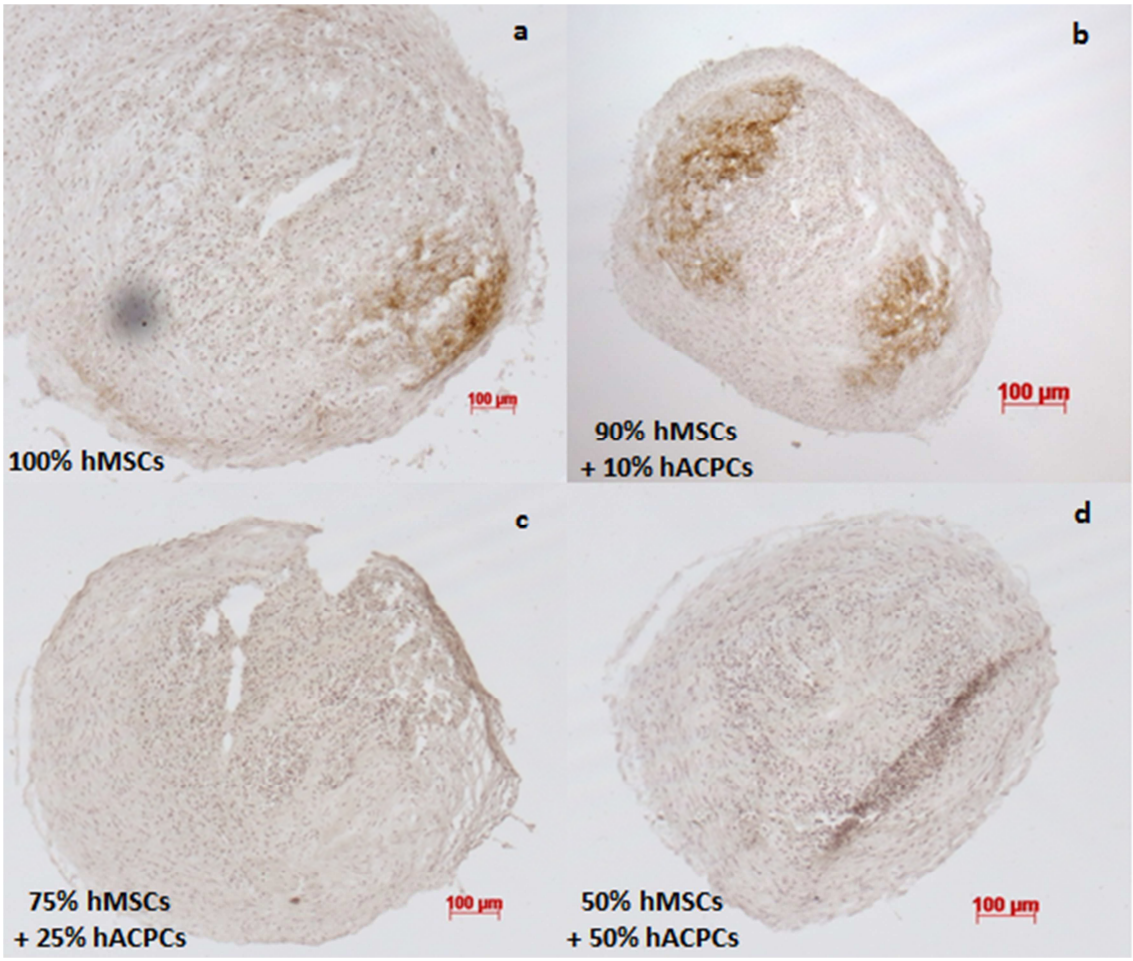

Figure 6-12: Col II immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and Col II immunohistochemistry was conducted using the CIICI antibody as primary antibody. The cell nuclei were counter-stained with haematoxyline. Representative images of the 100% hMSCs (a), the 90% hMSCs + 10% hACPCs (b), the 75% hMSCs + 25% hACPCs (c) and the 50% hMSCs + 50% hACPCs (d) group are shown. The dark spot on the bottom left side of figure 6-12 a is an air bubble that was created during cover-slipping. The images were taken with a total magnification of 6.3.

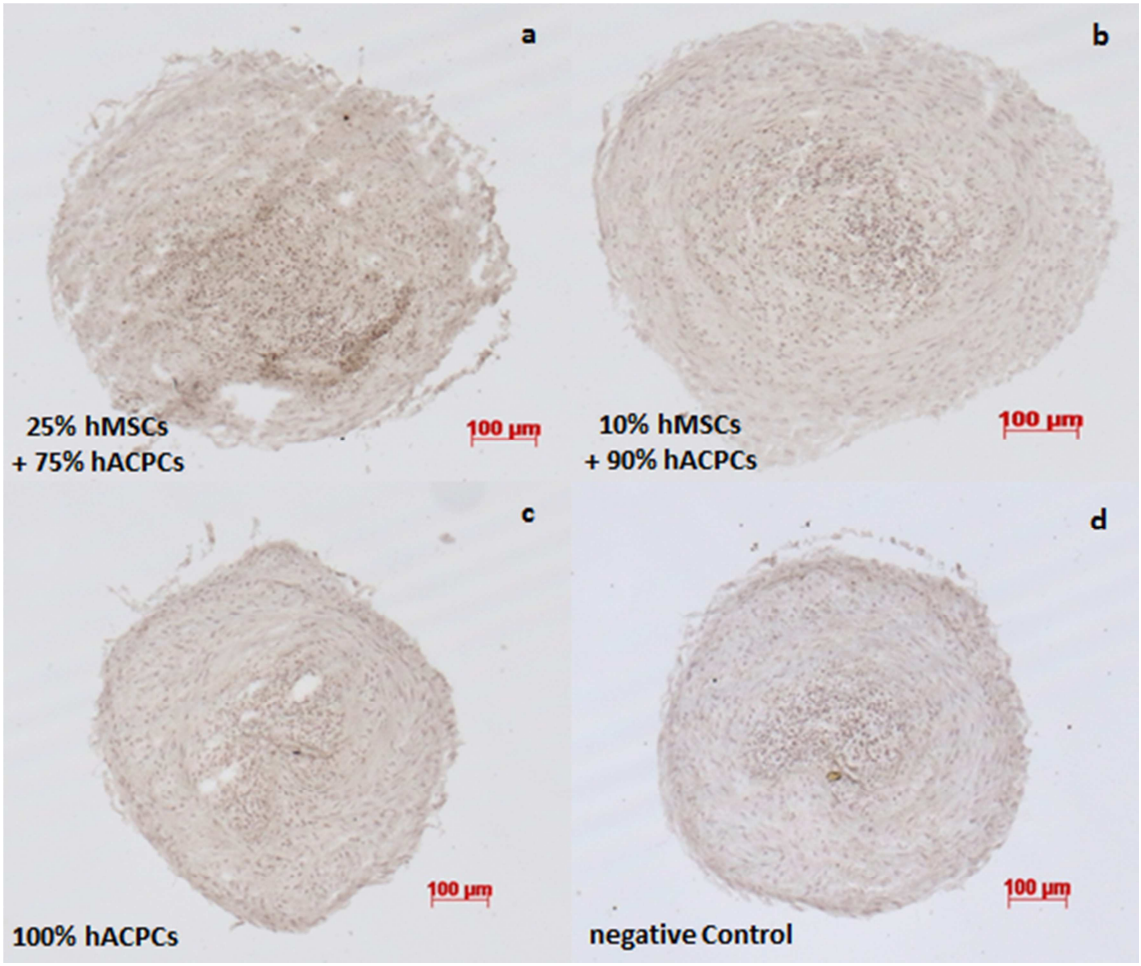

Figure 6-13: Col II immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and Col II immunohistochemistry was conducted using the CIICI antibody as primary antibody. Negative control sections were treated with PBS-T instead. The cell nuclei were counter-stained with haematoxyline. Representative images of the 25% hMSCs + 75% hACPCs (a), the 10% hMSCs + 90% hACPCs (b), the 100% hACPCs (c) and the negative control (d) group are shown.

Again, the background colour of the pellets was brown which complicated the analysis of the immunohistochemistry for the Col II protein. Distinct positive labelling was monitored in the 100% hMSCs (figure 6-12 a) and the 90% hMSCs + 10% hACPCs (figure 6-12 b) group. The remaining groups were negative for the Col II protein. In some of the images, especially in figures 6-12 d and 6-13 a, darker spots were detectable. Yet, they most likely represent clusters of cells in which the nuclei were stained black with haematoxyline according to Mayer.

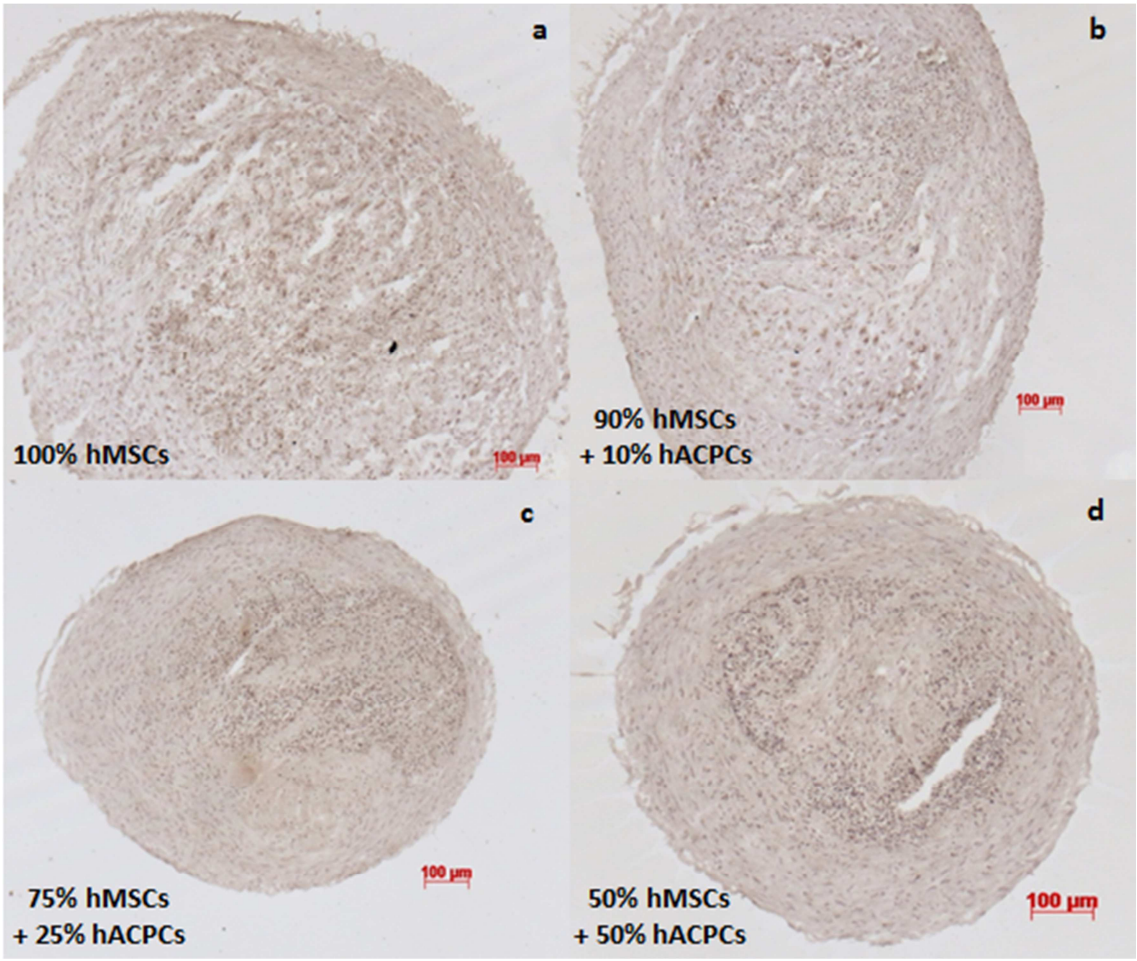

Figure 6-14: Col X immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and Col X immunohistochemistry was conducted using the Col-10 antibody as primary antibody. The cell nuclei were counter-stained with haematoxyline. Representative images of the 100% hMSCs (a), the 90% hMSCs + 10% hACPCs (b), the 75% hMSCs + 25% hACPCs (c) and the 50% hMSCs + 50% hACPCs (d) group are shown.

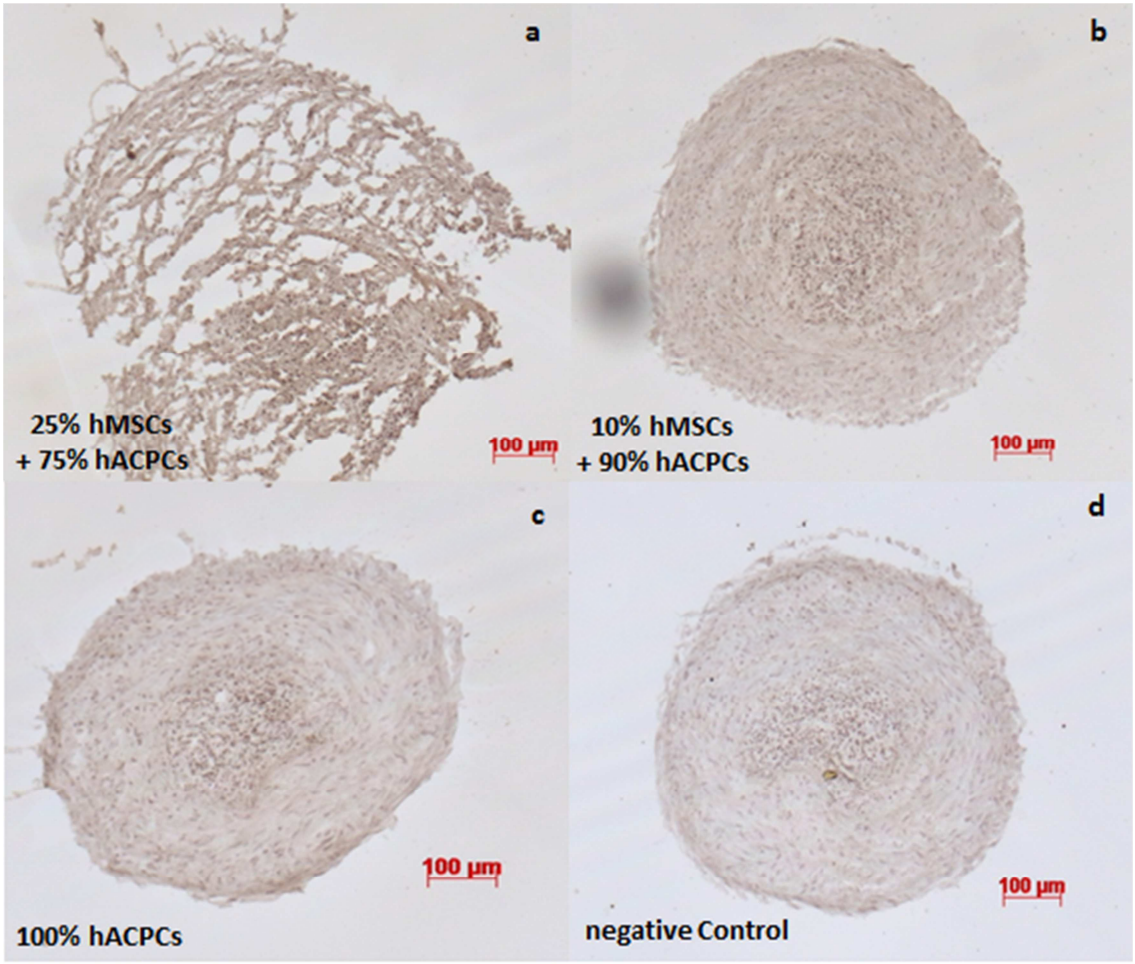

Figure 6-15: Col X immunohistochemistry of hMSCs and hACPCs, which were mixed in different ratios and cultured as pellets for 3 weeks. The total cell number per pellet was 250, 000. Pellets were cultured in 96-well V-bottom plates with 250µl of chondrogenic medium per well. Pellets were cryo-sectioned and Col X immunohistochemistry was conducted using the Col-10 antibody as primary antibody. Negative control sections were treated with PBS-T instead. The cell nuclei were counter-stained with haematoxyline. Representative images of the 25% hMSCs + 75% hACPCs (a), the 10% hMSCs + 90% hACPCs (b), the 100% hACPCs (c) and the negative control group (d) are shown. The dark spot on the bottom left side of figure 6-15 b is an air bubble that was created during cover-slipping.

Generally, immunohistochemical labelling for the Col X protein was least pronounced, when compared to the labelling for the proteins aggrecan and Col II. In the 100% hMSCs group (figure 6-14 a), labelling for the Col X protein was evident in the middle of the pellet. In the 90% hMSCs + 10% hACPCs group (figure 6-14 b), the labelling was much weaker. Yet, the location (middle of the pellets) was similar. In the other groups, the background colour of the pellets was fairly dark and no clear immunolabelling for the Col X protein was detectable. Unfortunately, the pellet in the 25% hMSCs + 75% hACPCs group was disintegrated and figure 6-15 a was the best image that could be obtained.

6.2.7. Generally, cytokine release of hMSCs is higher, when compared to hACPCs.

In order to conclude the analysis of the co-culture experiment, part of the secretome of hMSCs and hACPCs was analysed and compared. This was done in order to track differences between these cell types. Further, it was of interest to spot candidate cytokines that might be involved in the different chondrogenic response, monitored within both cell types. hMSCs and hACPCs were seeded using their respective growth medium into 12-well tissue culture plates, at a density that allowed for a confluent culture from the beginning of the experiment. After 24 hours, medium was changed to serum-free DMEM and cells were cultured for an additional 48 or 72 hours. Afterwards, medium was harvested and cells were digested in PK. The secretome analysis was conducted using the RayBio human cytokine antibody array G-series. DNA content was quantified in order to normalise results (figure 6-16). The processed raw data were obtained from THP Medical Products Vertriebs GmbH (Wien, Austria). Analysis was conducted, as described in section 2.2.31, from one sample of the 48 hour time-point for each donor and each cell type. The 72 hour time-point was not used as cells started to change their phenotype and the onset of cell death was monitored microscopically, after 72 hours under serum-free conditions. As suggested by the supplier of the assay, n-fold changes higher than 2 were considered as higher cytokine content in the secretome of hMSCs and n-fold changes lower than 0.5 were considered as lower cytokine content in the secretome of hMSCs, when compared to the secretome of hACPCs (table 6-3).

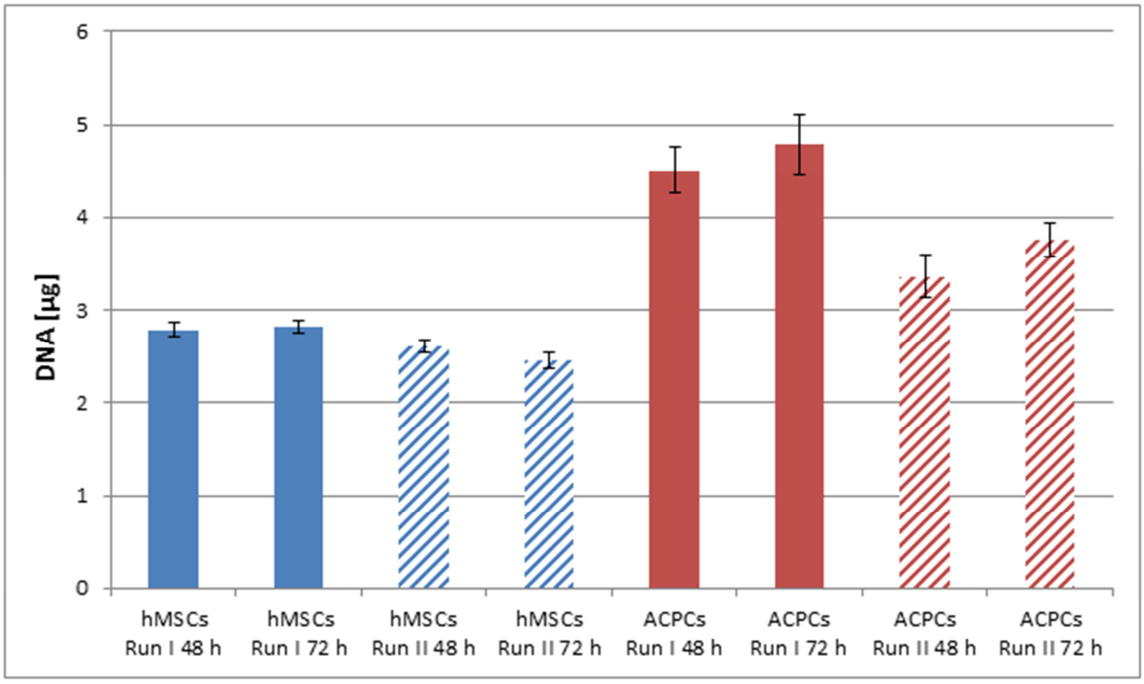

Figure 6-16: Total DNA content after 48 or 72 hours of serum-free culture. hMSCs and hACPCs were seeded at confluency into 12-well tissue culture plates and cultured for 24 hours in their respective growth medium. Subsequently, growth medium was replaced with serum-free DMEM and cells were cultured for an additional 48 or 72 hours. Cells were the PK-digested and DNA content was quantified using the Hoechst 33258 dye assay with calf thymus DNA as standard. Results are displayed as average ± standard deviation of triplicates from two donors.

For each individual cell type, the DNA content did not change between the 48 hours and the 72 hours time-points. The differences in DNA content, between the cell types and between the two donors for the hACPCs, originated from varying cell size. Generally, hACPCs are smaller, when compared to hMSCs. In order to reach confluency during seeding, a higher seeding density, which equals to more cells per well, for hACPCs was used. This higher cell seeding density then translates into a higher total DNA content.

Cytokine	Full name	n-fold difference
FGF-7	Keratinocyte growth factor	2.81
IFN-gamma	Interferon-gamma	3.35
IGFBP-2	Insulin-like growth factor-binding protein 2	5.49
IGFBP-4	Insulin-like growth factor-binding protein 4	2.32
IL-6	Interleukin 6	3.14
IL-7	Interleukin 7	2.85
MCP-2	Monocyte chemoattractant protein 2	2.16
MIG	Monokine induced by gamma interferon	2.10
Dtk	TYRO3/Tyrosine-protein kinase receptor	3.01
HGF	Hepatocyte growth factor	13.55
Osteoprotegerin	Osteoprotegerin	3.30
PIGF	Phosphatidylinositol-glycan biosynthesis class F protein	3.56
B7-1(CD80)	Cluster of Differentiation 80	2.04
MMP-1	Matrix metalloproteinase-1	2.05
IGFBP-3	Insulin-like growth factor-binding protein 3	0.38
MIF	Macrophage migration inhibitory factor	0.36
LAP	Leukocyte Alkaline phosphatase	0.39

Table 6-3: Overview of the cytokines that were elevated (green) or decreased (orange) in the secretome of hMSCs, when compared to the secretome of hACPCs.

The vast majority of cytokines analysed ware more abundant in the secretome of hMSCs, when compared to the secretome of hACPCs. Still, differences in the order of of two-fold (higher or lower) were only found for 17 out of 174 cytokines. Of these 17 cytokines, 14 were more abundant in hMSCs and only 3 of them were more abundant in hACPCs. The most pronounced differences were detected for hepatocyte growth factor (HGF) and macrophage migration inhibitory factor (MIF). HGF was 13.55-fold elevated in the secretome of hMSCs and MIF was 2.78-fold elevated in the secretome of hACPCs. It has to be mentioned that these information were only derived from single samples of two different donors (for hMSCs and hACPCs each). Therefore, these data can only give first hints. Unless the experiment is repeated with more samples from additional donors, conclusions about significant differences in the cytokine release profile between hMSCs and hACPCs cannot be drawn.

6.3 Co-culture of hMSCs and hACPCs – discussion.

For TE applications, aiming to regenerate articular cartilage, usually a single cell type, such as chondrocytes or hMSCs, is used. Yet, co-cultures allow for cell-cell signalling which may be regarded as an attractive alternative. These approaches can use one cell type in a different phenotypic state, for instance de-differentiated and primary chondrocytes (Gan and Kandel, 2007) or chondrocytes from different regions within articular cartilage (Sharma et al., 2007). Alternatively, two different cell types, for instance articular chondrocytes and MSCs, can be used (Liu et al., 2010; Meretoja et al., 2012; Wu et al., 2011). The discovery of progenitor cells within the superficial zone of articular cartilage led to a new candidate cell source for cartilage TE applications (Dowthwaite et al., 2004; Williams et al., 2010). Further, a direct comparison of equine ACPCs and equine MSCs using the pellet culture model revealed that both cell types were able to undergo chondrogenesis (McCarthy et al., 2011). Yet, ACPCs displayed a stable phenotype, whereas MSCs showed signs of hypertrophic differentiation. Based on these observations, it was of interest to determine if these results can be extrapolated to human cells. Therefore, the current study opted to compare the chondrogenic response and the progression towards hypertrophy of hMSCs and hACPCs. The pellet culture model and the "classical" serum-free chondrogenic medium, containing exogenous TGF-β1, were used. Further, both cell types were mixed in different ratios and cultured as co-culture pellets. It was hypothesised that both cell types influence each other during chondrogenic differentiation and that this will lead to a modulated chondrogenic response. The pellets, consisting of 250, 000 cells in varying ratios between hMSCs and hACPCs, were cultured for 3 weeks in "classical" serum-free chondrogenic medium. The medium was changed three times a week and collected for week 1, week 2 and week 3. In order to track if the ratio between hMSCs and hACPCs stays constant, both cell types were labelled with fluorescent dyes in one run of the experiment. The labelled pellets were harvested after 7 or 21 days, sectioned and monitored using fluorescent microscopy. Furthermore, biochemical analyses were carried out. The DNA content was determined in order to track differences in cell number between the groups. The matrix production was tracked by quantification of sulphated GAG, secreted into the culture medium. Next, the GAG production was normalised to DNA content in order to eliminate the skewing effect of varying cell numbers.

Moreover, gene expression analyses were conducted in order to track fibroblastic (Col I), chondrogenic (aggrecan and Col II) and hypertrophic (Col X) marker gene expression. Matrix production was evaluated on the protein level, using histology (Safranin-O/Fast Green staining) and immunohistochemistry (for the proteins aggrecan, Col II and Col X). Finally, part of the secretome of both cell types was analysed, as differences in these cell signalling molecules might explain for some of the monitored differences between both cell types.

Visualisation of the fluorescently labelled cells revealed that the proportion of hMSCs seemed to decrease with time, during long-term co-culture with hACPCs. The only exception was the 90% hMSCs + 10% hACPCs group. After 7 days of culture, the groups 90% hMSCs + 10% hACPCs, 75% hMSCs + 25% hACPCs and 50% hMSCs + 50% hACPCs still had the expected ratio between hMSCs and hACPCs. However, in the 25% hMSCs + 75% hACPCs group and the 10% hMSCs + 90% hACPCs group no hMSCs were detected. After 3 weeks of co-culture, the balance was further shifted towards hACPCs. The only group that demonstrated the expected ratio between the two cell populations was the 90% hMSCs + 10% hACPCs group. In the 75% hMSCs + 25% hACPCs group and the 50% hMSCs + 50% hACPCs group, few hMSCs were detected. In the other groups, again, the presence of hMSCs was no longer detectable. The interpretation of the fluorescent labelling can only be conducted under the assumption that both dyes have similar characteristics (stability, labelling efficiency) and that both cell populations divide with a similar rate. Under these assumptions, these results indicate that hMSCs did not seem to survive well during co-culture with other cell types, in this case hACPCs. Similar observations have been made during co-culture of rabbit MSCs and bovine chondrocytes (Meretoja et al., 2012) and co-cultures of hMSCs and bovine or human primary chondrocytes (Wu et al., 2011).

Quantification of cellular DNA, as indicator for total cell number, revealed no significant differences between hMSCs, hACPCs or their co-culture pellets. These data demonstrated that the total cell numbers, after 3 weeks of culture, was not different between the different groups.

Quantification of sulphated GAG, within the culture medium over the course of three weeks, revealed a tendency towards a decreased matrix production with increasing percentage of hACPCs within the co-culture pellets. The 100% hMSCs group produced the highest amount of GAG, among all groups investigated. This production was significantly higher, when compared to each other group, except the 90% hMSCs + 10% hACPCs and the 75% hMSCs + 25% hACPCs group. Further, these two groups also produced significantly more matrix, when compared to the 25% hMSCs + 75% hACPCs group, the 10% hMSCs + 90% hACPCs group and the 100% hACPCs group. The 100% hACPCs group produced the least amount of GAG between all groups investigated. GAG synthesis was significantly lower, when compared to all groups except the 10% hMSCs + 90% hACPCs group. As the amount of GAG retained within the co-culture pellets was not analysed, it cannot definitely be ruled out that these observed differences between the groups originate form varying GAG retention. Yet, as indicated by a lack of Safranin-O staining and aggrecan immunohistochemistry, it is highly unlikely that hACPCs are able to retain that much more GAG (up to 50µg) within their matrix, when compared to hMSCs. McCarthy et al., were able to demonstrate comparable immunolabelling for the chondrogenic matrix proteins Col II and aggrecan within pellets derived from equine MSCs and equine ACPCs (McCarthy et al., 2011). Yet, this information gives a hint about matrix retention within the pellets. The amount of GAG, secreted into the culture medium, was not tracked in the study of McCarthy and co-workers. Therefore, it cannot be confirmed or ruled out that a comparable amount of matrix proteins was additionally secreted into the culture medium.

The normalisation of matrix production to DNA content confirmed the observations which were performed when the amount of secreted GAG was analysed. Thereby, it was ruled out that a higher amount of matrix protein production, within certain groups, originated from higher cell numbers in these groups. The GAG/DNA ratio constantly decreased with increasing percentage of hACPCs within the co-culture pellets. The 100% hMSCs group and the 90% hMSCs + 10% hACPCs group had the highest GAG/DNA ratio, which was significantly higher, when compared to the 25% hMSCs + 75% hACPCs group, the 10% hMSCs + 90% hACPCs group and the 100% hACPCs group. The 100% hACPCs group had the lowest GAG/DNA ratio, among all groups investigated. Interestingly, the GAG/DNA ratio was higher if hMSCs were cultured in the fibrin-PU composite scaffold system, when compared to the standard pellet culture system. The GAG/DNA ratio, in µg per µg, was 16.47 ± 5.29 (pellet culture) and 37.3 ± 10.8 (fibrin-PU composite scaffold, control unloaded group; (Neumann et al., 2013a)) for hMSCs. For hACPCs, the difference in GAG/DNA ratio was marginal. It was 7.57 ± 2.91 (pellet culture) and 9.1 ± 5.6 (fibrin-PU composite scaffold, control unloaded group; see section 4.2.6). Nevertheless, it has to be stated that a direct comparison between both culture systems is not fully applicable as the culture medium (chondro-permissive medium, which does not contain exogenous TGF-β1, for fibrin-PU composite scaffolds and chondrogenic medium, which does contain exogenous TGF-β1, for pellets cultures) and the culture time (4 weeks for fibrin-PU composite scaffolds and 3 weeks for pellets cultures) were different between these studies.

Gene expression analyses revealed that Col II message was significantly elevated in the 100% hMSCs group, when compared to the 10% hMSCs + 90% hACPCs and the 100% hACPCs group. For the fibroblastic marker gene Col I, the 100% hMSCs group had the lowest expression, among all groups investigated. Col I message, in the 100% hMSCs group, was significantly lower than in the 50% hMSCs + 50% hACPCs group, in the 10% hMSCs + 90% hACPCs group and in the 100% hACPCs group.

On the protein level, chondrogenesis was only monitored in the 100% hMSCs and the 90% hMSCs + 10% hACPCs group. These groups showed abundant Safranin-O staining. Further, the aggrecan protein and the Col II protein were detected using immunohistochemistry. Unfortunately, these groups also demonstrated a weak positive immunolabelling for the hypertrophic matrix protein Col X. The other groups investigated showed no clear sign of chondrogenic differentiation, as evidenced by a lack of Safranin-O staining and a lack of immunolabelling for the chondrogenic matrix proteins aggrecan and Col II. Further, these groups displayed no immunolabelling for the Col X protein, indicating a lack of hypertrophic differentiation. These results conflict with the study of McCarthy and co-workers, where comparable immunolabelling for the proteins aggrecan and Col II, within pellets derived from either 100% equine MSCs or 100% equine ACPCs, was demonstrated (McCarthy et al., 2011). Further, the group was able to show that equine MSC pellet had abundant immunolabelling for the hypertrophic matrix protein Col X and the osteogenic transcription factor Runx2. In contrary, in this study, pellets generated from equine ACPCs were negative for both markers.

These conflicting results might originate from species differences. It cannot necessarily be assumed that cells from human origin behave similar to horse cells. Therefore, hACPCs might not be able to undergo *in vitro* chondrogenesis under these conditions. Another possible explanation is the slight difference in culture medium between the current experiment and the study from McCarthy and co-workers. In the current study, the "classical" serum-free chondrogenic medium was used. McCarthy et al., used 2% serum within their chondrogenic medium. The reason for serum addition was that serum, in this low concentration, enhances ACPC survival (personal correspondence). It cannot be excluded that the serum contains a bioactive factor that is necessary for their chondrogenic induction. This hypothetic factor might not be necessary for hMSCs or they might be able to produce it by themselves. This assumption would explain why hMSCs are able to undergo chondrogenesis, in the "classical" serum-free chondrogenic medium, whereas hACPCs are not. Therefore, in order to determine candidate factors that might explain for the observed differences between the cell types, it was of interest to compare part of the secretome of hMSCs and hACPCs.

The secretome analysis showed that differences, in an order of magnitude of 2-fold or higher, between both cell types were only detected for 17 out of 174 cytokines. Of these 17 cytokines, 14 were more abundant in hMSCs and only 3 were more abundant in hACPCs. Differences were seen in three IGF binding proteins. IGF binding protein 2 and 4 were more abundant in hMSCs and IGF binding protein 3 was more abundant in hACPCs. It was shown that IGF-binding proteins regulate the availability of different IGFs, such as IGF-1 (Bhakta et al., 2000) These differences in the IGF binding protein profile might explain for some of the observed differences between the two cell types. Another interesting candidate cytokine that might partially explain for the observed differences in chondrogenic response is HGF. HGF is over 13-fold more abundant in the secretome of hMSCs, when compared to the secretome of hACPCs. HGF has been demonstrated to increase mobility, proliferation and matrix synthesis in juvenile and foetal rabbit and rat chondrocytes (Takebayashi et al., 1995), suggesting it plays a role in chondrogenesis. Thus, it may have an influence on the differentiation of the progenitor cells used here. However, the role of HGF during matrix metabolism of human adult chondrocytes has been questioned (Bau et al., 2004). Yet, as the total sample size was too low to obtain statistical significant differences between groups, the secretome analysis was only able to provide first preliminary information. In order to confirm these observations, it would be necessary to repeat the secretome analysis with more samples from different donors.

In summary, the current study opted to investigate if hACPCs compare favourably to hMSCs, regarding chondrogenesis and progression towards hypertrophy, in the pellet culture model. Further, it was investigated if co-culturing these cells, in varying ratios, modulates their chondrogenic or hypertrophic response. As opposed to the study of McCarthy and co-workers using equine cells (McCarthy et al., 2011), it was not possible to induce stable chondrogenesis of hACPCs. Biochemical analyses demonstrated decreasing GAG/DNA ratios with increasing percentage of hACPCs within the co-culture pellets. Further, the chondrogenic gene Col II was significantly up-regulated in the 100% hMSCs group, when compared to the 10% hMSCs +90% hACPCs and the 100% hACPCs group.

These results were further confirmed on the protein level. Chondrogenesis was only monitored in the 100% hMSCs and the 90% hMSCs + 10% hACPCs group, as evidenced by Safranin-O/Fast Green staining and immunolabelling for the proteins aggrecan and Col II. Yet, these groups additionally showed positive immunolabelling for the Col X protein, indicating progression towards hypertrophy.

Fluorescent labelling of both cell types indicated that hMSCs might not have survived well if co-cultured with hACPCs. Even if only 25% of hACPCs were present within the co-culture pellets, hMSCs were barely detectable after 3 weeks of culture. Yet, as already mentioned, these conclusions are based on the assumption that both fluorescent dyes have similar characteristics (labelling efficiency, stability) and also that both cell types had a similar proliferation rate. Furthermore, a distinct chondrogenic response was only monitored in the 90% hMSCs + 10% hACPCs group. Yet, the 100% hMSCs group showed comparable results. Therefore, the data that was generated within this model, suggested that the enhanced effort, associated with the use of two different cell sources, is most likely not warranted.

Regarding the monitored differences between the current study and the work of McCarthy et al., (McCarthy et al., 2011), the most likely explanation is that the study of McCarthy et al., was conducted using 2% serum. The current study however, used the "classical" serum-free medium (Johnstone et al., 1998). Possibly, the serum contains a factor that is required for chondrogenic differentiation of equine/human ACPCs. On the other hand, hMSCs might be able to synthesise this factor endogenously or this factor might not be necessary for their chondrogenic induction. In order to gain more insight into this theory, part of the secretome of hMSCs and hACPCs was compared. Several candidate cytokines, such as IGF binding proteins or HGF, were differently abundant in the secretome of hMSCs or hACPCs. Still, the cytokine analysis can only provide a first insight into this problem and further work is warranted. It certainly would be interesting to perform the chondrogenic induction of hACPCs and hMSCs in medium containing 2% serum. Thereby, it could be analysed if hACPCs are able to undergo stable chondrogenesis (with no progression towards hypertrophy) under these conditions. If so, the next step would be to ascertain which factor(s), within the serum, is necessary for the chondrogenic induction. For instance, a first approach could be to determine the effect of exogenous addition of HGF and/or IGFs, such as IGF-1, to the serum-free chondrogenic medium in the 100% hACPCs group.

Chapter 7: hACPCs and load study.

7.1 hACPC and load study - introduction.

Hyaline, articular cartilage possesses a limited intrinsic repair capacity. Cartilage defects that are not, or not sufficiently, treated render the cartilaginous tissue prone to further degeneration. Ultimately, this might result in the development of secondary OA (Madry et al., 2011). Currently, available treatment options, such as microfracture (Steadman et al., 2002), mosaicplasty (Szerb et al., 2005), soft-tissue grafts (Homminga et al., 1990) or ACT (Brittberg et al., 1994) still fail to demonstrate reproducible success in articular cartilage regeneration.

TE represents a promising alternative treatment option for articular cartilage defects. In TE applications, three main questions have to be addressed, depending on the tissue that should be regenerated. These are; 1) which scaffold is appropriate for the given application? 2) from which starting cell source(s) should the tissue be regenerated? and 3) which stimuli are necessary to achieve tissue regeneration? For TE of articular cartilage, a plethora of different scaffold systems and stimulating factors are currently used (see also sections 1.4, 1.5 and 4.1). The two most commonly investigated cell sources are chondrocytes and MSCs (see also section 1.3).

Our group mainly focuses on a composite scaffold system, comprising of a fibrin hydrogel and a PU sponge. Additionally, the effect of complex mechanical stimulation on the chondrogenesis of hMSCs is studied with much courtesy. For administration of complex mechanical stimulation, a novel bioreactor system, based on tribological considerations, was designed (Wimmer et al., 2004). It was demonstrate that the fibrin-PU composite system is comparable to the "gold standard" pellet culture model and that it, additionally, compares favourably regarding expression of genes related to endochondral ossification (Li et al., 2009a). It was further shown that frequency and amplitude of mechanical stimulation influence chondrogenesis (Li et al., 2009c) and that mechanical stimulation enhances endogenous production of both TGF-β1 and TGF-β3 (Li et al., 2009b). This production then leads to chondrogenic induction of hMSCs, in medium lacking exogenous TGF-β1. More recently, it was demonstrated that shear plays an important role in chondrogenesis of hMSCs (Schatti et al., 2011).

Gene transfer represents an interesting alternative to the exogenous application of bioactive factors. The half-life of bioactive factors is usually very short and, therefore, their repeated administration is necessary. In a clinical setup, this is particularly disadvantageous. Gene transfer is based on the delivery of cDNA to a target cell, which is afterwards able to produce the desired transgene. Broadly, gene transfer vectors can be divided in non-viral vectors (less efficient, yet safe) and viral vectors (very efficient, yet their application still raises safety concerns) (Dinser et al., 2001; Steinert et al., 2008; Thomas et al., 2003). Viral vectors can be further sub-divided into integrating vectors, such as retrovirus or lentivirus, and non-integrating vectors, such as adenovirus and AAV. Adenoviral vectors are only able to provide a transient production of the transgene. Normally considered a disadvantage, this feature is beneficial for the treatment of musculoskeletal defects, where only a localised and short-term (days to weeks) production of a stimulating factor is necessary. This transgene production can then either initiate a repair response or enhance the endogenous repair. Lately, our group started to investigate the potential of combining viral-mediated over-expression of bioactive factors and complex mechanical stimulation, for TE of articular cartilage. It was shown that adenoviral-mediated over-expression of SOX9, combined with mechanical stimulation, is able to induce chondrogenesis in medium lacking exogenous dexamethasone and TGF-β1 (Kupcsik et al., 2010). Further, the beneficial effect of retroviral-mediated over-expression of BMP-2 and mechanical stimulation on the chondrogenesis of de-differentiated bovine chondrocytes was demonstrated (Salzmann et al., 2009). Recently, it was demonstrated that the combination of mechanical stimulation and adenoviral-mediated over-expression of BMP-2 harbours potential for *in vitro* chondrogenesis of hMSCs (Neumann et al., 2013a).

Besides MSCs and chondrocytes, there exist other possible cell sources, such as ESCs, iPSCs and ACPCs, which can be used for cartilage TE. Still, for varying reasons, these cell types are currently much less investigated, when compared to MSCs and chondrocytes. ACPCs represent a promising alternative cell source to MSCs and chondrocytes. They were first isolated, through differential adhesion to fibronectin, from the superficial zone of bovine articular cartilage (Dowthwaite et al., 2004). In later work of the same group, these cells were further characterised (Hayes et al., 2008; Khan et al., 2009). More recently, the same group demonstrated that these cells can be isolated from human articular cartilage (Williams et al., 2010).

In 2011, McCarthy et al., performed a first direct comparison between MSCs and ACPCs (McCarthy et al., 2011) in the horse. The pellet culture model, currently the "gold standard" for *in vitro* chondrogenesis, was used. In summary, it was shown that both cell types were able to undergo chondrogenesis, in the presence of low serum concentrations. Yet, MSCs seemed to undergo hypertrophy ("endochondral phenotype"). On the other hand, ACPCs appeared to undergo stable chondrogenesis, suggesting that they are superior to MSCs within this model.

Based on these encouraging results, it was opted to investigate the potential of hACPCs as cell source for articular cartilage TE. The following study aimed to investigate the effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2, alone and in combination, on the differentiation of monolayer-expanded hACPC. No exogenous growth factor was applied. It was hypothesised that mechanical stimulation will lead, as already demonstrated for hMSCs, to an enhanced endogenous production of TGF-β1 and, thereby, induce chondrogenesis. Transduction with Ad.BMP-2 will lead to the temporary production of BMP-2, which was hypothesised to further modulate the chondrogenic response. hACPCs were seeded into fibrin-PU composite scaffolds and either transduced with Ad.BMP-2, using a novel 3D transduction protocol (Neumann et al., 2013c), or left as untransduced controls. After 3 days of pre-culture, they were left as free-swelling controls or subjected to mechanical stimulation for 7 or 28 days. The production of TGF-β1 and BMP-2 was confirmed using quantitative ELISA. Biochemical analyses and gene expression analyses were conducted. The basic parameters of this study were similar to the Ad.BMP-2 and load study (chapter 4). Yet, as hACPCs were used as cell source, interesting questions regarding the chondrogenic potential of this cell type were addressed. Further, it allowed for a direct comparison of the chondrogenic response of the two cell types under identical conditions.

7.2 hACPC and load study - results.

For the hACPCs and load study, the same eight experimental groups (table 4-1) as in the Ad.BMP-2 and load study (chapter 4) were used. The experiment was separately repeated with cells from four different donors (♂ age 75, ♂ age 33, ♂ age 56 and ♀ age 30).

7.2.1 Transduction of hACPCs, in 3D with a MOI of 5, leads to the production of biologically relevant amounts of BMP-2.

It already has been demonstrated that hMSCs can be transduced with Ad.BMP-2 in a 3D environment (see enhanced adenoviral transduction in 3D study (section 3.4 – 3.6), Ad.BMP-2 and load study (Chapter 4) and (Neumann et al., 2013c)). Yet, these results are not necessarily transferable to other cell types. Thus, it is not guaranteed that this novel 3D gene transfer approach will also work for hACPCs. Therefore, it was tested if this technique was applicable for hACPCs. Cells were either left as untransduced controls or transduced with Ad.BMP-2 in 3D with a MOI of 5. The cell culture medium was collected and the BMP-2 concentration was determined using a quantitative ELISA kit for human BMP-2. In untransduced samples, under both loaded and unloaded conditions, the BMP-2 medium concentration was below the detection level of the assay (46.875pg/ml). Therefore, the groups control unloaded and control loaded were not included in figure 7-1.

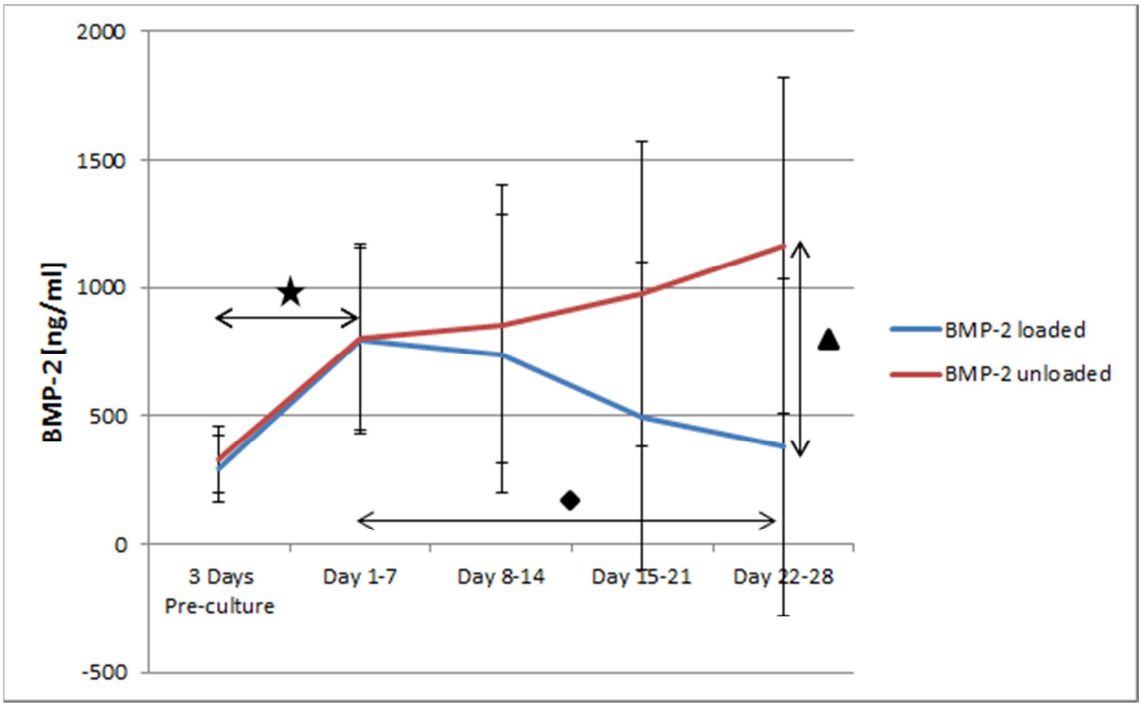

Figure 7-1: BMP-2 concentration within the cell culture medium of Ad.BMP-2 transduced hACPCs, which were cultured in fibrin-PU composite scaffolds. hACPCs were transduced with Ad.BMP-2 in 3D (5 MOI). Subsequently, they were cultured for up to 28 days. Either static culture conditions were applied (unloaded) or cells were cultured with application of 1 hour of mechanical stimulation each day for 6 days per week (loaded). Medium was changed and collected three times a week. Additionally, the medium of the 3 day pre-culture period was collected. Transgene concentration within the culture medium was determined using a quantitative ELISA for human BMP-2. Results are displayed as average ± standard deviation of triplicates from four donors.★significant difference between the 3 days pre-culture and the day 1-7 time-point for both the unloaded and the loaded group ($p \leq 0.05$).;◆ significant difference in the loaded day 1-7 vs. the loaded day 22-28 time-point ($p \leq 0.05$).;▲significant difference in the unloaded vs. the loaded group, after 4 weeks of culture ($p \leq 0.05$).

hACPCs that have been transduced with Ad.BMP-2, in 3D with a MOI of 5, already started to produce biologically relevant (100ng/ml or above) amounts of BMP-2 during the 3 day pre-culture period. At the first two time-points that were investigated (3 day pre-culture and day 1-7), the measured values in the BMP-2 unloaded and the BMP-2 loaded group were almost identical. Further, in both groups, there was a statistical significant increase in BMP-2 medium levels between these time-points (*p=0.019* for BMP-2 unloaded and *p=0.000* for BMP-2 loaded). Afterwards, both groups displayed opposite trends. In the unloaded group, BMP-2 medium levels stayed relatively constant (805.6ng/ml ± 362ng/ml on day 1-7 and 1164.8ng/ml ± 658.6ng/ml on day 22-28). In the loaded group however, the BMP-2 medium levels steadily and significantly (*p=0.000*) decreased from 793ng/ml ± 213ng/ml (day 1-7) to 376.7ng/ml ± 206.9ng/ml (day 22-28). After 4 weeks of culture, BMP-2 medium levels were significantly higher the unloaded group, if compared to the loaded group (*p=0.035*).

7.2.2 hACPCs produce TGF-β1 during culture in fibrin-PU composite scaffolds.

It already has been demonstrated that hMSCs are able to endogenously produce TGF-β1, if cultured in medium that lacks exogenous TGF-β1 (Li et al., 2009b). Further, it has been demonstrated that TGF-β1 production was increased, when these cells were subjected to mechanical stimulation and that this increased endogenous TGF-β1 production was able to induce chondrogenesis (Li et al., 2009b). For hACPCs this information is not yet available. Therefore, it was crucial to determine if these cells are able to endogenously produce TGF-β1 and how this production is influenced by mechanical stimulation. TGF-β1 concentration, within the cell culture medium, was analysed using a quantitative ELISA kit for human TGF-β1 (figure 7-2).

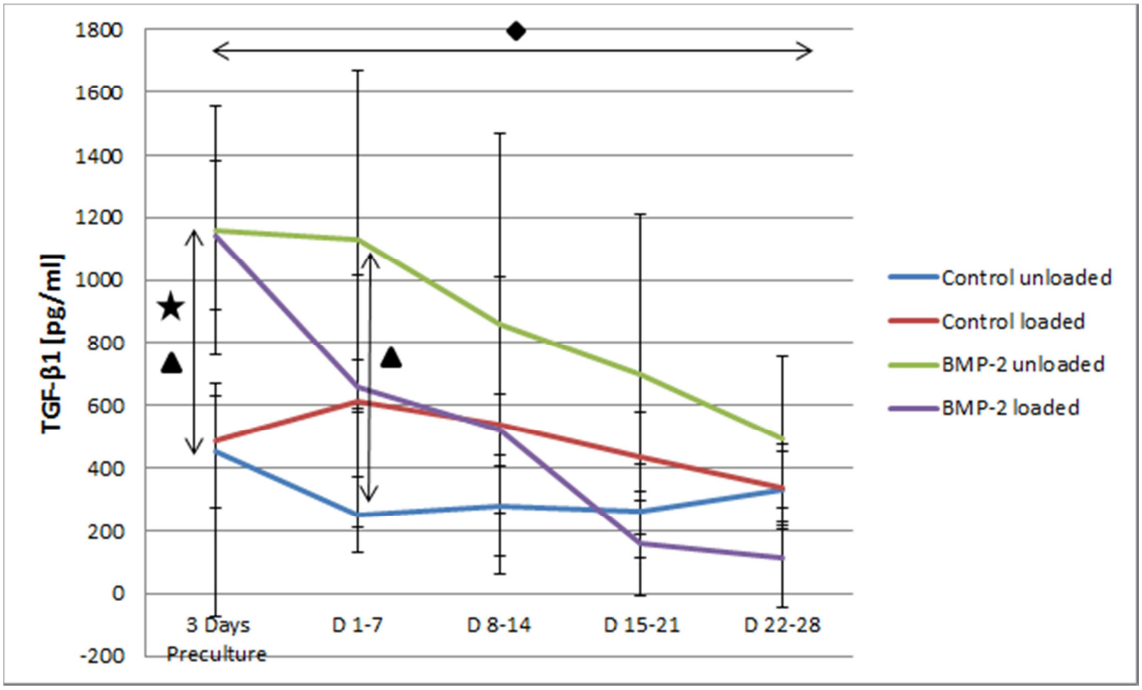

Figure 7-2: TGF-β1 concentration within the cell culture medium of hACPCs, which were cultured in fibrin-PU composite scaffolds. hACPCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2, in 3D with a MOI of 5, (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Medium was changed and collected three times a week. Additionally, the medium of the 3 day pre-culture period was collected. Transgene concentration, within the culture medium, was determined using a quantitative ELISA for human TGF-β1. Results are displayed as average ± standard deviation of triplicates from four donors. ★ significant difference between the control loaded and the BMP-2 loaded group ($p \leq 0.05$); ▲ significant difference between the control unloaded and the BMP-2 unloaded group ($p \leq 0.05$); ◆ significant difference between the 3 days pre-culture and the day 22-28 time-point for the BMP-2 loaded group ($p \leq 0.05$).

During the 3 day pre-culture period (static conditions), a statistically significant difference in TGF-β1 medium levels between the control and the Ad.BMP-2 transduced groups was detected. This difference was true for both the loaded (*p=0.027*) and the unloaded (*p=0.008*) group. Absolute concentrations were 453.1pg/ml ± 177.4pg/ml (control unloaded), 488.3pg/ml ± 180pg/ml (control loaded), 1158.8pg/ml ± 398pg/ml (BMP-2 unloaded) and 1143.8pg/ml ± 564.7pg/ml (BMP-2 loaded). Further, at the day 1-7 time-point, there was a significant difference between the control unloaded and the BMP-2 unloaded group (*p=0.016*). Generally, during the 4 weeks of culture, TGF-β1 medium levels showed a trend towards a decreased concentration in the Ad.BMP-2 transduced groups. This trend reached significance only in the Ad.BMP-2 transduced and loaded group (3 days pre-culture vs. day 21-28, *p=0.000*). In the control groups however, the TGF-β1 medium levels stayed relatively stable during the course of the experiment. After 4 weeks of culture, no significant differences in TGF-β1 medium concentration were detected between the different groups. Mechanical stimulation did not significantly influence TGF-β1 medium levels, in both the control and the Ad.BMP-2 transduced group. Absolute values, after 4 weeks of culture, were 334.5pg/ml ± 117pg/ml (control unloaded), 340pg/ml ± 197.5pg/ml (control loaded), 491pg/ml ± 263.4pg/ml (BMP-2 unloaded) and 114.2pg/ml ± 133.7pg/ml (BMP-2 loaded).

7.2.3 Mechanical stimulation leads to an up-regulation of aggrecan and collagen II, when compared to free-swelling controls. Transduction with Ad.BMP-2 is the predominant stimulus for the gene BMP-2. Further, it leads to an up-regulation of the hypertrophic marker gene collagen X (at the late time-point) and the early osteogenic marker gene ALP.

The effect of mechanical stimulation and transduction with Ad.BMP-2, on the gene expression profile of hACPCs, was investigated. Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, with 18s rRNA as an internal control. The unloaded control group at day 7 was used as normaliser and, therefore, set to 1. The following genes were analysed; Col I, Col X, Runx2, ALP and Notch 1 (figure 7-3) and aggrecan, SOX9, BMP-2 and PTHrP (figure 7-4). For the gene ALP, only three donors were used for RT-PCR analysis (in one of the four donors [♂ age 56] ALP message was not detectable). Additionally, RT-PCR was conducted for the chondrogenic marker gene Col II and the late osteogenic marker gene Osterix. Osterix gene expression was not detected in any of the experiments. Therefore, gene expression analysis for Osterix was not conducted. Col II message was only constantly (in each of the eight groups investigated) detected in one donor (♂ age 56). For this donor, a "normal" ($\Delta\Delta C_T$, statistics) Col II gene expression analysis was conducted (figure 7-5). It must be noted that, usually, a statistical analysis with samples from one donor is not warranted. Nevertheless, this donor showed clear response towards both stimuli and the statistical analysis was conducted to gain additional information. In the remaining three donors, Col II message was not constantly detectable in each of the eight groups. Therefore, it was not possible to conduct a reliable $\Delta\Delta C_T$ analysis for Col II in these three donors. Yet, these donors displayed the same trends, when compared to the donor where Col II message was constantly detected. Therefore, an overview about Col II gene expression, within these three donors, was carried out (table 7-1).

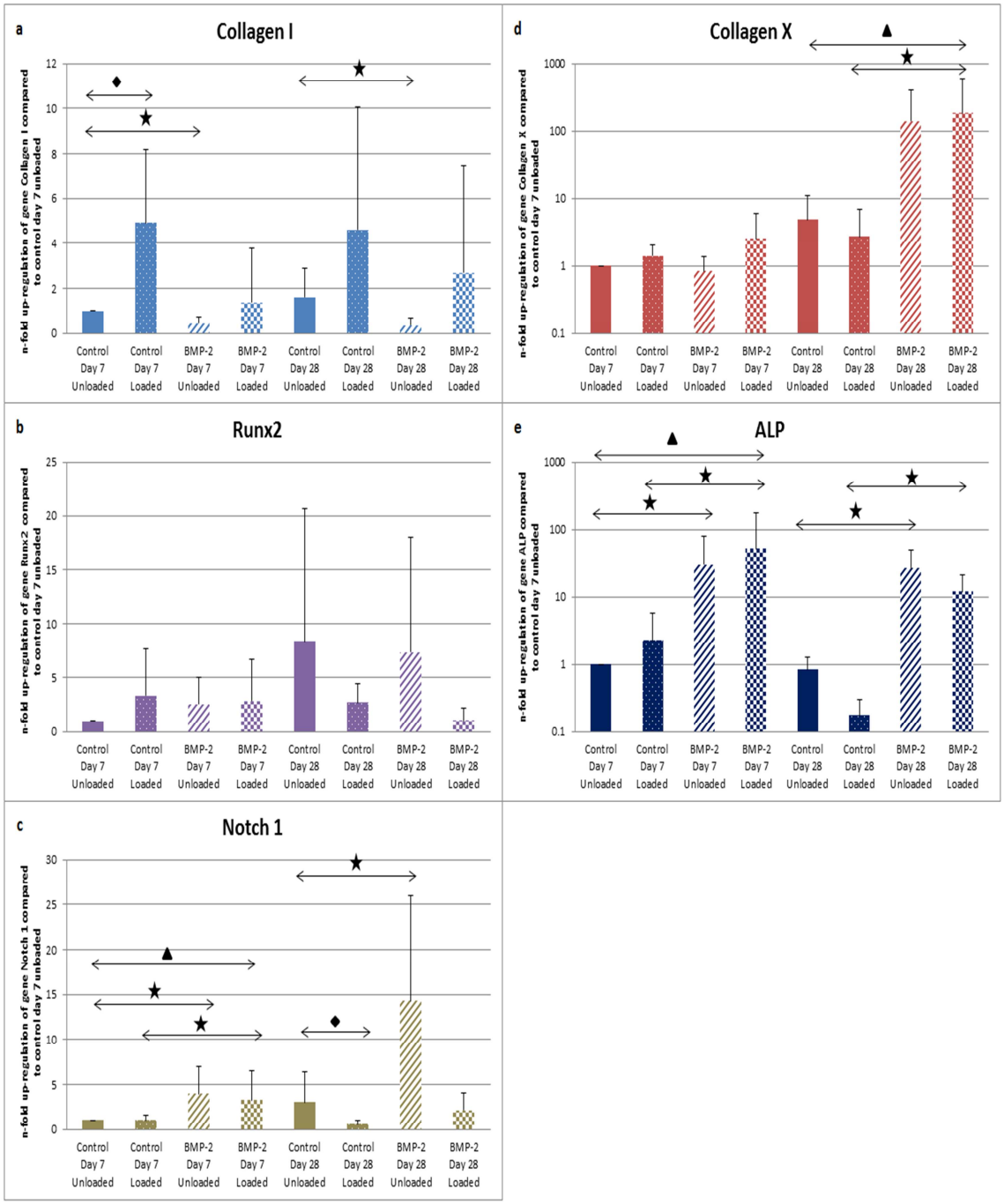

Figure 7-3: Relative gene expression of hACPCs, which have been cultured in fibrin-PU composite scaffolds for 7 or 28 days. They have been transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or were left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Col I (a), Runx2 (b), Notch 1 (c), Col X (d) and ALP (e). 18s rRNA was used as internal control. The unloaded control group at day 7 was used as calibrator and, therefore, set to 1. Results are displayed as average + standard deviation of triplicates from three donors (ALP) or four donors remaining genes).★ significant difference in the control vs. the Ad.BMP-2 transduced group ($p \leq 0.05$); ◆ significant difference in the unloaded vs. the loaded group ($p \leq 0.05$); ▲ significant difference in the control unloaded vs. the Ad.BMP-2 transduced loaded group ($p \leq 0.05$).

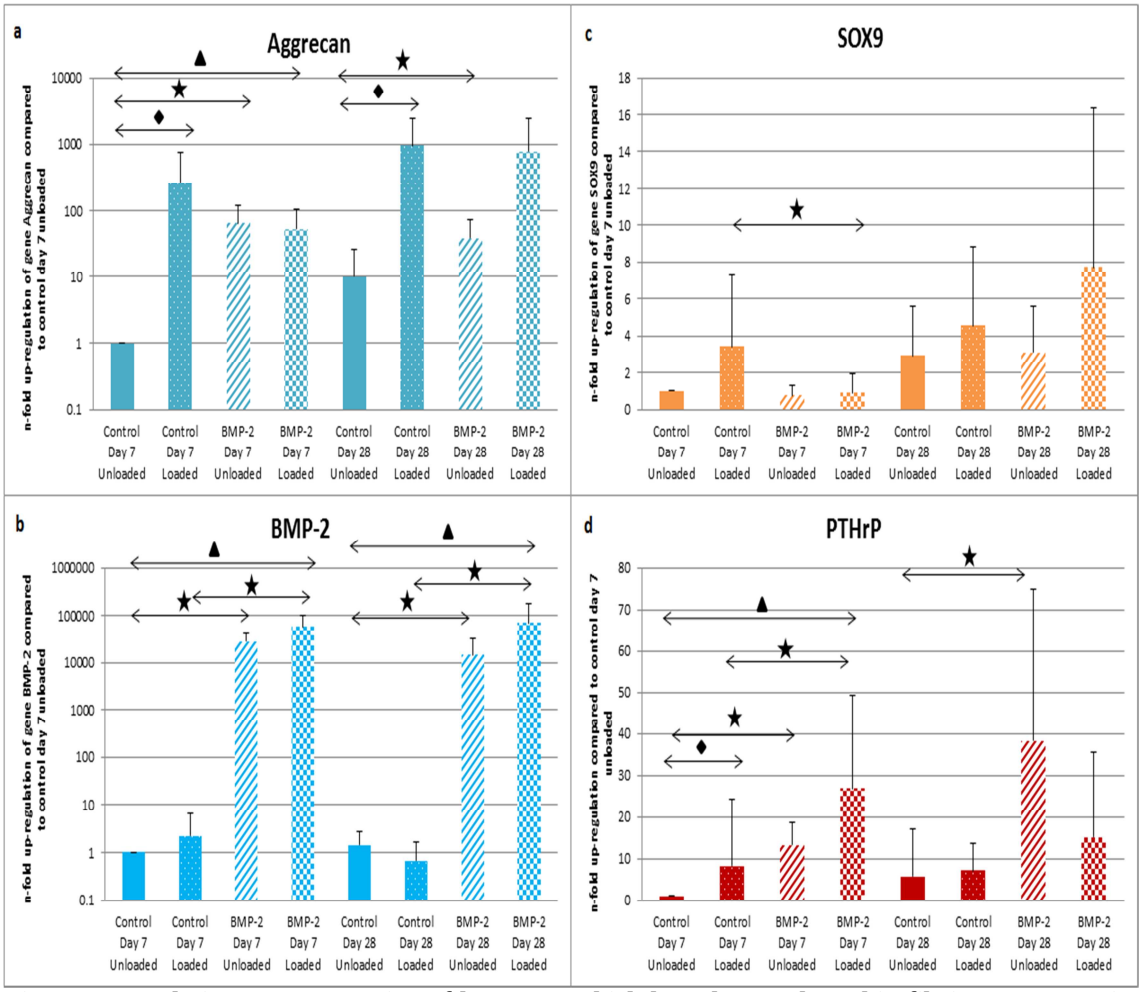

Figure 7-4: Relative gene expression of hACPCs, which have been cultured in fibrin-PU composite scaffolds for 7 or 28 days. They have been transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or were left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Gene expression analyses were conducted, using the comparative $\Delta\Delta C_T$ method, for genes Aggrecan (a), BMP-2 (b), SOX9 (c) and PTHrP (d). 18s rRNA was used as internal control. The unloaded control group at day 7 was used as calibrator and, therefore, set to 1. Results are displayed as average + standard deviation of triplicates from four donors.★significant difference in the control vs. the Ad.BMP-2 transduced group ($p \leq 0.05$); ◆significant difference in the unloaded vs. the loaded group ($p \leq 0.05$); ▲significant difference in the control unloaded vs. the Ad.BMP-2 transduced loaded group ($p \leq 0.05$).

The response (n-fold changes in gene expression compared to the control day 7 unloaded group) of the different genes investigated, towards both stimuli applied, can be broadly divided into three classes. The genes SOX9 (figure 7-4 c), Col I (figure 7-3 a), Runx2 (figure 7-3 b) and Notch1 (figure 7-3 c) showed little differences in gene expression. The n-fold changes in gene expression were usually below 10. The genes PTHrP (figure 7-4 d) and ALP (figure 7-3 e) showed an intermediate response. Maximal n-fold up-regulations monitored were below 100.

The genes Col X (figure 7-3 d) and, especially, aggrecan (figure 7-4 a) and BMP-2 (figure 7-4 b) were strongly regulated by both stimuli, depending on the group. A n-fold up-regulation in gene expression up to the range of several thousand-fold was detected.

The gene Runx2 (figure 7-3 b) was the only gene investigated where no significant changes in gene expression were detected. For the gene Col I (figure 7-3 a), mechanical stimulation led to a significant up-regulation in gene expression in the control group at day 7 ($p=0.001$). Further, transduction with Ad.BMP-2 led to a small, but significant, down-regulation of Col I message in the unloaded group, at both the early ($p=0.001$) and the late time-point ($p=0.013$). Notch 1 gene expression (figure 7-3 c) was down-regulated by mechanical stimulation in the control group at day 28 ($p=0.034$). On the other hand, transduction with Ad.BMP-2 led to a significant increase in Notch 1 message in both the unloaded ($p=0.002$) and the loaded group ($p=0.013$) at day 7 and in the unloaded group at day 28 ($p=0.000$). For the gene SOX9 (figure 7-4), transduction with Ad.BMP-2 led to a significant, yet small, down-regulation of SOX9 message in the loaded group at the early time-point ($p=0.035$). Mechanical stimulation did not lead to any significant changes in ALP gene expression (figure 7-3 e). Even though it led to a 80% decrease in ALP expression, in the control group at the late time-point (day 28), this trend failed to reach significance *($p=0.097$).* On the other hand, transduction with Ad.BMP-2 led to a statistically significant increase in ALP gene expression. This was true for both the unloaded ($p=0.006$ at day 7 and $p=0.013$ at day 28) and the loaded group ($p=0.047$ at day 7 and $p=0.004$ at day 28). At the early time-point (day 7), expression of the gene PTHrP (figure 7-4 d) was influenced by both stimuli applied. Both mechanical stimulation ($p=0.036$) and transduction with Ad.BMP-2 ($p=0.000$) led to a significant increase in PTHrP expression, when compared to the control unloaded group at day 7. Interestingly, transduction with Ad.BMP-2 seemed to be the predominant stimulus for PTHrP (at the early time-point, BMP-2 unloaded was not significantly different from BMP-2 loaded but control loaded was significantly lower than BMP-2 loaded). At the late time-point (day 28), only transduction with Ad.BMP-2 in the unloaded group still led to an up-regulation of PTHrP expression, when compared to the control group ($p=0.001$). After 7 days, both mechanical stimulation and transduction with Ad.BMP-2 did not significantly influence Col X expression (figure 7-3 d). Again, after 28 days, load had no effect on Col X message.

On the other hand, adenoviral-mediated over-expression of BMP-2 led to an over 100-fold, significant increase in Col X message in the loaded group on day 28 (*p=0.000*). Mechanical stimulation, strongly up-regulated the gene aggrecan (figure 7-4 a) in the control group at both time-points. In these groups, aggrecan message was significantly elevated, when compared to the respective unloaded control group (*p=0.000* at day 7 and *p=0.008* at day 28). Also, transduction with Ad.BMP-2 led to a significant up-regulation of aggrecan message, in the unloaded group on both the early (*p=0.000*) and the late time-point (*p=0.044*). The combined application of mechanical stimulation did not lead to a further increase in aggrecan message (the BMP-2 loaded group was not significantly different from the control loaded and the BMP-2 unloaded group, at both day 7 and day 28). Finally, the gene BMP-2 (figure 7-4 b) was un-responsive towards mechanical stimulation. However, when cells were transduced with Ad.BMP-2, BMP-2 message was massively and significantly elevated. This was true for both the unloaded (*p=0.000* at day 7 and *p=0.000* at day 28) and the loaded group (*p=0.000* at day 7 and *p=0.000* at day 28)

Col II gene expression detectable in								
Group → Run ↓	Control day 7 unloaded	Control day 7 loaded	BMP-2 day 7 unloaded	BMP-2 day 7 loaded	Control day 28 unloaded	Control day 28 loaded	BMP-2 day 28 unloaded	BMP-2 day 28 loaded
I	ND	ND	ND	ND	ND	Yes	ND	Yes
II	ND	Yes	ND	ND	ND	Yes	ND	ND
III	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
IV	ND	Yes	ND	ND	ND	Yes	ND	ND

Table 7-1: Overview of Col II gene expression within the ACPCs and load study. The table displays the groups, in which Col II message was detected within the four runs of the experiment. ND = not detectable.

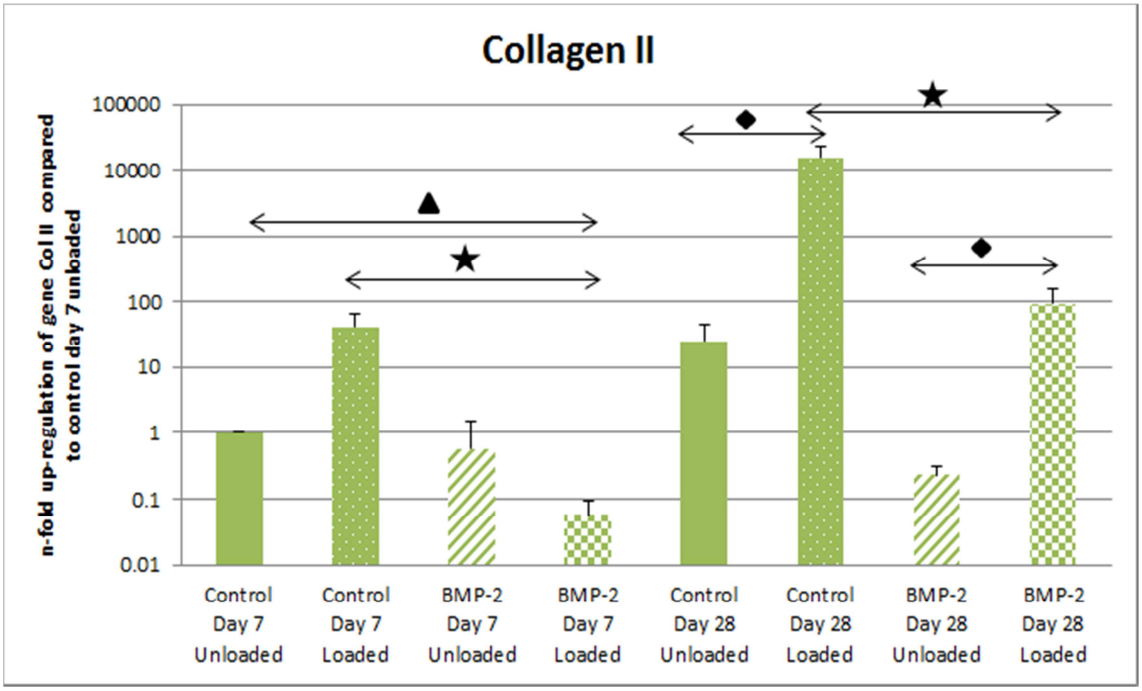

Figure 7-5: Relative gene expression of hACPCs, which have been cultured in fibrin-PU composite scaffolds for 7 or 28 days. They have been transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or were left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Gene expression analysis was conducted using the comparative $\Delta\Delta C_T$ method for the gene Col II. 18s rRNA was used as internal control. The unloaded control group at day 7 was used as calibrator and, therefore, set to 1. Results are displayed as average + standard deviation of triplicates from one donor.★Significant difference in the control vs. the Ad.BMP-2 transduced group ($p \leq 0.05$);◆significant difference in the unloaded vs. the loaded group ($p \leq 0.05$); ▲significant difference in the control unloaded vs. the Ad.BMP-2 transduced loaded group ($p \leq 0.05$).

The gene Col II was only constantly monitored (in each of the eight groups) in run III of the study (table 7-1). Further, the control loaded day 28 group was the only group that showed Col II expression in each of the four runs (table 7-1). In run I, II an IV, Col II message was only detectable in the control loaded day 7 group (run II and run IV), the control loaded day 28 group (run II, run III and run IV) and the BMP-2 loaded day 28 group (run I only). The results of run III demonstrated that both stimuli had a different effect on Col II message. On day 28, mechanical stimulation led to a significant increase in Col II message, in the control ($p=0.040$) and the Ad.BMP-2 transduced group ($p=0.014$). Interestingly, at the early time-point (day 7), application of both stimuli combined was detrimental for Col II expression. The control unloaded group was significantly higher, when compared to the BMP-2 loaded group ($p=0.035$). Transduction with Ad.BMP-2 led to a significant decrease in Col II expression in the loaded group ($p=0.001$ at day 7 and $p=0.012$ at day 28).

7.2.4 The DNA content of hACPCs samples, seeded into fibrin-PU composite scaffolds, decreases between week 1 and week 4 of culture.

In order to get a general idea of how the different stimuli influenced the total cell number, the DNA content was quantified using the Hoechst 33258 dye assay. Figure 7-6 displays the DNA content for each of the eight experimental groups. Figure 7-7 displays the average of day 7 and day 28 samples for each group. These values were used for the calculation of the GAG/DNA ratio in section 7.2.6.

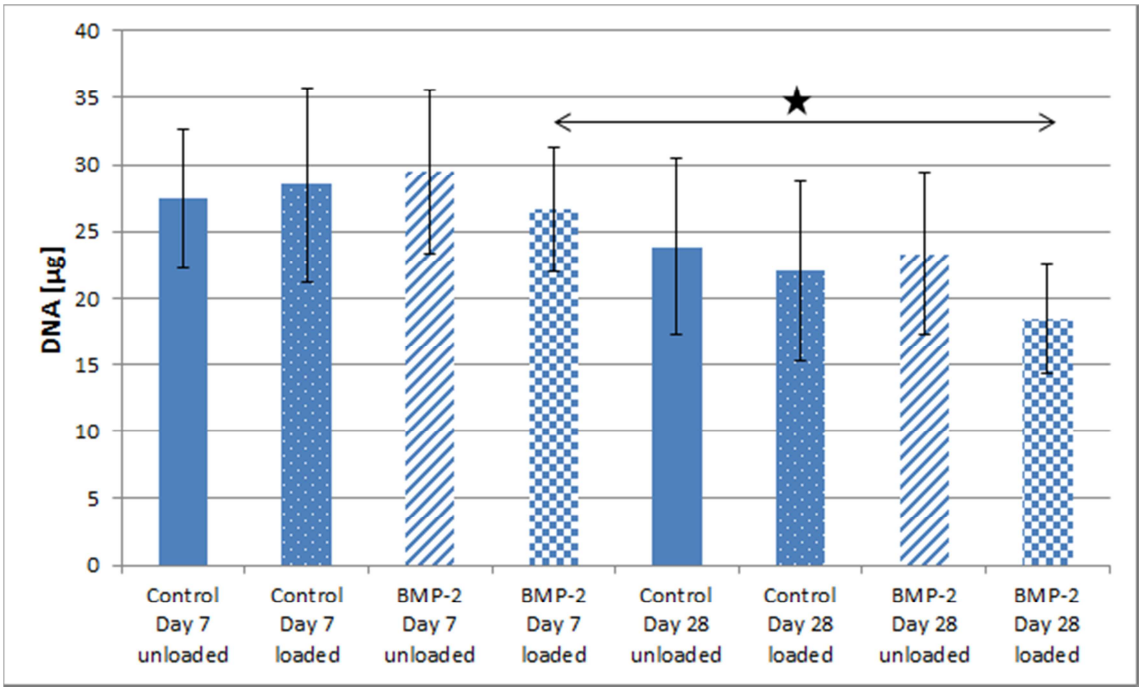

Figure 7-6: Total DNA content of hACPCs after 7 or 28 days of culture. hACPCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). DNA content was quantified from scaffolds using the Hoechst 33258 dye assay with calf thymus DNA as standard. Results are displayed as average ± standard deviation of triplicates from four donors; ★significant difference between the day 7 and the day 28 time-point for the BMP-2 loaded group ($p \leq 0.05$).

There was a general trend towards a decrease in DNA content between week 1 and week 4 of culture. Yet, this trend only reached statistical significance in the BMP-2 loaded group (*p=0.000*). The total DNA content for each group was 27.44µg ± 5.14µg (control day 7 unloaded), 28.5µg ± 7.27µg (control day 7 loaded), 29.46µg ± 6.15µg (BMP-2 day 7 unloaded), 26.65µg ± 4.6µg (BMP-2 day 7 loaded), 23.85µg ± 6.57µg (control day 28 unloaded), 22.05µg ± 6.73µg (control day 28 loaded), 23.33µg ± 6.09µg (BMP-2 day 28 unloaded) and 18.39µg ± 4.1µg (BMP-2 day 28 loaded).

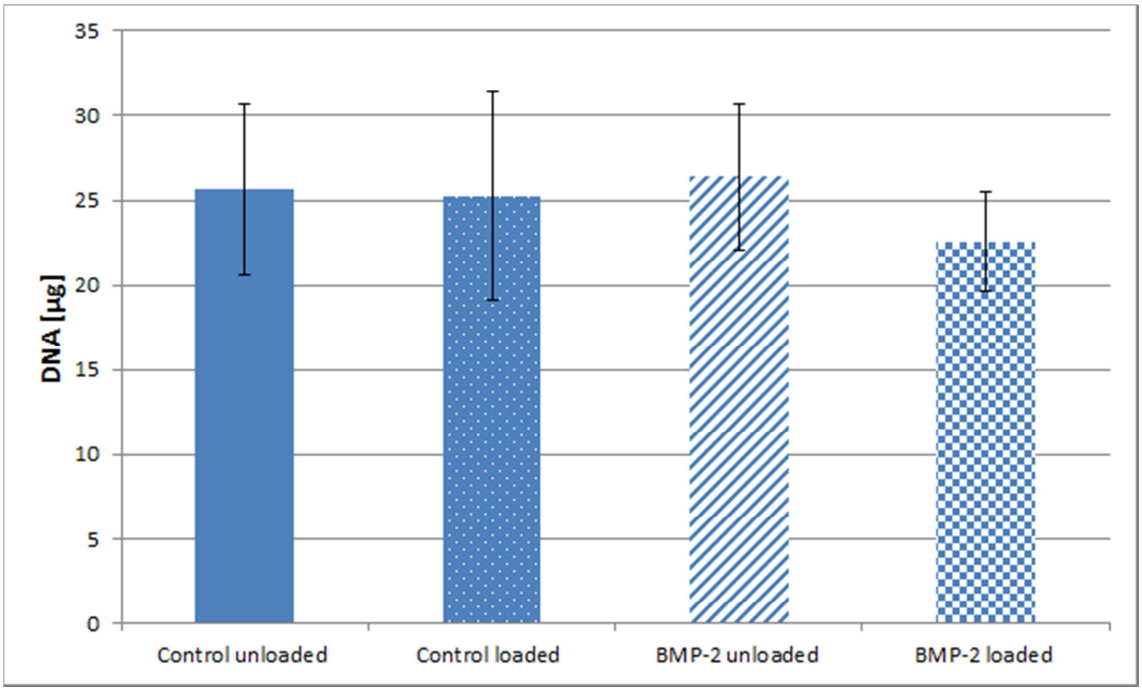

Figure 7-7: Average DNA content (calculated from day 7 and day 28 samples) of hACPCs. hACPCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). DNA content was quantified from scaffolds using the Hoechst 33258 dye assay with calf thymus DNA as a standard. Further, the average between the day 7 and the day 28 samples was individually calculated for each donor and each sample. Results are displayed as average ± standard deviation of triplicates from four donors.

When the average DNA content (independent from the time-point) was calculated there were no significant differences between the groups. Total values obtained were 25.65µg ± 5.08µg (control unloaded), 25.24µg ± 6.17µg (control loaded), 26.4µg ± 4.31µg (BMP-2 unloaded) and 22.52µg ± 2.92µg (BMP-2loaded).

7.2.5 The bulk amount of synthesised GAG is released into the culture medium.

In order to track matrix production of hACPCs, the cumulative amount of released GAG, over the course of 28 days, was quantified using the DMMB dye binding assay. Furthermore, the amount of GAG retained within the scaffolds was measured, after 4 weeks of culture (figure 7-8).

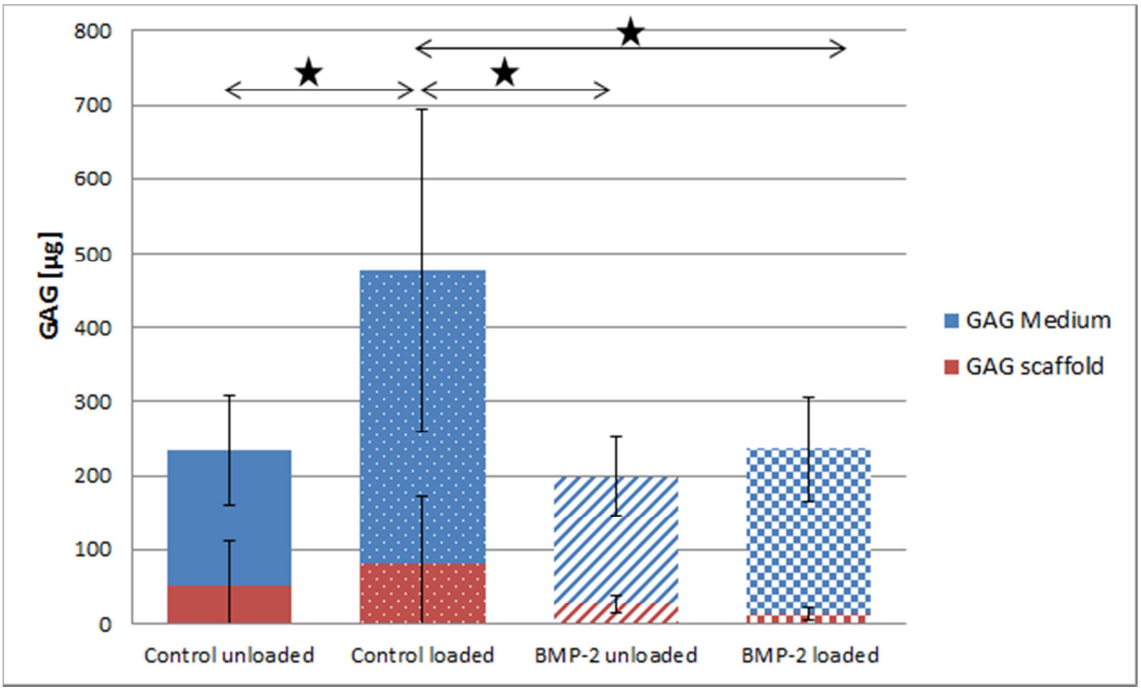

Figure 7-8: Total amount of GAG synthesised after 28 days. hACPCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). Medium was changed and collected three times a week. It was pooled for weeks 1, 2, 3 and 4. Additionally, the medium of the 3 day pre-culture period was collected. GAG content was quantified (both within the scaffold and within the culture medium) using the DMMB dye binding assay with chondroitin-4-sulfate as standard. Results are displayed as average ± standard deviation of triplicates from four donors. ★significantly different from the control loaded group, ($p \leq 0.05$).

As expected, the bulk amount of synthesised GAG was released into the culture medium. Only a small portion of the total amount of GAG produced was retained within the scaffolds. The control loaded group produced the highest amount of GAG, which was significantly higher, when compared to the other three groups (*p=0.011* vs. control unloaded, *p=0.003* vs. BMP-2 unloaded and *p=0.009* vs. BMP-2 loaded). In the Ad.BMP-2 transduced groups, mechanical stimulation led to a trend towards higher GAG production, which failed to reach statistical significance. Further, transduction with Ad.BMP-2 significantly reduced GAG production in the loaded group, when compared to the untransduced control group (*p=0.009*). Over the course of 4 weeks, a total of 231.4µg ± 107.7µg (control unloaded), 474.2µg ± 240.6µg (control loaded), 196.5µg ± 60.9µg (BMP-2 unloaded) and 233.7µg ± 768µg (BMP-2 loaded) of GAG was synthesised. The amount of GAG retained within the scaffolds was between 19.7% ± 14.3% (control unloaded) and 5.5% ± 3.3% (BMP-2 loaded).

7.2.6 The GAG/DNA ratio is higher in control vs. Ad.BMP-2 transduced and in loaded vs. unloaded samples.

Finally, in order to better compare matrix production between the different groups, the GAG/DNA ratio [in µg/µg] was calculated (figure 7-9). This procedure normalises the amount of synthesised matrix to a specific amount of DNA (indicator for total cell number). The total amount of GAG produced (scaffolds + medium over the course of 4 weeks) was divided by the average DNA content (from day 7 and day 28 samples).

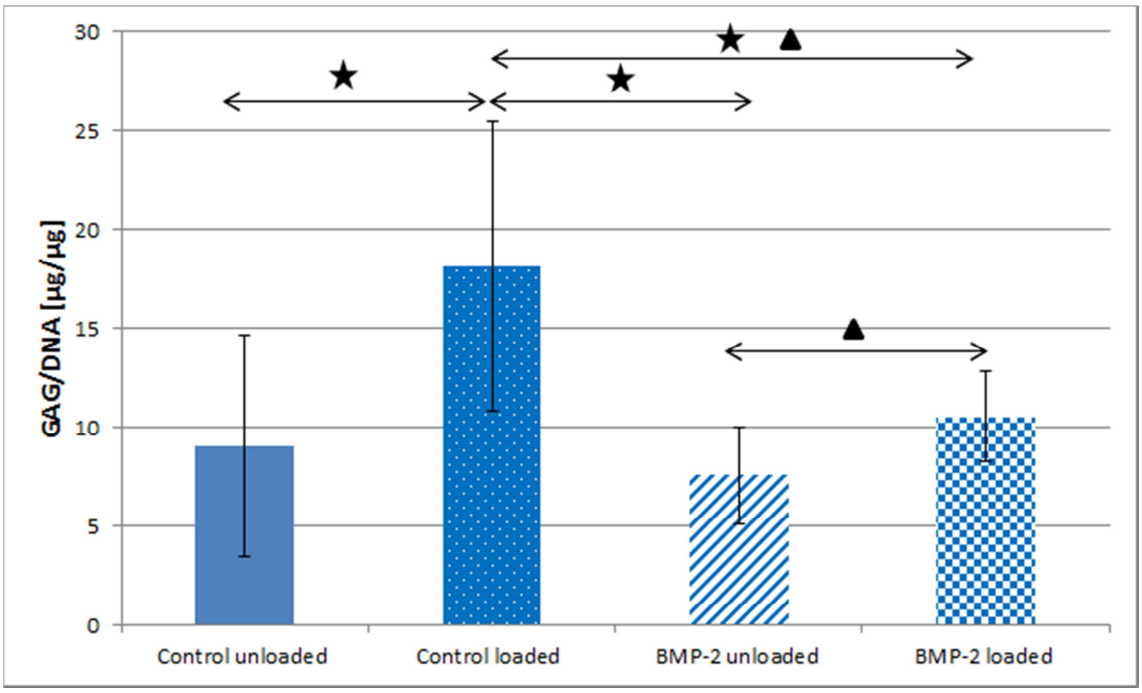

Figure 7-9: Total amount of GAG (scaffold + medium over the course of 4 weeks) normalised to average DNA content (of day 7 and day 28 samples). hACPCs were cultured in fibrin-PU composite scaffolds and either transduced with Ad.BMP-2 in 3D with a MOI of 5 (BMP-2) or left untreated (control). Subsequently, they were either cultured under static conditions (unloaded) or 1 hour of mechanical stimulation per day was applied for 6 days per week (loaded). The total amount of GAG and the amount of DNA was quantified. The GAG production was normalised to the DNA content. Results are displayed as averages ± standard deviation of triplicates from four donors.★Significantly different from the control loaded group ($p \leq 0.05$); ▲significantly different from the BMP-2 loaded group ($p \leq 0.05$).

For both the control (*p=0.007*) and the Ad.BMP-2 transduced group (*p=0.017*), mechanical stimulation led to a significant increase in the GAG/DNA ratio. The GAG/DNA ratio was highest in the control loaded group and significantly different from the three other groups that were investigated (*p=0.007* vs. control unloaded, *p=0.000* vs. BMP-2 unloaded and *p=0.007* vs. BMP-2 loaded). The GAG/DNA ratio, in µg/µg/, was 9.1 ± 5.6 (control unloaded), 18.16 ± 7.35 (control loaded), 7.56 ± 2.47 (BMP-2 unloaded) and 10.53 ± 2.27 (BMP-2 unloaded).

7.3 hACPC and load study - discussion.

ACPCs represent a new and potentially superior cell source, regarding potential stable chondrogenic induction, when compared MSCs, for TE of articular cartilage (McCarthy et al., 2011). Mechanical stimulation influences both the development and the maintenance of articular cartilage. Therefore, functional TE, which incorporates mechanical stimulation, more closely mimics the prevailing situation *in vivo*, when compared to classical TE approaches, which lack this stimulus. The current study opted to investigate the combined effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2 on the chondrogenesis of monolayer-expanded hACPCs. hACPCs were encapsulated in fibrin and seeded into PU scaffolds. A seeding density of 5 x 10^6 cells per sample was used. As mechanical stimulation was hypothesised to induce chondrogenesis of hACPCs, a chondro-permissive medium, which lacks exogenous growth factors, was used. The samples were cultured for 7 or 28 days. Mechanical stimulation and transduction with Ad.BMP-2 were applied alone or in combination. Further, an untransduced control group was included (cultured under free-swelling conditions). For hMSCs, it has been demonstrated that mechanical stimulation leads to an enhanced endogenous production of TGF-β1 and that this production was able to induce chondrogenesis, if exogenous TGF-β1 was omitted from the culture medium (Li et al., 2009b). It was hypothesised that hACPCs respond similarly towards mechanical stimulation. BMP-2 is a bioactive factor that is widely used for chondrogenic induction or enhancement of chondrogenesis in hMSCs (Majumdar et al., 2001; Schmitt et al., 2003). Further, it has been demonstrated that hMSCs can be transduced with Ad.BMP-2 using a novel protocol for 3D transduction (section 3.4 – 3.6, Ad.BMP-2 and load study (chapter 4) and (Neumann et al., 2013c)). This transduction then leads to the endogenous production of BMP-2. It was hypothesised that hACPCs can be transduced with Ad.BMP-2 in 3D and that they afterwards endogenously produce BMP-2. This production was believed to further modulate chondrogenesis. This combined approach has already demonstrated its potential, when hMSCs were used as cell source (Neumann et al., 2013a). Outcome parameters measured were the concentrations of TGF-β1 and BMP-2 within the cell culture medium. Further, biochemical analyses (GAG, DNA and GAG/DNA) and gene expression analyses were conducted.

After 3D transduction with Ad.BMP-2, hACPCs were able to produce BMP-2. This result confirmed the feasibility of the novel 3D transduction protocol for hACPCs, seeded into fibrin-PU composite scaffolds. Further, when compared to hMSCs, higher amounts of transgene product were produced. The peak BMP-2 concentration that was measured when hMSCs were transduced was 339.8ng/ml ± 186ng/ml (Neumann et al., 2013a). For hACPCs, the peak concentration generated was 1164.8ng/ml ± 658.6ng/ml. Interestingly, BMP-2 medium levels were always 100ng/ml or above (even during the 3 days of pre-culture). This concentration is considered biologically relevant and commonly used when BMP-2 is applied exogenously (Majumdar et al., 2001; Sailor et al., 1996; Toh et al., 2007). During the first week of culture, almost identical BMP-2 concentrations were generated in both the unloaded and the loaded group. Also, in both groups, the amount of BMP-2 produced significantly increased between the 3 days of pre-culture and the first week of culture. Afterwards, both groups showed opposing trends. In the loaded group, BMP-2 medium levels steadily decreased between week 1 and week 4. This decrease resulted in a significant difference between the week 1 and the week 4 time-point. In the unloaded group, the BMP-2 medium concentrations stayed relatively constant. After 4 weeks of culture, the BMP-2 concentration within the unloaded group was significantly higher, when compared to the loaded group. In hMSCs, the exact opposite trend was detected (Neumann et al., 2013a). For hMSCs, it was proposed that, in the unloaded group, BMP-2 can leave the scaffold only by means of passive diffusion. In the loaded group however, the synthesised BMP-2 is expelled from of the scaffold. For hACPCs, different effect(s) seem to be predominant which probably masked this phenomenon. It is possible that mechanical stimulation negatively affected BMP-2 production within hACPCs. Another explanation may be that mechanical stimulation influenced the ECM, which might have prevented the synthesised BMP-2 from leaving the scaffold. Yet, without further investigation, this conflicting observation is difficult to interpret.

It was confirmed that hACPCs endogenously produce TGF-β1, if cultured in medium lacking exogenous growth factors. After 3 days of pre-culture, significantly more TGF-β1 was produced in the Ad.BMP-2 transduced samples. This result was true for both the unloaded and the loaded group and indicates that the presence (or production) of BMP-2 enhances endogenous TGF-β1 production. After 7 days however, this observation was only confirmed in the unloaded and not in the loaded group. In the control groups (both loaded and unloaded), TGF-β1 medium levels stayed relatively constant over the course of 4 weeks and no significant differences between the different time-points were monitored. In the Ad.BMP-2 transduced groups, TGF-β1 medium levels constantly decreased, especially in the loaded group. After 4 weeks, the TGF-β1 medium concentration in the BMP-2 loaded group was significantly lower, when compared to the same group at the 3 days pre-culture time-point. Also, starting from week 2 of culture, no significant differences between the groups were detected. Interestingly, after 7 days of culture, comparable amounts of TGF-β1 were generated in both the unloaded and the loaded control group (no transduction with Ad.BMP-2), when compared to hMSCs cultured under identical conditions (Li et al., 2009b). Li and co-workers did not further track TGF-β1 concentrations over time. Therefore, it cannot be validated that both cells types produced comparable amounts of TGF-β1 over the course of the study.

Between week 1 and week 4 of culture, there was a significant decrease in the DNA content in the BMP-2 loaded group. This result suggests that, when both stimuli were applied simultaneously, cell death, over proliferation, dominated the cellular response. Interestingly, hACPCs seem to survive better and/or display an enhanced proliferation capacity within the fibrin-PU composite scaffold system, when compared to hMSCs. In the Ad.BMP-2 and load study (chapter 4, (Neumann et al., 2013a)), the exact same parameters (seeding density, scaffold system, culture medium and stimuli) were applied. Still, the DNA content (indicator for total cell number), after both 1 week and 4 weeks of culture, was higher when hACPCs were used, when compared to hMSCs. For hMSCs, the DNA content was between 11.81µg ± 3.83µg (BMP-2 day 28 unloaded) and 16.72µg ± 4.71µg (control day 28 loaded) (Neumann et al., 2013a). For hACPCs however, the DNA content was between 18.39µg ± 4.1µg (BMP-2 day 28 loaded) and 29.46µg ± 6.15µg (BMP-2 day 7 unloaded).

The GAG quantification revealed that most of the synthesised GAG was not retained within the scaffold. Instead, the bulk amount of synthesised GAG was released into the culture medium. Similar observations were made using fibrin-PU composite scaffolds and hMSCs or bovine chondrocytes (Li et al., 2009b; Li et al., 2009c; Neumann et al., 2013a). For both the control loaded and the Ad.BMP-2 transduced and loaded group, the percentage of GAG retained within the scaffold was lower, when compared to the corresponding unloaded group. Generally, the amount of matrix that can be retained depends on the pericellular matrix, the mechanical environment and the porosity of the scaffolds. The pericellular matrix of the hACPCs might not have been developed enough to retain most of the synthesised GAG. This task was further complicated by application of mechanical stimulation and the properties of the PU scaffold (pore size, highly porous scaffold structure). Regarding total GAG production, the two stimuli applied resulted in a different response. In the control group, mechanical stimulation led to a significant increase in GAG production. On the other hand, adenoviral-mediated over-expression of BMP-2 led to trend towards a decrease in GAG production. This observation was true for both the unloaded and the loaded group, yet it failed to reach statistical significance. The control loaded group produced significantly more GAG, when compared to the remaining three groups. When matrix production was compared between hACPCs and hMSCs, hMSCs were able to produce more total GAG, when compared to hACPCs. In each group, except the control loaded group, hMSCs produced more than twice the amount of total GAG, when compared to hACPCs (Neumann et al., 2013a). In the control loaded group, hMSCs produced approximately 1.5-fold more total GAG, when compared to hACPCs. Still, the general response towards the stimuli applied was similar for both cell types. Mechanical stimulation increased GAG production and transduction with Ad.BMP-2 led to a trend towards a decreased GAG production.

The normalisation of matrix production (GAG synthesis) to cell number (DNA content) revealed the same trends that were monitored for the total GAG production. Mechanical stimulation led to a significant increase in the GAG/DNA ratio, in both the control and the Ad.BMP-2 transduced group. Furthermore, in the loaded group, adenoviral-mediated over-expression of BMP-2 led to a significant decrease in GAG/DNA ratio. In each of the four groups investigated, the GAG/DNA ratio was lower in hACPCs, when compared to hMSCs. hMSCs had approximately 3-4 fold higher GAG/DNA ratios, depending on the individual group investigated (Neumann et al., 2013a). These results suggest that, under the monitored conditions, hMSCs have a higher baseline in total matrix production, when compared to hACPCs. Yet, both cell types displayed the same general response towards both stimuli applied. Load led to an increase, whereas transduction with Ad.BMP- 2 led to a trend towards a decrease, in GAG/DNA ratio. Interestingly, if the control unloaded group was used as baseline, the increase in GAG/DNA ratio in the control loaded group was higher in hACPCs (approximately 2-fold), when compared to hMSCs (approximately 1.4-fold). For the two remaining groups, the differences in GAG/DNA ratio were similar between both cell populations.

Gene expression analyses revealed interesting data that conflicted with the biochemical analysis. In the BMP-2 unloaded group, aggrecan message was significantly up-regulated, when compared to the control unloaded group. Yet, the total GAG production and the GAG/DNA ratio were not different between the control unloaded and the BMP-2 unloaded group. Further, on the gene level, there were no differences between the control loaded and the BMP-2 loaded group. Still, the control loaded group had a significantly higher total GAG production and a significantly higher GAG/DNA ratio. The same conflicting results were observed for hMSCs (Neumann et al., 2013a). Further, this discrepancy between aggrecan gene expression and matrix protein production has already been observed within our group and by other investigators (Barry et al., 2001; Kisiday et al., 2008; Kupcsik et al., 2010). The genes Runx2, SOX9, Notch 1 and Col I were either un-responsive (Runx2) or small changes in gene expression were monitored (other genes), when the stimuli were applied. For the gene SOX9, as it is a transcription factor, it is likely that even these small increases in gene expression led to a significant cellular response.

For the gene BMP-2, it was observed that adenoviral-mediated over-expression of BMP-2 massively and significantly up-regulated BMP-2 gene expression, independent of mechanical stimulation. For the gene PTHrP, mechanical stimulation (only in the control group at day 7) and, more predominantly, transduction with Ad.BMP-2 (except the loaded group at day 28) led to a significant increase in gene expression. This result indicates that both stimuli, to some extent, increased PTHrP production which can be interpreted as an attempt to counteract a possible hypertrophic differentiation of the cells. The combined results of the genes aggrecan, Col II, Col X and ALP were very interesting. Aggrecan message was massively up-regulated through both stimuli, which indicated an enhanced chondrogenic matrix production. Yet, the combined application of both stimuli did not further up-regulate aggrecan message, when compared to the sole application of each stimulus. It has to be emphasised that when mechanical stimulation was applied solely, a huge (several hundred-fold) and significant up-regulation in aggrecan message was monitored. For the gene Col II, mechanical stimulation led to a significant increase in gene expression after 28 days (for both the control and the Ad.BMP-2 transduced group). Contrary, transduction with Ad.BMP-2 led to a significant decrease in the loaded group on both time-points. However, these results have to be interpreted with caution, as Col II message was not constantly detected. Analysis of the hypertrophic marker gene Col X and the early osteogenic marker gene ALP revealed that mechanical stimulation had no significant effect on their gene expression. For the gene ALP, transduction with Ad.BMP-2 led to a significant up-regulation of gene expression in both the loaded and the unloaded group at both time-points. For the gene Col X, exposure to supraphysiological doses of BMP-2, resulted in a massive increase in gene expression after 28 days. This was true for both the unloaded and the loaded group. Taken together, the gene expression profile of these four genes indicated that both stimuli are, at least to some extent, able to induce a chondrogenic response (significant up-regulation of aggrecan message and up-regulation of Col II message in the loaded groups). Yet, mechanical stimulation resulted in a more stable chondrogenic gene expression profile (aggrecan high, Col II high, Col X and ALP low). On the contrary, transduction with Ad.BMP-2, independent of the application of load, resulted in a more hypertrophic gene expression profile (aggrecan, Col X and ALP all high).

Summarised, the current study opted to investigate the effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2 on hACPCs chondrogenesis and progression towards hypertrophy. It was shown that both stimuli differently influenced the cells. As opposed to the working hypothesis of the study, supra-physiological doses of BMP-2 were not enhancing the chondrogenic response. Biochemical analysis revealed that the DNA content, the total GAG synthesis and the GAG/DNA ratio were significantly reduced in the Ad.BMP-2 transduced and loaded group, when compared to its corresponding untransduced control. Further, transduction with Ad.BMP-2 led to a significant increase in hypertrophic (Col X) and osteogenic (ALP) marker gene expression, independent of mechanical stimulation. On the other hand, mechanical stimulation led to a significant increase in both the total GAG production and the GAG/DNA ratio (2-fold higher, when compared to the unloaded control group). Further, the gene expression profile of the control loaded group was very promising, especially concerning Col II data. Compared to the unloaded control group (baseline), the chondrogenic marker genes aggrecan and Col II were massively and significantly up-regulated, after 4 weeks of culture. Yet, no sign of an increase in hypertrophic (Col X) or osteogenic (ALP, Runx2, Osterix) marker gene expression was evident.

Finally, it has to be emphasised that, of all the four groups investigated, the control loaded had the highest GAG production, the highest GAG/DNA ratio and the most promising gene expression profile, as evidenced by a significant increase in chondrogenic marker gene expression (aggrecan and Col II) and no changes in hypertrophic (Col X) or osteogenic (ALP and Runx2) marker gene expression. The progression towards hypertrophy is one of the main draw drawbacks of hMSCs, as potential cell source for cartilage TE (Bosnakovski et al., 2006; Pelttari et al., 2006; Yoo et al., 1998). Further, even though mechanical stimulation and transduction with Ad.BMP-2 were able to enhancing the chondrogenic response, when hMSCs were used as cell source (Neumann et al., 2013a), the current study suggest that hACPCs are superior within the fibrin-PU scaffold system, regarding stable chondrogenic differentiation. Even though total matrix production was not as high as in hMSCs (hMSCs have a higher baseline level of GAG production) and Col II gene expression was not constantly observed, the cells seemed to undergo stable chondrogenic differentiation with no signs of hypertrophic differentiation.

Chapter 8: General Discussion.

The current thesis had two main foci. First, gene transfer methods, using different viral vectors, were investigated and optimised (chapter 3). This chapter was divided into three main parts. In the first part of chapter 3, it was investigated how retrovirally-mediated over-expression of BMP-2 influenced hMSCs during monolayer proliferation. In the second part of chapter 3, a modified enhanced 3D transduction protocol for adenoviral vectors was introduced and compared to the routine 2D transduction protocol. This comparison was conducted using hMSCs, which were seeded into three different hydrogels (alginate, agarose and fibrin-PU). The last part of chapter 3 introduced a fast and easy way to conduct a timed transduction of hMSCs, seeded into fibrin-PU composite scaffolds, with Ad.BMP-2. These investigations were needed to prepare for the main part of the thesis, which opted to investigate how gene transfer can be utilised to modulate mechanically-driven chondrogenesis in 3D constructs. In the second part of the thesis, the effect of different stimuli on the chondrogenic and hypertrophic differentiation of chondroprogenitor cells (hMSCs and hACPCs; chapter 4, 5, 6 and 7), was investigated. Chapter 4 focussed on the application of mechanical stimulation and adenoviral-mediated over-expression of BMP-2, alone and in combination, and its effect on the chondrogenic differentiation and the progression towards hypertrophy of monolayer-expanded hMSCs. In chapter 5, the effect of dorsomorphin, a small molecule that was suggested to supress hypertrophic differentiation by blocking BMP signalling, was examined. hMSCs were seeded into fibrin-PU composite scaffolds and chondrogenic differentiation was induced by exogenous application of TGF-β1. Then, it was determined how dorsomorphin altered chondrogenic and hypertrophic differentiation, within this culture system. Work in chapters 6 and 7 investigated the potential of hACPCs, as a cell source for cartilage TE. In chapter 6, the pellet culture model was used to compare hMSCs, hACPCs and co-cultures of both cell types. Serum-free conditions were used to analyse these cells during TGF-β1-driven chondrogenic and hypertrophic differentiation. In chapter 7, it was investigated how mechanical stimulation and adenoviral-mediated over-expression of BMP-2, alone and in combination, affect the chondrogenic and hypertrophic differentiation of monolayer-expanded hACPCs. Further, these combined results allowed for a direct comparison between hMSCs (chapter 4) and hACPCs (chapter 7).

Viral gene transfer.

Retroviral vectors integrate into the host genome. As a consequence, they are passed to the daughter cell during cell division. With the exception of inducible vectors systems, the production of the transgene product is constitutively active. For basic research applications, usually, a small number of cells are transduced with the retroviral vector. The transduced cells are then further propagated until the desired cell numbers are reached. Most of the time, the gene of interest encodes for a bioactive factor and control cells are usually transduced with a non-bioactive transgene (Kuroda et al., 2006; Salzmann et al., 2009). Hence, during the initial monolayer proliferation step, cells are already exposed to the virally encoded bioactive factor. It can be assumed that the cells are influenced by this exposure. Still, it is rarely, if ever, investigated how this exposure influences the starting cell population. Therefore, the first part of chapter 3 focussed on the effect of Rv.BMP-2 transduction on hMSCs during monolayer proliferation. hMSCs were transduced with either Rv.BMP-2 or Rv.eGFP. Subsequently, they were sub-cultured and further proliferated, until they reached 70-80% confluency. The gene expression profile, of both cell populations, was investigated and additionally compared to the gene expression profile of cells that were exposed to recombinant human BMP-2 (Neumann et al., 2013b). Further, it was examined if retroviral transduction itself is detrimental for hMSCs. It was shown that, in three out of four donors, retroviral transduction itself did not affect the cells. The cells of the last demonstrated a change in phenotype and confluency following retroviral transduction. This was probably due to the high age of the donor (81 years). Yet, the transgene product (BMP-2) itself had a pronounced effect. The gene expression profile between Rv.eGFP and Rv.BMP-2 transduced cells differed. Aggrecan message was significantly elevated almost 9-fold in Rv.BMP-2 transduced cells, if compared to Rv.eGFP transduced cells. If cells were exposed to 25-fold higher concentrations of exogenous BMP-2 (Rv.BMP-2 transduced cells produced between 3-4ng/ml of BMP-2), similar changes in gene expression were monitored. Col X message was significantly down-regulated by approximately 50% and aggrecan message was significantly up-regulated over 1000-fold. These results indicate that the response towards BMP-2 seemed to be threshold-dependent for the gene Col X and is dose-dependent for the gene aggrecan.

The combined results suggest that controls need to be carefully planned, if integrating vectors are used, and cells are further propagated after viral transduction. It has to be ruled out that differences, obtained during later culture, are due to the initial differences during cell proliferation. A possible approach to solve this problem can be the application of an inducible vector system, such as Tet-On. In this system, the expression of the gene of interest can be tightly regulated. Basal expression of the transgene product is virtually absent. The aforementioned Tet-On system comprises two vectors; a regulator vector and a response vector. The regulator vector constitutively expresses the tetracycline-controlled transactivator rtTA-Advanced (Urlinger et al., 2000). The response vector contains an inducible promoter which controls the expression of the gene of interest and does not contain any binding site for endogenous mammalian transcription factors. As a result, it is virtually silent without prior induction. The induction can be conducted through application of the tetracycline derivative doxycycline. Once induced, rtTA-Advanced binds to the inducible promoter on the response vector and transcription of the gene of interest is activated. With this kind of system, cells can be transduced with the inducible vector and monolayer-proliferated without addition of doxycycline. Thereby, the cells will not be exposed to the gene of interest during proliferation. After seeding the cells into a 3D environment, doxycycline can be added to the culture medium. This process will enable transcription of the gene of interest and, thereby, induce transgene product production. In control cells, doxycycline can be omitted from the culture medium and, hence, no transgene is produced. As a consequence, the same starting cell population, which is transduced with the inducible vector system, can be used for both control and treated cells. Further, the exposure of treated cells, to the gene of interest, is limited to the time of 3D culture.

Gene transfer using adenoviral vectors allows for an effective local, albeit transient, expression of the transgene product. Still, adenoviral transduction protocols are far from perfect and require further optimisation. The adenoviral vector itself and the transduced cell can be immunogenic. Further, at very high doses, adenoviral vectors are cytotoxic (Brunetti-Pierri et al., 2004). A more efficient transduction protocol will allow to significantly reducing the amount of viral vector needed to generate a specific amount of transgene product. This feature thereby might help to overcome the safety concerns that are still present, regarding a clinical application of adenoviral vectors.

Further, for a clinical application, a transduction protocol that is no longer dependent on a cell culture step is highly desirable. Therefore, in the second part of chapter 3, a novel 3D transduction protocol for adenoviral vectors was introduced. Its efficiency was compared to the routine adenoviral transduction protocol, which is conducted on cell culture plastic (2D environment). Both transduction protocols were compared using Ad.BMP-2 as vector, hMSCs as the targeted cell type, three different hydrogels (alginate, agarose and fibrin-PU) for cell encapsulation and two different vector doses (5 MOI and 100 MOI) (Neumann et al., 2013c). It was demonstrated that the 3D protocol is clearly superior, when compared to standard transduction in 2D. This was true for each condition investigated and indicated by higher amounts of transgene product produced per cell. At the same time, it was excluded that the 3D transduction protocol is detrimental for hMSCs (no significant changes in DNA content were monitored, when compared to both the routine 2D protocol and untransduced control cells) or that these differences originate from differences in BMP-2 retention within the hydrogels. Strikingly, it was further shown that, in agarose and fibrin-PU composite scaffolds, application of the 3D transduction protocol with only 5% of the initial vector dose led to a comparable or higher transgene product production, when compared to the routine 2D transduction protocol using a MOI of 100. The modified protocol has already demonstrated its value during other work within the thesis. In the Ad.BMP-2 and load study (chapter 4) and the hACPCs and load study (chapter 7) it was used to successfully transduce cells in 3D. In both cases, transduction was successful and biologically relevant amounts of BMP-2 (100ng/ml or above) were generated, using only 5 infectious viral particles per cell.

It is also worth noting that, when using the 3D transduction protocol, the duration of transgene expression was more prolonged, when compared to classical 2D transduction. For example, in 2005, Palmer et al., transduced rabbit MSCs on tissue culture plastic with different doses of an adenovirus encoding for BMP-2. The transgene product concentration peaked already at day 3 (in a 3 week experiment) and steadily declined thereafter (Palmer et al., 2005).

Recent work of other groups demonstrated that 3D transduction can occur for non-viral vectors (He et al., 2011) or lentiviral vectors (Aviles and Shea, 2011). However, the present study has directly compared the efficiency of 3D adenoviral transduction to standard 2D protocols. He et al., compared classical transfection, reverse transfection and reverse transfection in 3D using a non-viral gene delivery system (pullulan-spermine/DNA complexes) (He et al., 2011). Reverse transfection was shown to be superior, under serum-containing conditions, when compared to classical transfection. Furthermore, it was demonstrated that reverse transfection can be conducted in 3D and that transgene expression is significantly higher at day 9, when compared to transduction in 2D. At the other time-points (day 2, day 5, day 14 and day 21), there were no significant differences in transgene expression. In the current system, an adenoviral vector was used to transduce hMSCs (as opposed to rat cells) and a constant and significant improvement of transgene product production, over the course of 4 weeks, was demonstrated. It was further demonstrated that, if 3D transduction is used, clinically relevant amounts of bioactive factors can be produced with a much lower vector dose (compared to classical transduction in 2D). This feature might increase safety, if the method is transferred into the clinic.

Also in 2011, Aviles et al., demonstrated that PLG scaffolds can be combined with either collagen or fibrin hydrogels and that these composite scaffolds can be used to entrap lentiviral particles, coding for green fluorescent protein (under the control of a CMV promoter) or CMV-luciferase (Aviles and Shea, 2011). No cells were added to the constructs. The scaffolds were subsequently implanted into Cd1 male mice. *In vivo* transgene expression peaked between day 3 and day 7 and steadily declined thereafter. Adenoviral vectors (non-integrating) have already been approved for clinical trials (Danthinne and Imperiale, 2000; Penny and Hammond, 2004), whereas lentiviral vectors (integrating) will probably never be used in the orthopaedic field. In addition, the current study demonstrated a prolonged high level of transgene expression, over the course of 4 weeks, (instead of an early peaked expression which steadily declined afterwards).

Apart from its establishment and its successful application in work within the current thesis, the 3D adenoviral transduction protocol might prove beneficial for both *in vitro* work and, additionally, for a possible clinical application. If applied in the cell-culture laboratory, it requires less time and a lower amount of work, when compared to classical 2D transduction. Further, a whole monolayer passaging step can be conserved, which is a desirable feature when working with MSCs. Increasing passage number has been associated with a decline in proliferation rate, multilineage differentiation capacity and potential for activation (Banfi et al., 2000; Bonab et al., 2006; Crisostomo et al., 2006). For a possible clinical application, it is no longer dependent on a time-consuming and money-consuming cell culture step. Additionally, due to its increased efficiency, a much lower viral vector dose is needed to generate a certain amount of transgene product. This feature directly translates into increased safety. Additionally, the amount of administrative work, required to get clinical approval, would be significantly reduced. It is much easier to achieve approval for a technique that does not leave the operating theatre, due to the different regulatory rules that are applied.

These exciting observations leave room for further investigation. It would be interesting to confirm that this novel transduction protocol also works for chondrocytes. Together with MSCs, chondrocytes represent the most commonly used cell type in cartilage TE applications. On top of that, work within this thesis (hACPCs and load study) has already demonstrated its applicability for hACPCs, another promising cell source in cartilage TE. Besides, it would be interesting to confirm that the novel transduction protocol works for other viral vectors, especially non-integrating vectors such as AAV. Since the untimely and tragic death of Jessy Gelsinger in 1998, the administration of adenoviral vectors, for human gene therapy applications, is viewed with more caution. The working group that investigated this incident concluded that "human gene transfer experiments using adenoviral vectors should continue with caution." (2002 NIH report). Still, it has to be kept in mind that during the study in which Gelsinger was a participant, extremely high doses of virus were being injected directly into the hepatic artery. This is a highly unusual protocol which led to a cytokine storm and ultimate organ failure. This is not the normal immunogenic response being described when discussing adenovirus, which in nature has the common cold as the most severe event. In addition, articular cartilage represents a more immunoprivileged environment, due to its intrinsic biology (no lymphatic system and no blood supply).

If the adenoviral vector ("*in vivo* approach") or the transduced cells ("*ex vivo* approach") can be restricted to the cartilaginous tissue and macrophage infiltration can be avoided, immunogenicity of the vector, respectively the transduced cell, might not represent such a challenge. Nevertheless, AAV possess some distinct advantages (reviewed e.g. in: (Nixon et al., 2007; Steinert et al., 2008; Young et al., 2006)), when compared to adenovirus. It is not known to cause any human disease and also does not express any viral proteins within the infected cell. Lately, our group is also starting to investigate the potential of this promising gene transfer vector.

Timing of administration is critical for the effect of many bioactive factors. Most viral vectors are constitutively active. That means that their transgene expression cannot be regulated. Inducible vectors are an exception to this rule. Yet, these vectors possess other disadvantages, such as low expression levels, high price or impaired availability. With the new 3D transduction protocol, it is possible to transduce cells within different hydrogels (Neumann et al., 2013c). Assuming that the adenoviral vector is able to penetrate the hydrogel, it should be possible to conduct transduction after a certain amount of time in 3D culture. The last part of chapter 3 opted to investigate if hMSCs, seeded into fibrin-PU composite scaffolds, can be transduced with Ad.BMP-2 during 3D culture. Cells were either left as untransduced controls or were transduced with Ad.BMP-2, after one week or two weeks of 3D culture. Transduction was conducted by simply dripping the adenoviral vector on top of the cell-seeded scaffolds. A fast onset (2 days after transduction) of transgene product production was monitored for both the week 1 and the week 2 group. Control cells and cells prior to timed adenoviral transduction did not produce BMP-2. On the contrary, after delayed Ad.BMP-2 transduction, cells were able to produce biologically relevant amounts of BMP-2 (100ng/ml or above). Further, it was shown that this procedure did not negatively influence the total cell number. Even though it was not used for further work within the thesis, this brief study introduced a new potential tool for certain basic research applications. It opens the possibility to investigate the effect of a timed administration of a bioactive factor, without the need to apply it exogenously. Of course, these results open further questions such as, is that procedure also feasible for other viral vectors, hydrogels or cells? Answering these questions was beyond the scope of this short study and they have to remain open for now.

The results of the timed Ad.BMP-2 transduction study (part of chapter 3 and (Neumann et al., 2013c)) suggest that this protocol might also be applicable for alginate and agarose. Further, it would be interesting to test if the viral vector is able to completely penetrate the hydrogel or if only cells in the surface layer of the scaffold were successfully transduced. An easy way to answer this question would be to use an adenoviral vector, which expresses a fluorescent protein, such as enhanced green fluorescent protein. After transduction, scaffolds could be sectioned and the distribution of labelled (transduced) cells could be visualised using fluorescent microscopy. Possible shortcomings of this system are that it is difficult to apply in an *in vivo* setup and that the transgene production, after delayed adenoviral transduction, cannot be controlled or switched off.

The effect of mechanical stimulation and adenoviral-mediated over-expression of BMP-2 on human chondroprogenitor cell chondrogenesis and hypertrophy.

Articular cartilage is challenged with loading forces on a daily basis. Further, it is widely accepted that mechanical forces play a role in both development and maintenance of articular cartilage. Functional TE tries to better mimic the "*in vivo* situation" by applying mechanical stimulation as additional stimulus (reviewed for example in (Estes et al., 2004; Grad et al., 2011; Kelly and Jacobs, 2010; Potier et al., 2010). Yet, as the physiological movement of the joint is very complex, the application of a single stimulus, such as compression, fluid-flow or hydrostatic pressure, will not adequately reflect the situation that prevails *in vivo*. This is why, a novel bioreactor system, based on tribological considerations, was developed (Wimmer et al., 2004). This bioreactor system is able to generate up to three different mechanical stimuli (compression, shear and flexion), which can be applied simultaneously or individually. It already has been used to gain more insight into *in vitro* chondrogenesis of hMSCs without the need to apply TGF-β1 exogenously (Kupcsik et al., 2010; Li et al., 2009b; Li et al., 2009c; Li et al., 2011; Schatti et al., 2011). Further, the combined application of complex mechanical stimulation and retroviral-mediated over-expression of BMP-2 demonstrated a beneficial, synergistic effect on the re-differentiation of de-differentiated bovine chondrocytes (Salzmann et al., 2009).

These combined results led to the development of two studies within the thesis. In chapter 4, it was investigated how complex mechanical stimulation and adenoviral-mediated over-expression of BMP-2, alone and in combination, influence the differentiation of monolayer-expanded hMSCs. In chapter 7, the question how the application of the two stimuli (alone and in combination) influence the differentiation of hACPCs was addressed. Further, by using the results of both studies, it was possible to directly compare the chondrogenic/hypertrophic potential of these cell types, under the investigated conditions (fibrin-PU scaffold system, no exogenous TGF-β1, four weeks of culture),

In chapter 4, the chondrogenic differentiation and the progression towards hypertrophy of hMSCs was investigated (Neumann et al., 2013a). Cells were either transduced with Ad.BMP-2, in 3D using a MOI of 5, or were left as untransduced controls. After 3 days of pre-culture, the samples were subjected to mechanical stimulation (1 hour per day, 6 days per week) or left as free-swelling controls. After 7 or 28 days, samples were harvested. Outcome measurements were BMP-2 concentration in the culture medium, DNA content (indicator for total cell number), GAG production (indicator for matrix synthesis) and gene expression analyses (chondrogenic and hypertrophic/osteogenic genes were investigated). It was confirmed that Ad.BMP-2 transduced cells produced biologically relevant amounts of BMP-2 (100ng/ml or above). Load had no significant effect on BMP-2 production, even though a trend towards a higher release in the loaded groups was investigated. The total cell number was not influenced by time, mechanical stimulation or adenoviral-mediated over-expression of BMP-2. This result indicates that neither proliferation nor cellular death was the predominant response towards the culture environment. Consistent with previous work within our group, the bulk amount of synthesised GAG was released to the culture medium (Li et al., 2009c). Further, in the control group, the total amount of GAG produced and the GAG/DNA ratio was significantly elevated by mechanical stimulation. On the other hand, transduction with Ad.BMP-2 led did not significantly influence the GAG production or the GAG/DNA ratio.

The sole application of mechanical stimulation (no Ad.BMP-2 transduction) led to a general trend towards an increase in gene expression. Yet, for most genes these differences were small and are most likely not of any biological relevance. Exceptions were the hypertrophic matrix protein Col X (moderately up-regulated) and the chondrogenic matrix proteins Col II and aggrecan (massively up-regulated). In addition, it was shown that both stimuli had a synergistic effect on Col II message, on the early time-point, and that Ad.BMP-2 transduction was the predominant stimulus for the gene aggrecan, which is probably differently regulated, when compared to the other genes investigated. Finally, it was of interest that the increase in aggrecan gene expression, in the Ad.BMP-2 transduced groups, was not reflected at the protein level. This discrepancy has already been described in work from our group and in work from other groups (Barry et al., 2001; Kisiday et al., 2008; Kupcsik et al., 2010; Schatti et al., 2011). It must be noted that Ad.BMP-2 transduction also led to a significant increase in SOX9 message at the early time point (for both unloaded and loaded samples). This increase was rather small (around 10-fold to 15-fold). Yet, as SOX9 is a transcription factor, this increase might well have a strong biological effect and indicated a positive effect of Ad.BMP-2 transduction on chondrogenesis.

In chapter 7, the chondrogenic differentiation and the progression towards hypertrophy of hACPCs, under the same conditions as described for hMSCs (chapter 4), was investigated. The same outcome parameters were analysed. Additionally, the amount of TGF-β1 in the culture medium was quantified. Further, a greater panel of genes were analysed. First, it was shown that hACPCs are able to endogenously produce TGF-β1, in medium lacking exogenous growth factors. In Ad.BMP-2 transduced samples, a trend towards increases TGF-β1 medium concentration was evidenced, when compared to untransduced controls. This trend was significant during the pre-culture period (both loaded and unloaded) and during the first week of culture (unloaded only). During the four weeks of culture, TGF-β1 medium levels declined in the Ad.BMP-2 transduced samples but stayed rather constant in untransduced controls. As opposed to hMSCs, mechanical stimulation had no significant effect on TGF-β1 medium levels (Li et al., 2009b).

Analysis of the BMP-2 concentration within the culture medium revealed that biologically relevant amounts of BMP-2 (100ng/ml or above) were constantly generated in Ad.BMP-2 transduced samples. Interestingly, in loaded samples, BMP-2 medium levels significantly declined with time. In unloaded samples, however, no significant changes were observed, which resulted in a significant difference between the loaded and the unloaded group, after 4 weeks of culture. Quantification of total DNA content revealed that, when both stimuli were simultaneously applied, the total cell number significantly decrease between week 1 and week 4 of culture. Similar to hMSCs, most of the GAG produced was released into the culture medium (Neumann et al., 2013a). Also, the general response towards mechanical stimulation and Ad.BMP-2 transduction was similar between both cell populations. Mechanical stimulation significantly increased the total amount of GAG produced in the control group and, further, the GAG/DNA ratio in the control group and in the Ad.BMP-2 transduced group. On the contrary, in the loaded group, transduction with Ad.BMP-2 led to a significant decrease in total GAG production and in the GAG/DNA ratio.

The genes Col I, Notch I, SOX9 and Runx2 were the genes that were the least influenced by the stimuli applied, if compared to the other genes that were investigated. For Runx2, no significant differences in gene expression were detected. The influence of Ad.BMP-2 transduction on the remaining genes that were investigated was very interesting. It led to a massive and significant increase in BMP-2 message. Further, it led to a significant increase in aggrecan expression in the unloaded groups, at both day 7 and day 28. In the repeat of the experiment where Col II was constantly detected, Ad.BMP-2 transduction led to a significant decrease in gene expression within the loaded group (at both time-points). In addition, adenoviral-mediated over-expression of BMP-2 resulted in a massive and significant increase in ALP message (early osteogenic marker) in all groups investigated (unloaded and loaded at both day 7 and day 28) and to a significant increase in Col X message (hypertrophic marker) in the loaded group, at the late time-point. On the other hand, mechanical stimulation led to a significant increase in aggrecan message in the control group at both time-points. In the repeat of the experiment where Col II message was constantly detected, load led to a massive and significant up-regulation of Col II in the control group at day 28. Yet, neither the hypertrophic marker gene Col X, nor the osteogenic marker genes ALP and Runx2 were up-regulated, when compared to the respective unloaded control.

In summary, the loaded control group, at day 28, had the most promising gene expression profile, when compared to the other three groups investigated, regarding genes related to chondrogenesis and progression towards hypertrophy. In this group, the chondrogenic genes (Col II and aggrecan) were significantly up-regulated, whereas no significant changes in hypertrophic (Col X) or osteogenic (Runx2, ALP) marker gene expression were detected.

When the results of both studies were taken together and directly compared, several differences between the cell populations (hMSCs and hACPCs) became obvious. Following Ad.BMP-2 transduction, hACPCs were able to generate higher BMP-2 medium levels, when compared to hMSCs. These differences might originate from the fact that hACPCs had a higher total cell number per sample, when compared to hMSCs. The peak BMP-2 concentration generated was almost 3.5-fold higher in hACPCs. Further, hACPCs produced biologically relevant amounts of BMP-2 (100ng/ml or above) during the whole duration of the study, including the pre-culture period. For hMSCs, this was only true starting from week 1 (BMP-2 loaded) or week 2 (BMP-2 unloaded) of culture (Neumann et al., 2013a). Regarding TGF-β1 production, initial differences between the control and the Ad.BMP-2 transduced groups were detected in hACPCs. The Ad.BMP-2 transduced groups produced significantly more TGF-β1, during the three day pre-culture period, when compared to untransduced controls. After 7 days, this difference was only significant in the unloaded group. Later during the study, no significant differences between groups were detected. Strikingly, after 7 days of culture, TGF-β1 medium levels in the controls groups (no Ad.BMP-2 transduction) were comparable between hACPCs and hMSCs (see: (Li et al., 2009b)). Li et al., did not further track TGF-β1 production and therefore it was not possible to compare TGF-β1 production between hMSCs and hACPC at later time-points.

Quantification of the total DNA content indicated that hACPCs had a higher total cell number than hMSCs, after both one week and four weeks of culture. Yet, they displayed a trend towards decreasing cell numbers between week 1 and week 4 of culture. This trend reached statistical significance in the BMP-2 and loaded group only. In hMSCs, for every group that was investigated, the total cell numbers stayed constant between week 1 and week 4 of culture (Neumann et al., 2013a).

When matrix production was compared between both cell types, they showed a similar response towards both stimuli. For both hMSCs and hACPCs, the bulk amount of synthesised GAG was released into the culture medium. Also, mechanical stimulation consistently led to a significant increase in both total GAG production and GAG/DNA ratio, in the control group. On the contrary, in the loaded group, transduction with Ad.BMP-2 led to a significant decrease in total GAG production and GAG/DNA ratio in both cell types. The baseline level of matrix production was higher in hMSCs, when compared to hACPCs. Depending on the group investigated, the GAG/DNA ratio was between approximately 3-fold and up to almost 4.5-fold higher in hMSCs. Yet, for hACPCs, mechanical stimulation led to a stronger increase in matrix synthesis, as indicated by the GAG/DNA ratio, when compared to hMSCs. For hACPCs, the GAG/DNA ratio was doubled when the control unloaded and the control loaded group were compared. For hMSCs, the increase in GAG/DNA ratio, between these groups, was only around 1.4-fold (Neumann et al., 2013a).

On the gene level, both cell types responded differently. Mechanical stimulation led to an increase in chondrogenic and hypertrophic marker gene expression in hMSCs. Transduction with Ad.BMP-2 displayed a synergistic effect on Col II message, at the early time-point, and, further, was the predominant stimulus for aggrecan gene expression. For hACPCs, mechanical stimulation significantly increased aggrecan and Col II message, by day 28, in the control group. Furthermore, it did not influence hypertrophic or osteogenic marker gene expression. In hACPCs, transduction with Ad.BMP-2 led to a significant up-regulation of the hypertrophic marker gene Col X (at the late time-point) and the osteogenic marker gene ALP (at both time-points). Further, it did not led to an increase in Col II gene expression. Even though over-expression of BMP-2 significantly increased the expression of aggrecan in the unloaded group, it was not the predominant stimulus, as opposed to hMSCs. Summarised, hMSCs had a higher baseline level of chondrogenic response. In the control group, mechanical stimulation increased matrix production (total GAG and GAG/DNA ratio) and the expression of chondrogenic marker genes Col II and aggrecan. Yet, it also led to an increase of the hypertrophic marker gene Col X. Over-expression of BMP-2 displayed a synergistic effect on Col II message and was the predominant stimulus for the gene aggrecan. Yet, the total GAG production was significantly decreased in Ad.BMP-2 transduced and loaded samples, when compared to untransduced controls.

hACPCs displayed a lower chondrogenic baseline level but responded more robustly, when compared to hMSCs. Sole application of mechanical stimulation led to an increase in matrix production (total GAG and GAG/DNA ratio) and to an increase in chondrogenic marker gene expression (aggrecan and Col II). Yet, hypertrophic (Col X) and osteogenic (Runx2, ALP) marker gene expression were not elevated. On the other hand, transduction with Ad.BMP-2 decreased the total GAG production and the GAG/DNA ratio in the loaded group. Further, it led to an up-regulation of the hypertrophic marker Col X (at the late time-point) and the osteogenic marker ALP (at both time-points).

The results of both studies leave room for further work. In my opinion, the chondrogenic differentiation of both cell types still needs to be improved, yet with different emphasis. The current results suggest that, for hMSCs, it would be necessary to focus on how to prevent hypertrophic differentiation. For instance, a follow up study could focus on the effect of mechanical stimulation and adenoviral-mediated over-expression of PTHrP. For hACPCs, the main emphasis should be on how to further improve their matrix production. Examples would be the combination of mechanical stimulation and adenoviral-mediated over-expression of SOX9 or IGF-1. SOX9 is the master transcription factor for chondrogenesis. Using this combined approach, it already has been demonstrated that it possible to induce the chondrogenic differentiation of hMSCs without the exogenous application of dexamethasone and TGF-β1. (Kupcsik et al., 2010). IGF-1 is one of the main anabolic factors for cartilage matrix production and is widely utilised in cartilage TE (Balcom et al., 2012; Gelse et al., 2003; Madry et al., 2013; Palmer et al., 2005; Steinert et al., 2009a). Finally, it is interesting to note that the time-frames investigated are actually quite short for the development of chondrogenesis from progenitors.

Effect of dorsomorphin on hMSC hypertrophy.

The unwanted progression towards hypertrophy, during TGF-β-driven *in vitro* chondrogenesis, is the main obstacle for a successful application of MSC in cartilage TE (Pelttari et al., 2006; Yoo et al., 1998). One prime candidate to block this progression towards hypertrophy is PTHrP. It can be either applied exogenously or it can be provided by articular chondrocytes during co-culture (Fischer et al., 2010; Kim et al., 2008; Lee and Im, 2012; Mueller et al., 2013). Recently, promising data were generated using a different approach. Hellingman and co-workers showed that both phosphorylated SMAD 2/3-mediated TGF-β and phosphorylated SMAD 1/5/8-mediated BMP signalling play crucial roles during early stages of chondrogenic differentiation in hMSCs (Hellingman et al., 2011). Further, at later stages during chondrogenic differentiation, inhibition of phosphorylated SMAD 2/3-mediated TGF-β signalling inhibited chondrogenesis. On the contrary, during these later stages of chondrogenesis, the inhibition of phosphorylated SMAD 1/5/8-mediated BMP signalling impeded terminal differentiation and mineralisation.

The Hellingman study utilised the pellet culture model and 10µM of dorsomorphin was applied to block phosphorylated SMAD 1/5/8 (BMP) signalling. The pellet culture model is currently the most commonly used model to study *in vitro* chondrogenesis. Yet, the fibrin-PU composite system possesses several beneficial features, when compared to the pellet culture model. For instance, it is possible to apply mechanical stimulation, the size of the scaffold (2 x 8 mm or 4 x 8 mm) better reflects the clinical demand and it compares favourably regarding gene expression related to endochondral ossification (Li et al., 2009a). Recent data, which investigated chondrogenesis of hMSCs in monolayer culture, gave further evidence that BMP-2 induces hypertrophic differentiation of hMSCs (Caron et al., 2013). Therefore, it was of interested to determine how blocking of phosphorylated SMAD 1/5/8-mediated BMP signalling influences the differentiation of hMSCs, within the fibrin-PU composite system. hMSCs were seeded at 2 x 10^6 cell per sample into 2 x 8 mm fibrin-PU composite scaffolds. In order to induce chondrogenic differentiation, the samples were cultured for two weeks in "classical" serum-free chondrogenic medium with 10ng/ml of exogenous TGF-β1. Afterwards, samples were divided into two groups.

In the control group, samples were further cultured under identical conditions. In the dorsomorphin group, 10µM of dorsomorphin were additionally added to block BMP signalling. After 4 weeks of culture, samples were harvested and biochemical analyses, gene expression analyses, histology and immunohistochemistry were conducted.

The combined result showed that exposure to 10 µM of dorsomorphin blocked the general differentiation potential of hMSCs (both chondrogenic and hypertrophic), in the fibrin-PU composite system. This diminished differentiation potential was likely due to the cytotoxic effect monitored, when cells were exposed to 10 µM of dorsomorphin, within this system. This cytotoxic effect was evidenced by a significant decrease in the total DNA content in the dorsomorphin group, when compared to the control group. Further, biochemical analyses revealed that the total amount of GAG, secreted into the culture medium, was significantly lower in the dorsomorphin group, when compared to the control group. When the GAG secretion was monitored on a weekly basis, it became evident that these differences originate from diminished GAG production during the third and, especially, during the fourth week of culture. Interestingly, the GAG/DNA ratio, after 4 weeks of culture, was not different between the groups. Yet, as already discussed in section 5-3, the study design was not optimal and it could not be definitely determined if, in the dorsomorphin group, a smaller amount of cells produced the same amount of GAG, when compared to cells in the control group or if a smaller amount of cells additionally produced less GAG, when compared to cells in the control group.

Gene expression analyses revealed that the chondrogenic marker genes Col II and aggrecan and the hypertrophic marker gene Col X were significantly lower in the dorsomorphin group, when compared to the control group. These results indicated that exposure to 10µM of dorsomorphin had a negative effect on general differentiation of hMSCs (both chondrogenic and hypertrophic), within the fibrin-PU composite system. As already mentioned, this is probably due to the cytotoxic effect observed, after exposure to 10µM of dorsomorphin. This observation was further confirmed by histology (Safranin-O/Fast Green) and immunohistochemistry (for the matrix proteins Col II, aggrecan and Col X). Depending on the particular donor, Safranin-O staining was either significantly diminished or completely absent in the dorsomorphin group, as opposed to the control group. The same observation was made, when the chondrogenic matrix proteins Col II and aggrecan, as well as the hypertrophic matrix protein Col X, were specifically detected using immunohistochemistry.

The observation that exposure to 10µM of dorsomorphin leads to a cytotoxic effect and, thereby, blocks the general differentiation of hMSCs conflicts, at least to some degree, with the findings of Hellingman and co-workers (Hellingman et al., 2011). This group was able to demonstrate that blocking of phosphorylated SMAD 1/5/8 signalling, by exposure to 10µM of dorsomorphin, inhibited terminal differentiation without affecting chondrogenesis. Yet, the outcome of their study was partially dependent on the chondrogenic capacity of the individual donor. If the chondrogenic capacity of the donor was rather low, as evidenced by a lack of Col II protein deposition after 2 weeks of culture, exposure to dorsomorphin blocked or significantly diminished further Col II protein incorporation (Hellingman et al., 2011). Besides the cytotoxic effect, there exist other, less likely, explanations for the observed differences between this study and the Hellingman study. They might also have originated from differences in culture time (4 weeks in this study, 5 weeks in the Hellingman study) or chondrogenic inducer (TGF-β1 in this study, TGF-β2 in the Hellingman study). Further, the differences in culture system (fibrin-PU composite scaffolds in this study, pellet culture in the Hellingman study) might have influenced the cellular response. In the pellet culture model, cells are in close contact to each other and can communicate by direct cell-cell contact or cell-matrix contact and not only by paracrine signalling. In the fibrin-PU composite system, cells are separated from each other and the main route of cell-cell interaction is via paracrine signalling molecules. Yet, it has to be emphasised that the dorsomorphin concentration of 10µM was chosen according to the study of Hellingman et al., (Hellingman et al., 2011). When compared to the densely packed pellets, the fibrin-PU composite system allows for a better medium penetration. This potentially translates to a higher dorsomorphin concentration within the scaffold, especially in the centre. As indicated by the DNA data, exposure to 10µM of dorsomorphin had a detrimental effect on the total cell number, within the fibrin-PU composite scaffolds. This decrease in total cell number was due to a lower cellular proliferation and/or an increase in cell death. This feature then likely diminished the general differentiation potential of hMSCs within the dorsomorphin group, when compared to the control group. In retrospect, it would have been crucial to perform a dose-response test first. Thereby, a non-toxic dorsomorphin concentration could have been determined. This concentration then could have been used to analyse the effect of dorsomorphin, on the chondrogenic and hypertrophic differentiation potential of hMSCs, within the fibrin-PU composite system.

Further, it might have been helpful to increase the culture time before addition of dorsomorphin. Thereby, cells would have more time to produce a mature matrix. Dorsomorphin has been suggested to prevent the phosphorylation of SMAD 1/5/8 by selectively inhibiting the BMP type I receptors ALK 2, ALK3 and ALK6 (Yu et al., 2008). Recent work of Vogt and co-workers questioned the specificity of dorsomorphin for BMP signalling (Vogt et al., 2011). Among other substances, the group tested dorsomorphin and LDN-193189 (a dorsomorphin derivate) against a panel of up to 123 protein kinases. It was shown that dorsomorphin and LDN-193189 are able to non-specifically inhibit several protein kinases and caution was suggested, especially for the application of dorsomorphin (Vogt et al., 2011). There exist several other options for the specific blocking of BMP signalling apart from application of dorsomorphin. Examples are, for instance, secreted BMP-2 antagonists, such as Noggin or Chordin, or other molecules that act on the receptor level, such as the inhibitory SMAD 6. Noggin and Chordin form complexes with BMPs (extracellular) and prevent them from binding to their receptor (reviewed e.g. in: (Pogue and Lyons, 2006; Wu et al., 2007). SMAD 6 acts intercellularly and seems to selectively block BMP signalling (Goto et al., 2007; Hanyu et al., 2001; Horiki et al., 2004). It competes with receptor activated SMADs for the binding onto the phosphorylated type I BMP receptors.

Co-culture of hMSCs and hACPCs.

In TE of articular cartilage, co-cultures of more than one cell type are increasingly considered as attractive alternative to the sole application of a single cell type (Fischer et al., 2010; Gan and Kandel, 2007; Meretoja et al., 2012; Wu et al., 2011). This approach allows for cell-cell signalling and, thereby, is hypothesised to have a positive effect, such as increased proliferation or enhanced differentiation. The most commonly investigated cell sources are MSCs and chondrocytes. Other options can be, for instance, the use of chondrocytes in a different phenotypic state or the use of chondrocytes harvested from different zones within articular cartilage. In 2004, it has been demonstrated that a cell population with progenitor-like characteristics can be isolated from the superficial zone of bovine articular cartilage (Dowthwaite et al., 2004). These cells were termed ACPCs and later further characterised (Hayes et al., 2008; Khan et al., 2009). More recently, it has been shown that hACPCs can be isolated from the superficial zone of equine cartilage and from full-depth human articular cartilage (McCarthy et al., 2011; Williams et al., 2010). Recent work of McCarthy and co-workers directly compared the chondrogenic and hypertrophic potential of equine MSCs and equine ACPCs using the pellet culture model (McCarthy et al., 2011). The group demonstrated that ACPCs were able to undergo stable chondrogenesis, whereas MSCs showed distinct signs of hypertrophic differentiation, as evidenced by the absence of immunolabelling for the proteins Col X, Runx2 and matrilin-1. Furthermore, Williams and co-workers showed that hACPCs did not express Col X or ALP, when induced towards the chondrogenic phenotype, in chondrogenic medium containing 2% serum (Williams et al., 2010). These results led to the development a study where the chondrogenic and hypertrophic potential of hMSCs and hACPCs was directly compared (chapter 6). In addition, it was investigated if co-cultures of both cell populations have a modulating effect on their chondrogenic differentiation. The pellet culture model with 250, 000 cells per pellet, using varying ratios between hMSCs and hACPCs, was used. Cells were cultured for three weeks in "classical", serum-free chondrogenic medium containing 10µg/ml of exogenous TGF-β1.

Outcome measurements were biochemical analyses (GAG, DNA and GAG/DNA), RT-PCR, histology (Safranin-O/Fast Green) and immunohistochemistry (for the matrix proteins Col II, aggrecan and Col X). Furthermore, in one run of the experiment, both cell populations were labelled with different fluorescent dyes. The labelled pellets were harvested, after 7 or 21 days, and analysed using fluorescent microscopy. Thereby, it was tracked if the ratio between hMSCs and hACPCs stayed constant during culture. Finally, part of the secretome of both cell populations was analysed in order to track differences in their cytokine release profile.

The combined results were unexpected and showed that, in this particular model, hACPCs were not able to undergo chondrogenesis. Further, co-culture had no beneficial effect on chondrogenic differentiation, if compared to the 100% hMSCs group. Chondrogenesis was only induced in the 100% hMSCs and in the 90% hMSCs + 10% hACPCs group. This was evidenced by a significant increase in total GAG production and a significantly higher GAG/DNA ratio, when compared to the 25% hMSCs + 75% hACPCs group, the 10% hMSCs + 90% hACPCs group and the 100% hACPCs group. Furthermore, the expression of the chondrogenic marker gene Col II was significantly higher in the 100% hMSCs group, when compared to the 10% hMSCs + 90% hACPCs group and the 100% hACPCs group. These results were confirmed on the protein level. Safranin-O staining was only evident in the 100% hMSCs and 90% hMSCs + 10% hACPCs group. Further, these groups were the only groups where the chondrogenic matrix proteins Col II and aggrecan were detected immunohistochemically. Unfortunately, these groups also demonstrated positive labelling for the hypertrophic matrix protein Col X, indicating progression towards hypertrophy.

The analysis of fluorescently labelled cells indicated that hMSCs did not seem to survive well, if co-cultured with hACPCs. If the amount of hACPCs was 25% or 50%, almost no hMSCs were detected after 3 weeks of culture. If the amount of hACPCs was 75% or 90%, no hMSCs were evident, after both 7 and 21 days of. Similar observations were already made when rabbit or human MSCs were co-cultured with chondrocytes from bovine or human origin (Meretoja et al., 2012; Wu et al., 2011). Still, after 21 days, there were no significant differences in DNA content between the groups and it has to be emphasised that this conclusions can only be drawn under the assumptions that both fluorescent dyes have similar properties (labelling-efficiency, stability) and that both cell population divide at a similar rate.

Summarised, these results conflicted with the study of McCarthy et al., (McCarthy et al., 2011). One explanation might be species differences. It is possible that hACPCs behave differently, when compared to equine ACPCs. Yet, there exist a more likely explanation; the presence of a low amount (2%) of serum within the chondrogenic medium used during the equine study. Serum contains many bioactive factors and it might contain one factor that is necessary for the chondrogenic induction of equine and human ACPCs. On the other hand, hMSCs might either not need this factor or they might produce it by themselves. The lack of this hypothetical factor would consequently explain why hMSCs are able to undergo chondrogenesis, under serum-free conditions, whereas hACPCs are not.

The secretome analysis revealed that around 10%, of a total of 174 analysed cytokines, were more abundant (2-fold difference or more) in the secretome of hMSCs or in the secretome of hACPCs. The most interesting candidate cytokines that might partially explain for the observed differences were IGF binding proteins and HGF. IGF binding proteins regulate the availability of IGF´s, such as IGF-1 (Bhakta et al., 2000). IGF-1 has been shown to increase cartilage matrix production (van der Kraan et al., 2002; McQuillan et al., 1986) and over-expression of IGF-1, mediated by genetically modified chondrocytes, improved the repair of an osteochondral defect in the rabbit (Madry et al., 2013). HGF was over 13-fold more abundant in the secretome of hMSCs. Interestingly, it has been shown to increase proliferation, migration and matrix synthesis in foetal and juvenile lapine and murine chondrocytes (Takebayashi et al., 1995).

One next step could be to rule out that the observed differences, between hACPCs and equine ACPCs, are simply due to species differences. Thus, it would be interesting to investigate if hACPCs are able to undergo chondrogenesis in chondrogenic medium containing 2% of serum. The results of the hACPCs and load study (increase in GAG production, GAG/DNA ratio and chondrogenic gene expression profile) indeed indicate that hACPCs have the potential to undergo stable chondrogenesis. Additionally, if their chondrogenic induction is possible, it would be interesting to track which factor, within the serum, is crucial for this process. This would then allow using a chemical-defined, serum-free medium for chondrogenic induction. As serum contains a plethora of bioactive factors, this feature would better allow comparing results between different studies. The secretome analysis already provided hints and suggested possible starting candidate molecules.

One possibility would be to test the effect of HGF. Additionally, the observed differences in IGF binding proteins point towards a role of IGF-1 and/or insulin. Previous work, using bovine cartilage explants, demonstrated that IGF-1 is the key factor within serum that is responsible for the stimulation of proteoglycan synthesis (McQuillan et al., 1986). Further, it was demonstrated that the degree of proteoglycan synthesis is dependent on IGF-1 or insulin concentration. Due to the high cost of recombinant IGF-1, IGF-1 is usually replaced by insulin within the standard chondrogenic medium. Yet, the insulin concentration, used within the classical "Johnstone medium", was optimised for MSCs (Johnstone et al., 1998). It might be possible that hACPCs either need a higher dose of insulin or that they need IGF-1 for chondrogenic differentiation. The modification in IGF binding proteins expression between the two cell types may offer some insight in this direction.

Further, by using the current study design, it was not possible to confirm that co-culture of hMSCs and hACPCs has a modulating effect on their chondrogenic differentiation. Thus, if only the results of the present study are taken into account, the combined application of these cell sources is not warranted. In retrospect, as the in *vitro* chondrogenic induction of hACPCs was not successful in this model, this observation was not very surprising. Once this problem is solved, the picture might change. Then, it might be warranted to repeat the co-culture experiment. It would be interesting to determine if a similar degree of chondrogenesis, or even an enhanced chondrogenic potential, can be obtained by replacing some of the hACPCs with hMSCs. hMSCs might then act as trophic mediators and further increase the chondrogenic capacity of hACPCs. Similar observations have already been made using bovine MSCs and bovine chondrocytes (Meretoja et al., 2012). In contrast to mature chondrocytes, hACPCs maintain their differentiation potential even at high PDs (Khan et al., 2009). This feature allows generating significantly more cells from the same amount of starting material, when compared to articular chondrocytes. Further, chondrocytes tend to de-differentiate during monolayer proliferation (von der Mark et al., 1977). Even though re-differentiation is possible (Benya and Shaffer, 1982; Bonaventure et al., 1994; Stoddart et al., 2006), it would be easier to obtain the required amount of cells, using hACPCs, especially if allogeneic cells are used. The application of allogenic cells would eliminate the need to perform a cartilage biopsy from each individual donor/patient and thereby would allow for an easier translation into clinical practise.

Future clinical application.

All experiments within the current thesis were *in vitro* work, focussing on basic research questions. It was, among others, investigated how complex, multiaxial load and adenoviral gene transfer influences the chondrogenesis of chondroprogenitor cells. Thus, a possible clinical translation of these results is not as far away as it might seem at first sight. On the contrary, very valuable results were gained which, regulatory problems aside, in theory can be directly implemented into clinical practise.

The application of the modified 3D transduction protocol for adenoviral vectors dramatically reduced the amount of viral vector needed to generate the desired amount of transgene product. As the acute inflammatory response towards adenoviral vectors is dependent on the vector dose, this feature will directly translate into increased safety. Further, the 3D transduction protocol is fast, easy to apply and no longer dependent on a cell culture step. This opens the door for a direct application within the operating theatre.

Another benefit that makes a possible clinical translation much easier is that most components of the current work have already been approved for clinical use and are nowadays already widely used. The approach to encapsulate cells in fibrin resembles the microfracture procedure, which is a commonly used first line treatment for cartilage defects (Steadman et al., 2002). The additional use of the PU scaffold provides mechanical stability. Yet, depending on the mechanical environment after surgery, which is dependent on the rehabilitation protocol applied, it might not even be necessary to use a scaffold. The European Medicines Agency has recently approved an AAV gene therapy protocol (for web reference see: (Johnstone et al., 2013)) and this is likely to open the door to more viral gene therapy approaches as more safety data is obtained.

The currently used bioreactor system is able to mimic the mechanical situation that prevails *in vivo* much more precisely, when compared to other bioreactors that are only able to apply a single stimulus. Thereby, the interaction and emphasis of several mechanical stimuli can be investigated and compared. In theory, this bioreactor system might help to optimise currently available rehabilitation protocols, which are applied after acute cartilage injury, microfracture or ACT. Of course, the decision which protocol is used depends on the surgical procedure that was applied to treat the defect. Still, the exact parameters of the rehabilitation protocol are usually strongly biased by the experience and the preference of the respective surgeon/physiotherapist. The bioreactor system is partially able to simulate the different steps during rehabilitation. Culture under free-swelling conditions corresponds to the time where the patient is resting (no mechanical stimulation). Sole application of shear forces corresponds to the time where the patient undergoes constant passive motion. The combined application of shear and compression corresponds to the time where the patient is allowed partial weight-bearing on crutches. The exact time-frame of these three individual stimuli can be varied and optimised, regarding improved chondrogenesis of the desired cell type. Then, it might be possible to transfer these results into an adapted rehabilitation protocol. This would allow to dramatically reducing the number of experimental animals needed. Further, as it is very difficult to simulate these different conditions in most animal models, it might even be possible to directly translate the results into the clinics without the need for additional in *vivo* work.

Summarised, the combined results within the thesis might help to develop a new one-step approach to treat cartilage defects. In theory, a surgeon could harvest bone-marrow directly in the operating theatre and isolate the mononuclear fraction, which contains hMSCs, through Ficoll gradient centrifugation. Alternatively, the surgeon could use autologous or allogenic hACPCs. Then, these cells could be mixed with fibrin and a viral vector, such as adenovirus or AAV, expressing the desired transgene. The cell/fibrin/virus mix could then either be directly injected into the defect or, alternatively, first seeded into PU scaffolds and then implanted. Finally, the patient would be able to undergo a pre-tested and optimised rehabilitation protocol.

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Appendix

A.1 Images effect of exposure of hMSCs to 8µg/ml of "Polybrene" for 2 hours.

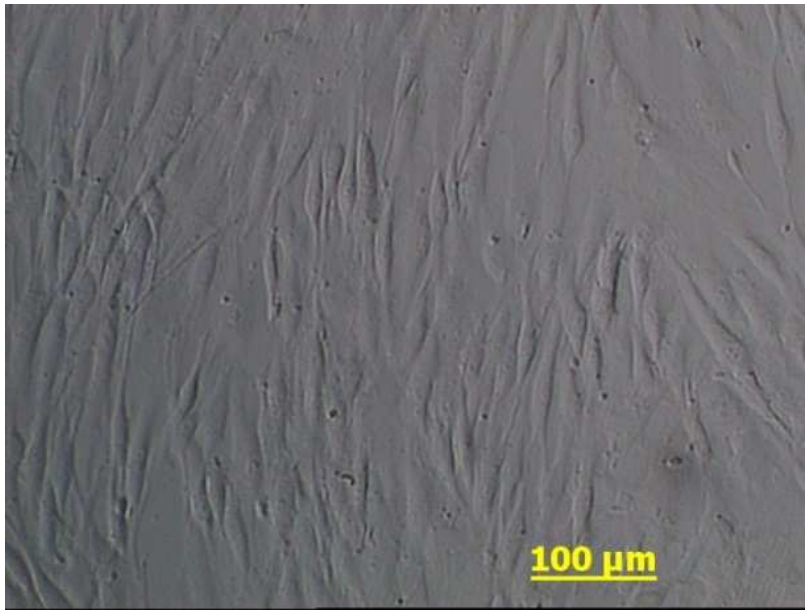

Figure A-1: Control hMSCs at day 1. The image was taken at the side of the well.

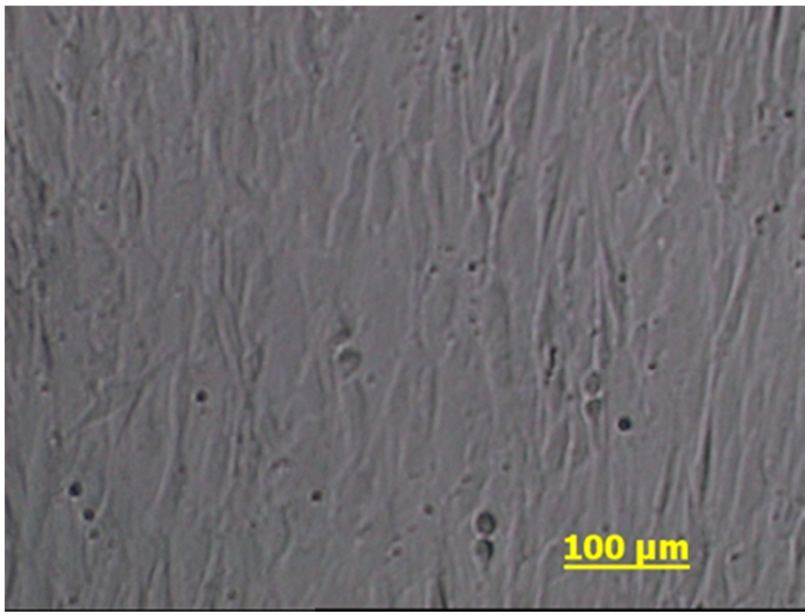

Figure A-2: hMSCs exposed to 8µg/ml of "Polybrene" at day 1. The image was taken at the side of the well.

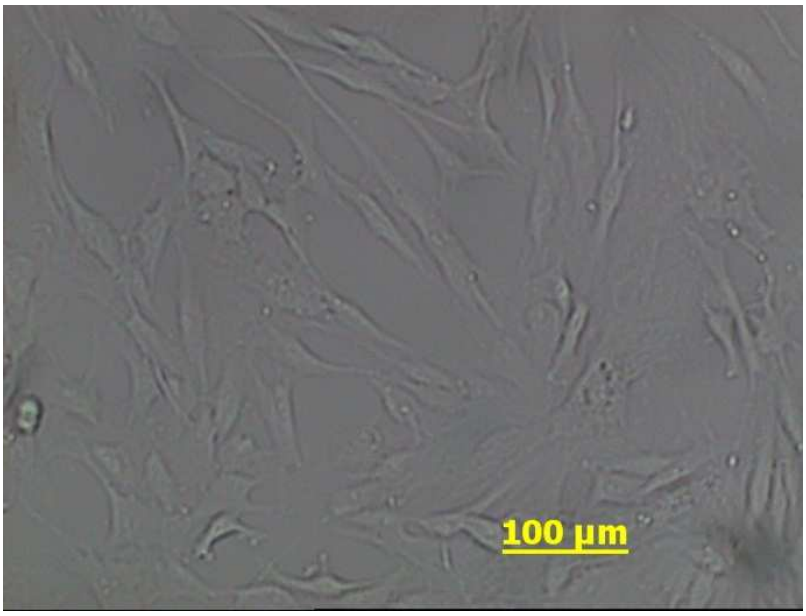

Figure A-3: Control hMSCs at day 1. The image was taken in the middle of the well.

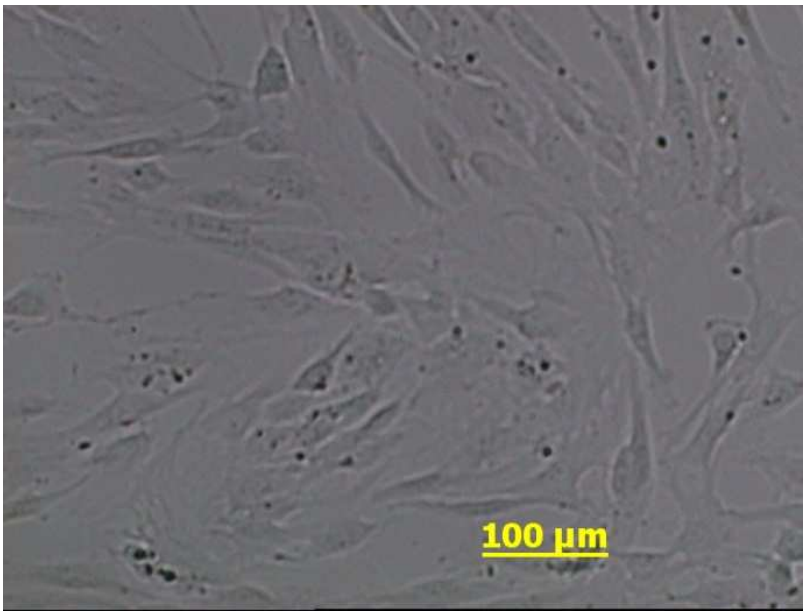

Figure A-4: hMSCs exposed to 8µg/ml of "Polybrene" at day 1. The image was taken in the middle of the well.

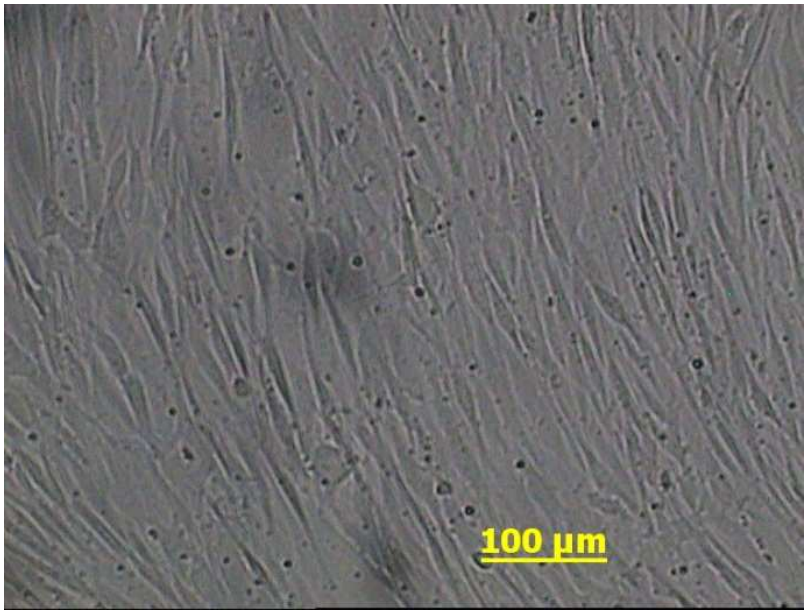

Figure A-5: hMSCs exposed to 8µg/ml of "Polybrene" at day 3. The image was taken at the side of the well.

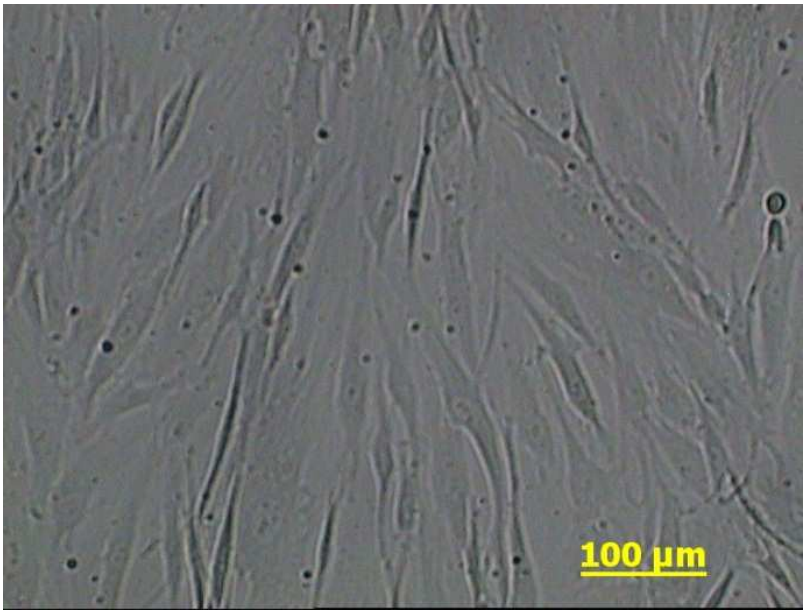

Figure A-6: hMSCs exposed to 8µg/ml of "Polybrene" at day 3. The image was taken in the middle of the well.

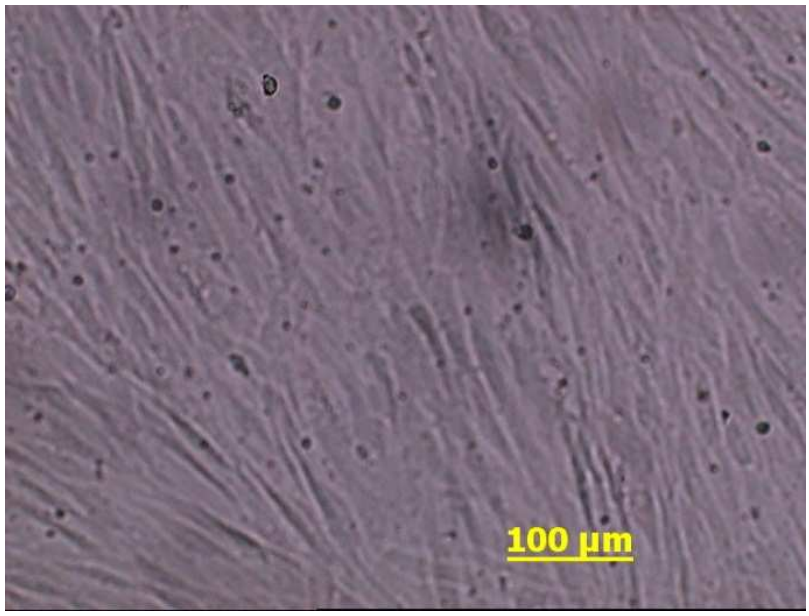

Figure A-7: hMSCs exposed to 8µg/ml of "Polybrene" at day 6. The image was taken at the side of the well.

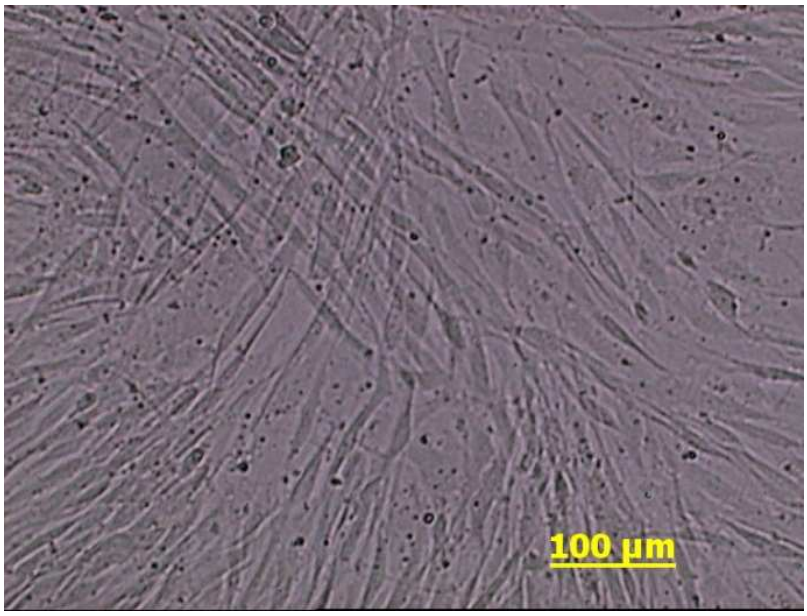

Figure A-8: hMSCs exposed to 8µg/ml of "Polybrene" at day 6. The image was taken in the middle of the well.

A.2 Images effect retroviral transduction on hMSCs.

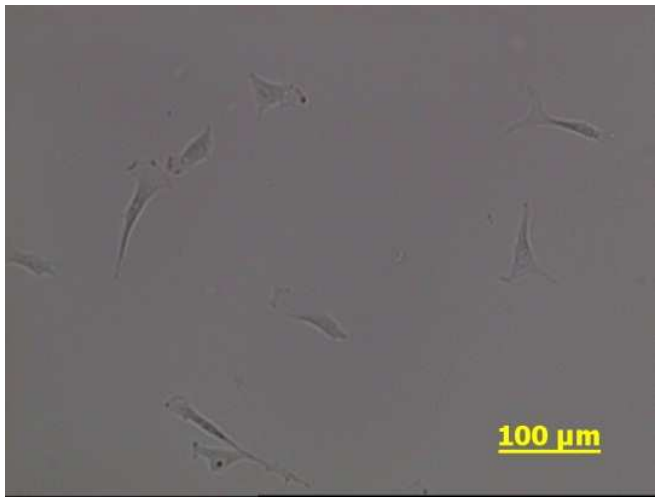

Figure A-9: Mock-treated hMSCs 3 days after treatment.

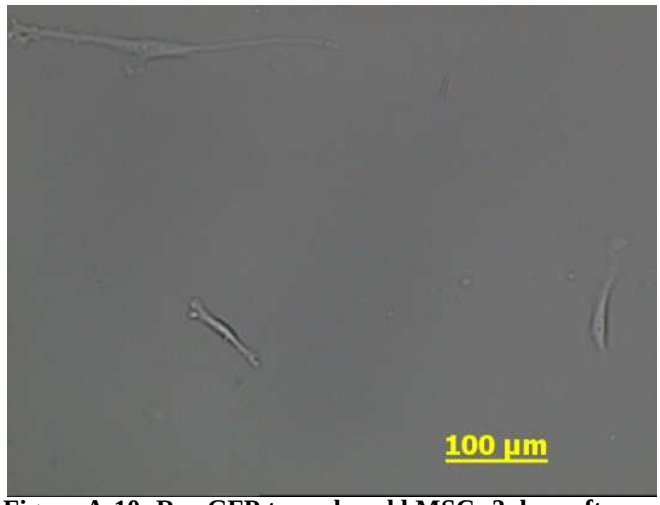

Figure A-10: Rv.eGFP transduced hMSCs 3 days after transduction

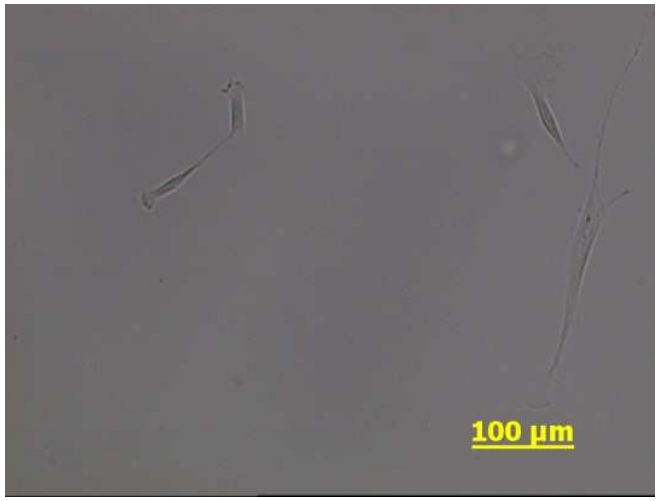

Figure A-11: Rv.BMP-2 transduced hMSCs 3 days after transduction

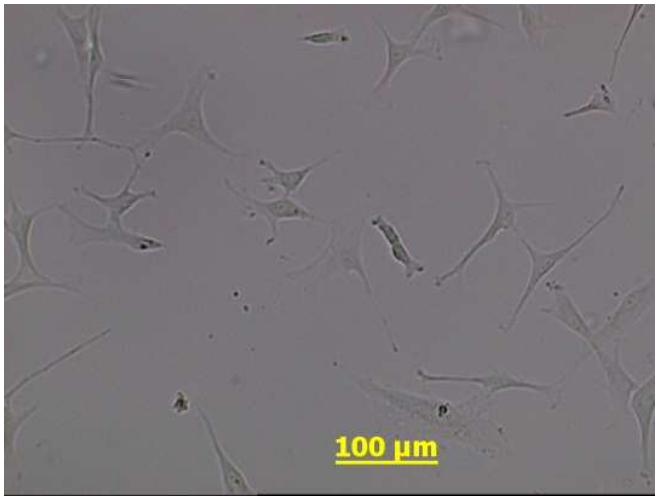

Figure A-12: Mock-treated hMSCs 5 days after treatment

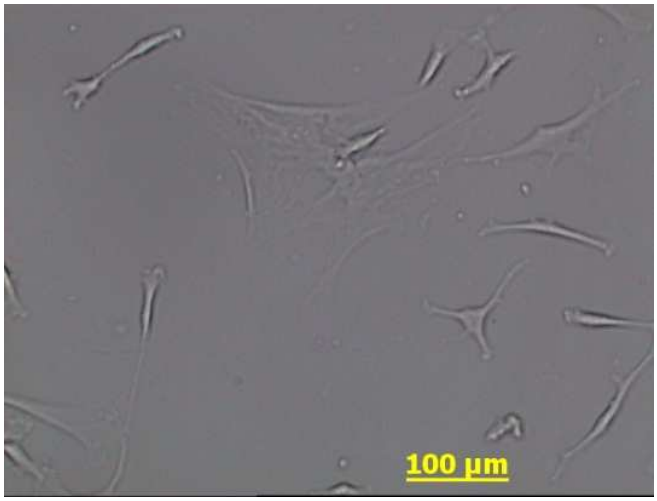

Figure A-13: Rv.eGFP transduced hMSCs 5 days after transduction

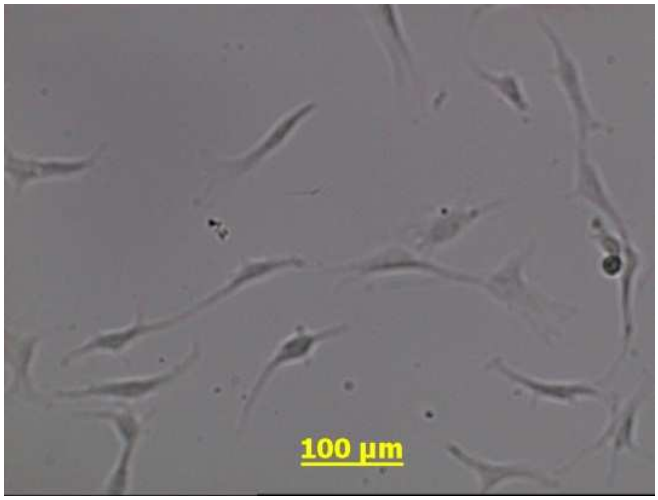

Figure A-14: Rv.BMP-2 transduced hMSCs 5 days after transduction

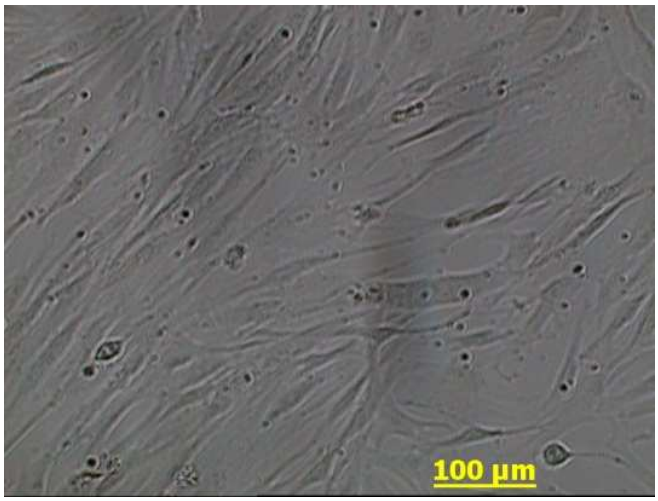

Figure A-15: Mock-treated hMSCs 7 days after treatment

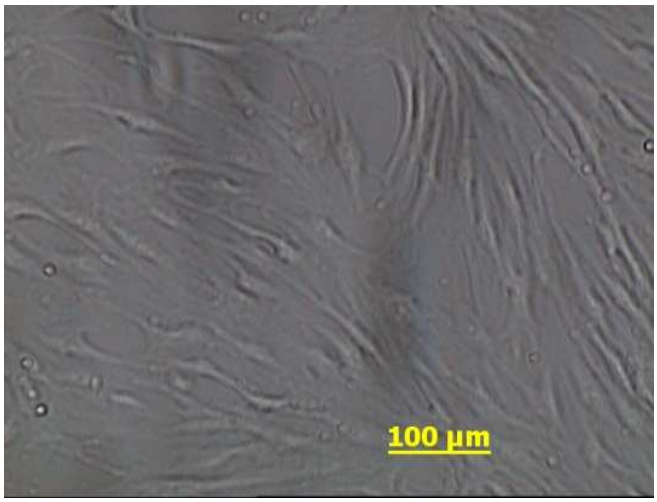

Figure A-16: Rv.eGFP transduced hMSCs 7 days after transduction

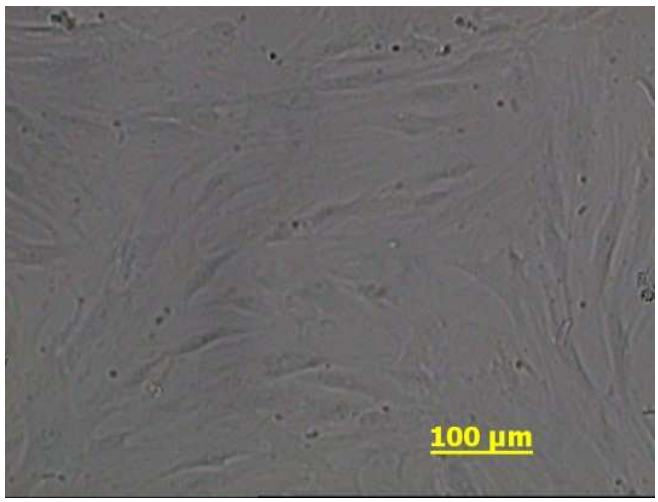

Figure A-17: Rv.BMP-2 transduced hMSCs 7 days after transduction

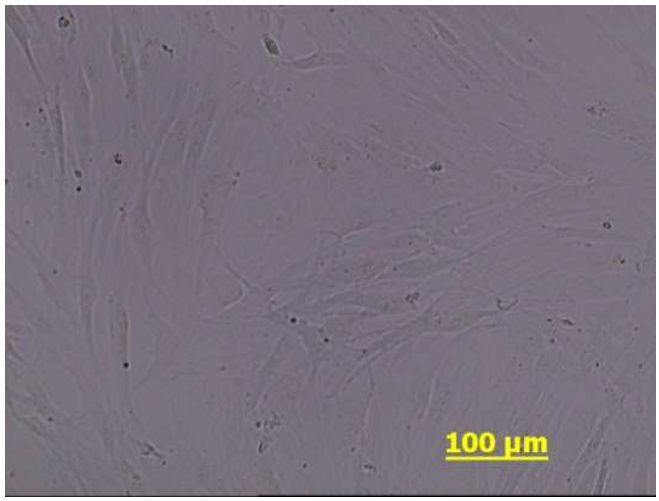

Figure A-18: Mock-treated hMSCs 8 days after treatment

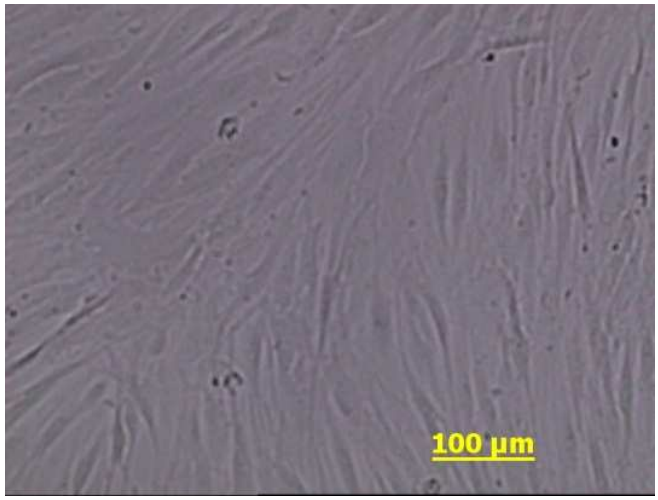

Figure A-19: Rv.eGFP transduced hMSCs 8 days after transduction

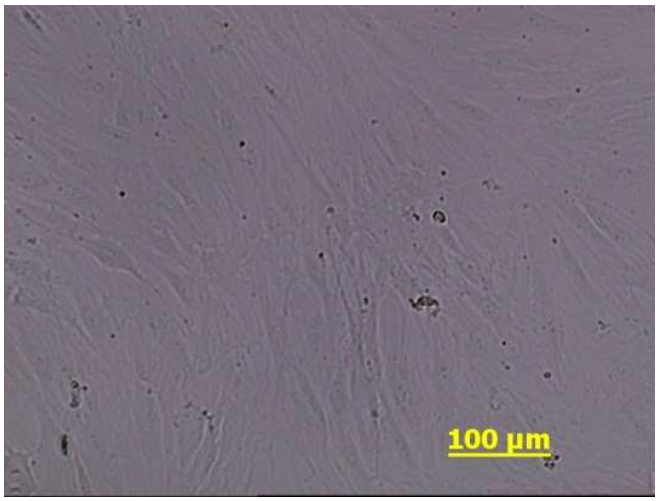

Figure A-20: Rv.BMP-2 transduced hMSCs 8 days after transduction

A3. Array maps of the different sub-arrays from the Ray Bio human Cytokine array G-series.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP-4	BMP-6	Ckβ 8-1	CNTF	EGF	CCL11
2	POS1	POS2	POS3	NEG	NEG	ANG	BDNF	BLC	BMP-4	BMP-6	Ckβ 8-1	CNTF	EGF	CCL11
3	CCL24	CCL26	FGF6	FGF7	Flt-3L	CX3CL1	GCP-2	GDNF	CSF2	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
4	CCL24	CCL26	FGF6	FGF7	Flt-3L	CX3CL1	GCP-2	GDNF	CSF2	I-309	IFN-γ	IGFBP1	IGFBP2	IGFBP4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP1	MCP2	MCP3	CSF1	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	NT-4	PARC
8	Leptin	LIGHT	MCP1	MCP2	MCP3	CSF1	MDC	MIG	MIP-1δ	MIP-3α	NAP-2	NT-3	NT-4	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β3	TNF-α	TNF-β	NEG	NEG	NEG	NEG	NEG
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β3	TNF-α	TNF-β	NEG	NEG	NEG	NEG	NEG

Figure A-21: Array map of sub-array G-Series 6. Figure adapted from the official user manual provided by RayBiotech.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	β-NGF	BTC	CCL28
2	POS1	POS2	POS3	NEG	NEG	Acrp30	AgRP	ANGPT2	AREG	Axl	bFGF	β-NGF	BTC	CCL28
3	CTACK	Dtk	EGFR	ENA-78	Fas	FGF4	FGF9	CSF3	GITRL	GITR	GRO	GRO-α	HCC-4	HGF
4	CTACK	Dtk	EGFR	ENA-78	Fas	FGF4	FGF9	CSF3	GITRL	GITR	GRO	GRO-α	HCC-4	HGF
5	ICAM1	ICAM3	IGFBP3	IGFBP6	sIGF-1R	IL1R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Rα	sIL-6R	IL-8
6	ICAM1	ICAM3	IGFBP3	IGFBP6	sIGF-1R	IL1R4	IL-1 R1	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Rα	sIL-6R	IL-8
7	I-TAC	XCL1	MIF	MIP-1α	MIP-1β	MIP-3α	MSP-α	NT-4	OPG	OSM	PLGF	sgp130	sTNFR2	sTNFR1
8	I-TAC	XCL1	MIF	MIP-1α	MIP-1β	MIP-3α	MSP-α	NT-4	OPG	OSM	PLGF	sgp130	sTNFR2	sTNFR1
9	TECK	TIMP1	TIMP2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF-A	VEGF-D	NEG	NEG	NEG	NEG	NEG
10	TECK	TIMP1	TIMP2	THPO	TRAIL R3	TRAIL R4	uPAR	VEGF-A	VEGF-D	NEG	NEG	NEG	NEG	NEG

Figure A-22: Array map of sub-array G-Series 7. Figure adapted from the official user manual provided by RayBiotech.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS1	POS2	POS3	NEG	NEG	Activin A	ALCAM	B7-1 (CD80)	BMP-5	BMP-7	CTF1	CD14	CXCL16	DR6
2	POS1	POS2	POS3	NEG	NEG	Activin A	ALCAM	B7-1 (CD80)	BMP-5	BMP-7	CTF1	CD14	CXCL16	DR6
3	Endoglin	ErbB3	SELE	FASLG	ICAM2	IGF-II	IL-1 R2	IL-10 Rβ	IL-13 Rα2	IL-18 BPα	IL-18 Rβ	MMP3	IL-2 Rβ	IL-2 Rγ
4	Endoglin	ErbB3	SELE	FASLG	ICAM2	IGF-II	IL-1 R2	IL-10 Rβ	IL-13 Rα2	IL-18 BPα	IL-18 Rβ	MMP3	IL-2 Rβ	IL-2 Rγ
5	IL-21 R	IL-5 Rα	IL-9	IP-10	LAP	Leptin R	LIF	SELL	CSF1R	MMP1	MMP 13	MMP9	MPIF1	NGFR
6	IL-21 R	IL-5 Rα	IL-9	IP-10	LAP	Leptin R	LIF	SELL	CSF1R	MMP1	MMP 13	MMP9	MPIF1	NGFR
7	PDGF-AA	PDGF-AB	PDGF Rα	PDGF Rβ	PECAM1	Prolactin	SCFR	SDF-1 β	Siglec-5	TGF-α	TGF-β2	Tie-1	Tie-2	TIMP4
8	PDGF-AA	PDGF-AB	PDGF Rα	PDGF Rβ	PECAM1	Prolactin	SCFR	SDF-1 β	Siglec-5	TGF-α	TGF-β2	Tie-1	Tie-2	TIMP4
9	CDH5	VEGF R2	VEGF R3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
10	CDH5	VEGF R2	VEGF R3	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

Figure A-23: Array map of the sub-array G-Series 8. Figure adapted from the official user manual provided by RayBiotech.

A4. Complete overview of all 174 cytokines from the Ray Bio human Cytokine array G-series.

Acrp30	GCP-2	IL-2 Rbeta	NT-4
Activin A	GCSF	IL-2 Rgamma	Oncostatin M
AgRP	GDNF	IL-21R	Osteoprotegerin
ALCAM	GITR	IL-3	PARC
Amphiregulin	GITR-Ligand	IL-4	PDGF AA
Angiogenin	GM-CSF	IL-5	PDGF Ralpha
Angiopoietin-2	GRO	IL-5 Ralpha	PDGF Rbeta
Axl	GRO-alpha	IL-6	PDGF-AB
B7-1(CD80)	HCC-4	IL-6 R	PDGF-BB
BDNF	HGF	IL-7	PECAM-1
bFGF	I-309	IL-8	PIGF
BLC	ICAM-1	IL-9	Prolactin
BMP-4	ICAM-2	IP-10	RANTES
BMP-5	ICAM-3	I-TAC	SCF
BMP-6	IFN-gamma	LAP	SCF R
BMP-7	IGFBP-1	Leptin	SDF-1
b-NGF	IGFBP-2	Leptin R	SDF-1beta
BTC	IGFBP-3	LIF	sgp130
Cardiotrophin-1	IGFBP-4	LIGHT	Siglec-5
CCL-28	IGFBP-6	L-Selectin	sTNF RII
CD14	IGF-I	Lymphotactin	sTNF-RI
CK beta 8-1	IGF-I SR	MCP-1	TARC
CNTF	IGF-II	MCP-2	TECK
CTACK	IL-1 R II	MCP-3	TGF beta2
CXCL- 16	IL-1 R4/ST2	MCP-4	TGF-alpha
DR6 (TNFRSF21)	IL-1 RI	M-CSF	TGF-beta 1
Dtk	IL-10	M-CSF R	TGF-beta 3
EGF	IL-10 Rbeta	MDC	Thrombopoietin
EGF-R	IL-11	MIF	Tie-1
ENA-78	IL-12 p40	MIG	Tie-2
Endoglin	IL-12 p70	MIP-1alpha	TIMP-1
Eotaxin	IL-13	MIP-1beta	TIMP-2
Eotaxin-2	IL-13 Ralpha2	MIP-1-delta	TIMP-4
Eotaxin-3	IL-15	MIP-3-alpha	TNF-alpha
ErbB3	IL-16	MIP-3beta	TNF-beta
E-Selectin	IL-17	MMP-1	TRAIL R3
Fas Ligand	IL-18 BPalpha	MMP-13	TRAIL R4
Fas/TNFRSF6	IL-18 Rbeta	MMP-9	uPAR
FGF-4	IL-1alpha	MPIF-1	VE-Cadherin
FGF-6	IL-1beta	MSP-alpha	VEGF
FGF-7	IL-1ra	NAP-2	VEGF R2
FGF-9	IL-2	NGF R	VEGF R3
Flt-3 Ligand	IL-2 Ralpha	NT-3	VEGF-D
Fractalkine	IL-2 Rapha		

Figure A-24: Overview of the 174 cytokines which were analysed, using the Ray Bio human Cytokine array G-series, for the secretome analysis of hMSCs and hACPCs. Cytokines highlighted in green were more abundant (2-fold or higher) in hMSCs, cytokines highlighted in orange were more abundant (2-fold or higher) in hACPCs

A5. Immunohistochemical detection of the matrix proteins aggrecan, collagen II and collagen X, within fibrin-PU composite scaffolds, seeded with cells from donor Pat 52 and donor Pat 53.

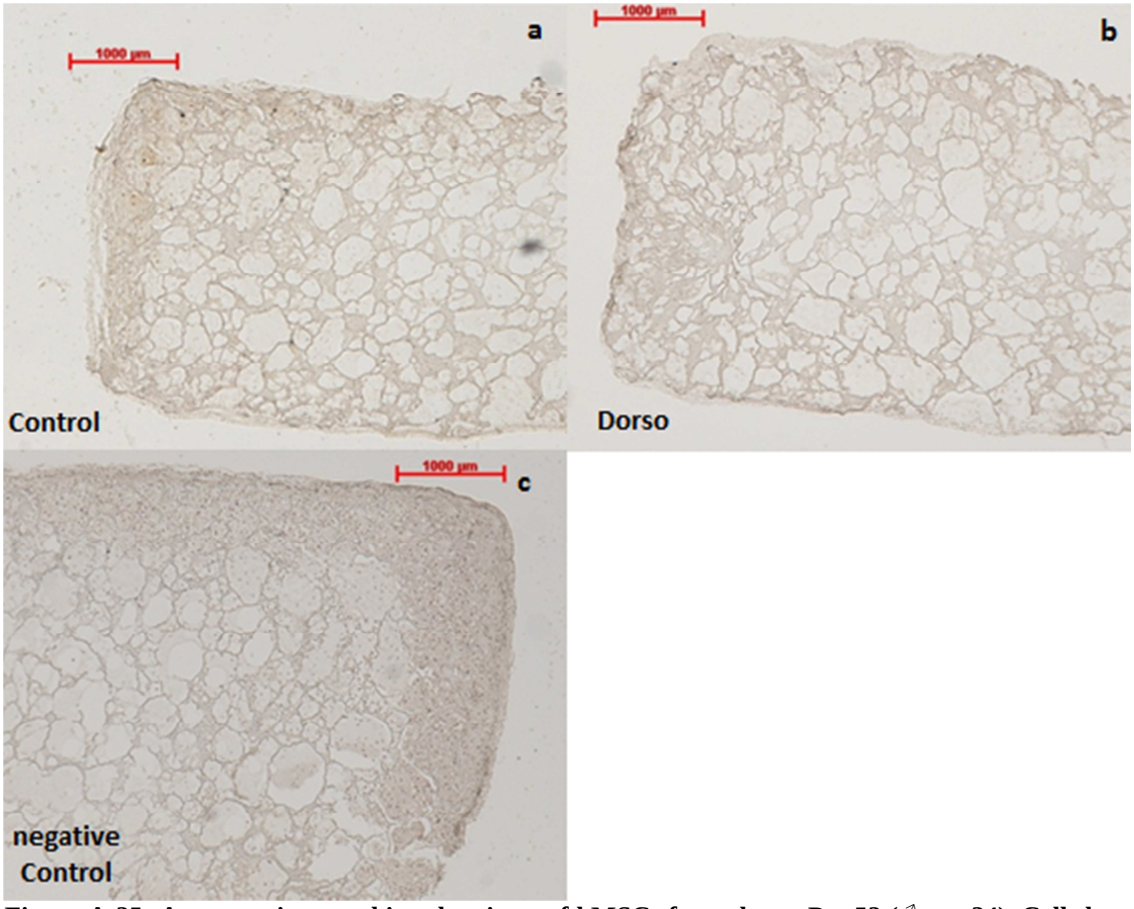

Figure A-25: Aggrecan immunohistochemistry of hMSCs from donor Pat 52 (♂ age 24). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Aggrecan immunohistochemistry was conducted using the 1-C-6 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control a, Dorso b and negative control c) are shown.

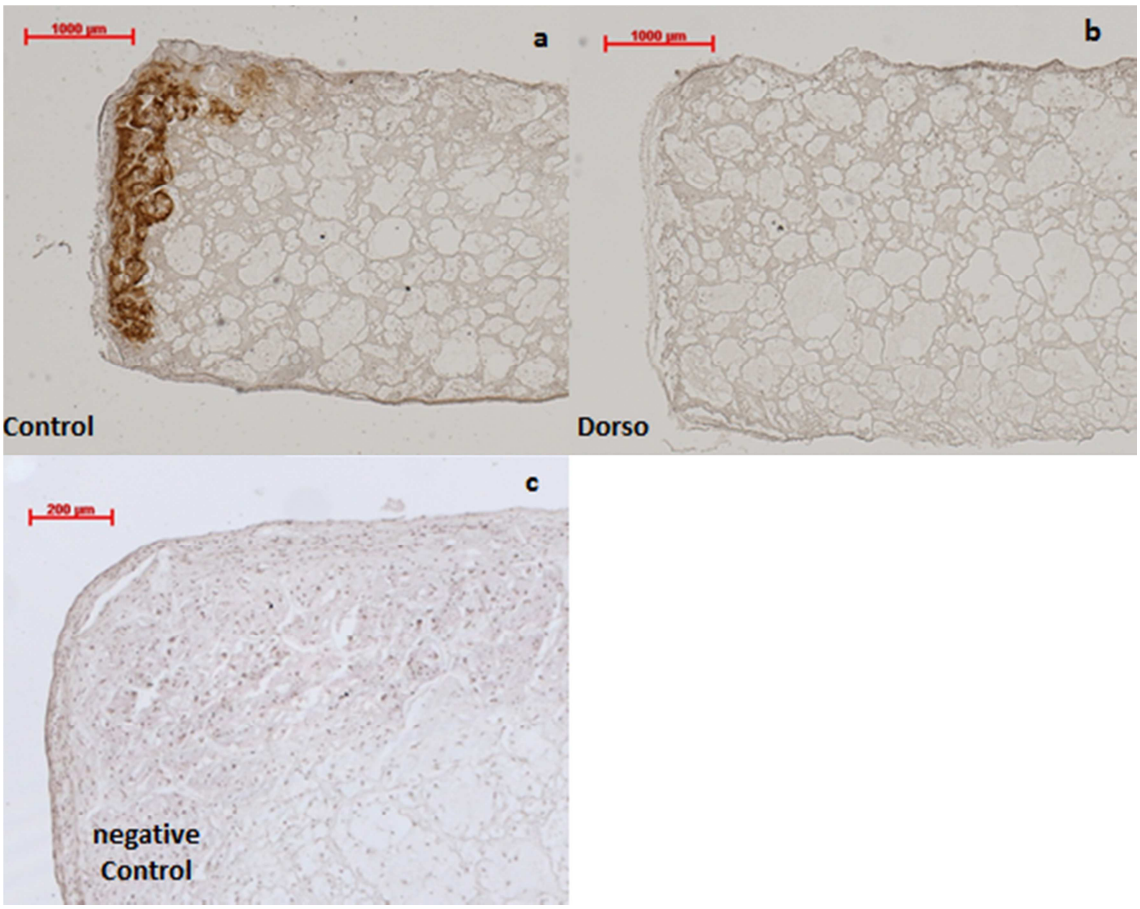

Figure A-26: Col II immunohistochemistry of hMSCs from donor Pat 52 (♂ age 24). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col II immunohistochemistry was conducted using the CIICI antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control a, Dorso b, and negative control c) are shown. Images of the negative control sections were taken with a higher magnification.

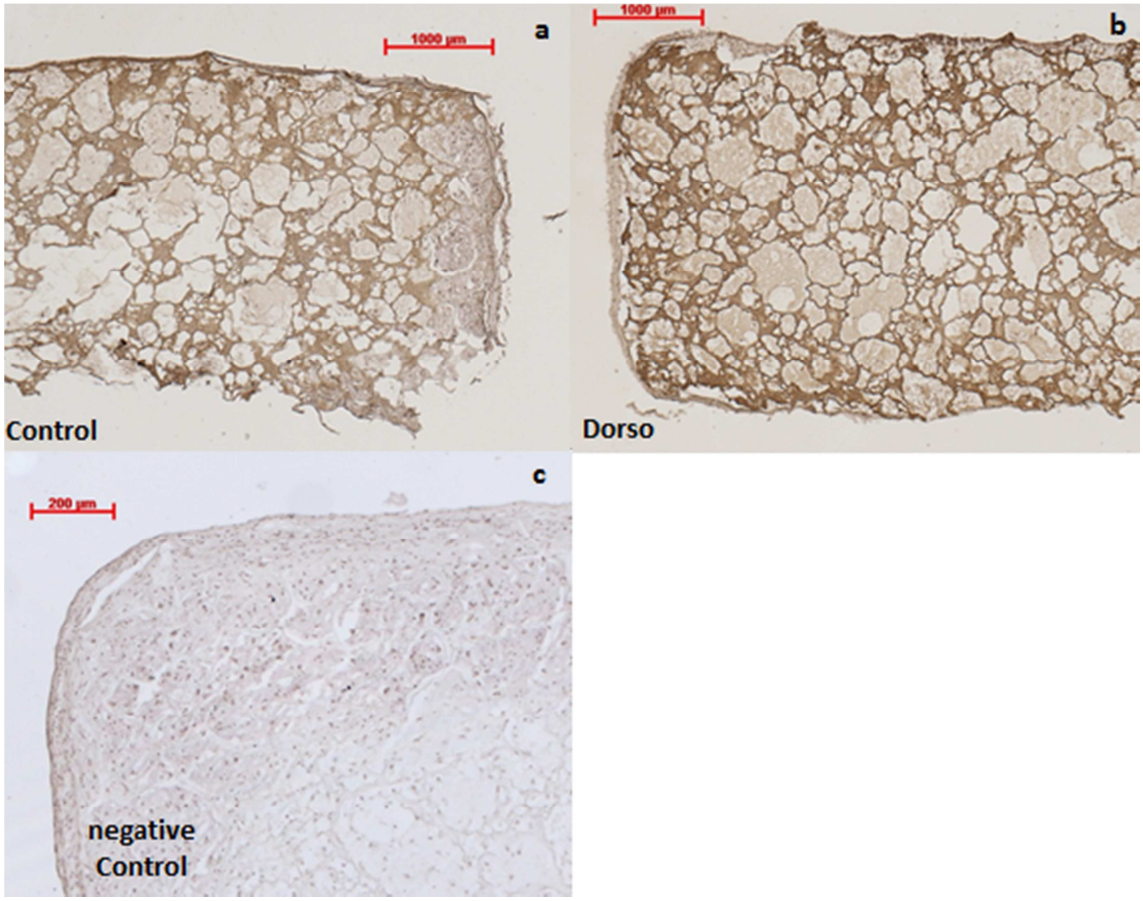

Figure A-27: Col X immunohistochemistry of hMSCs from donor Pat 52 (♂ age 24). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col X immunohistochemistry was conducted using the Col-10 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images, (control a, Dorso b and negative control c) are shown. Images of the negative control sections were taken with a higher magnification.

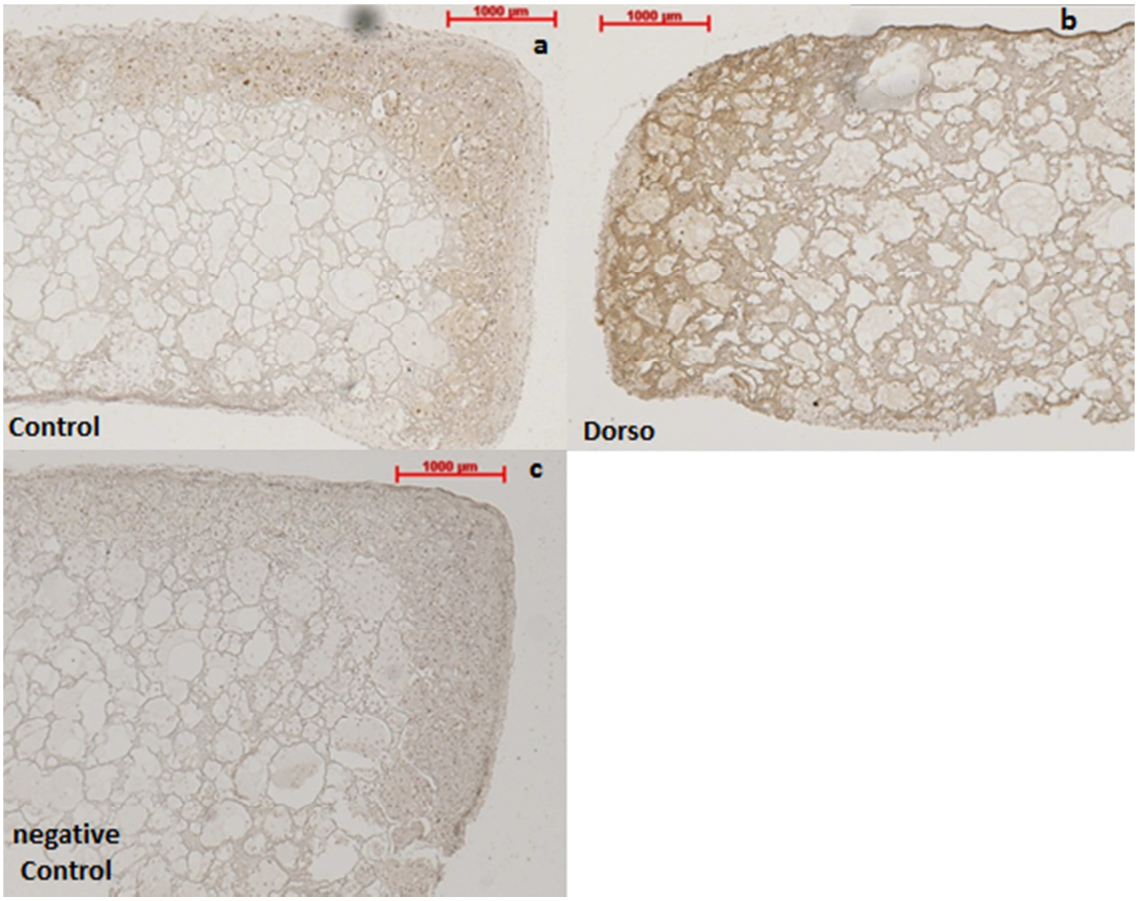

Figure A-28: Aggrecan immunohistochemistry of hMSCs from donor Pat 53 (♀age 20). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Aggrecan immunohistochemistry was conducted using the 1-C-6 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control a, Dorso b and negative control c) are shown. The dark spot on top of figure A-28 a is an air bubble that was created during cover-slipping.

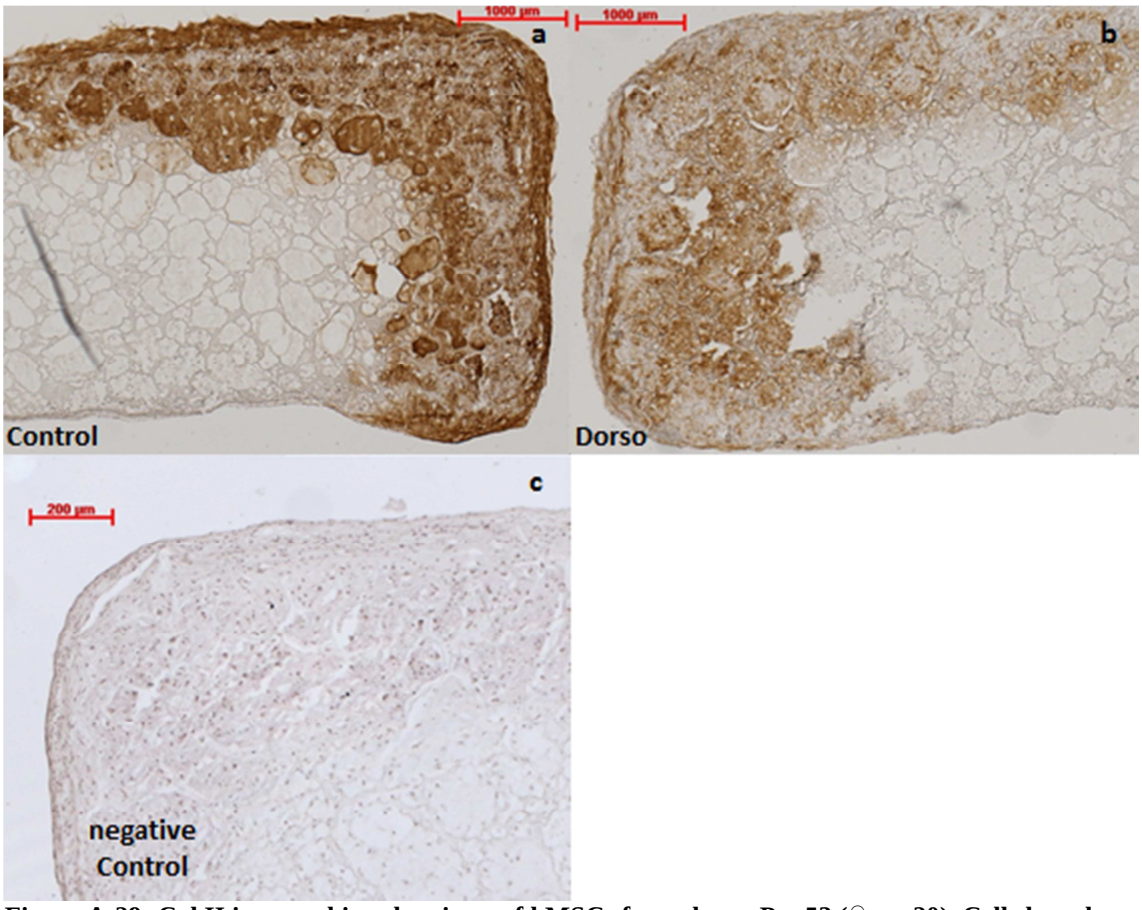

Figure A-29: Col II immunohistochemistry of hMSCs from donor Pat 53 (♀age 20). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col II immunohistochemistry was conducted using the CIICI antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control a, Dorso b and negative control c) are shown. Images of the negative control sections were taken with a higher magnification.

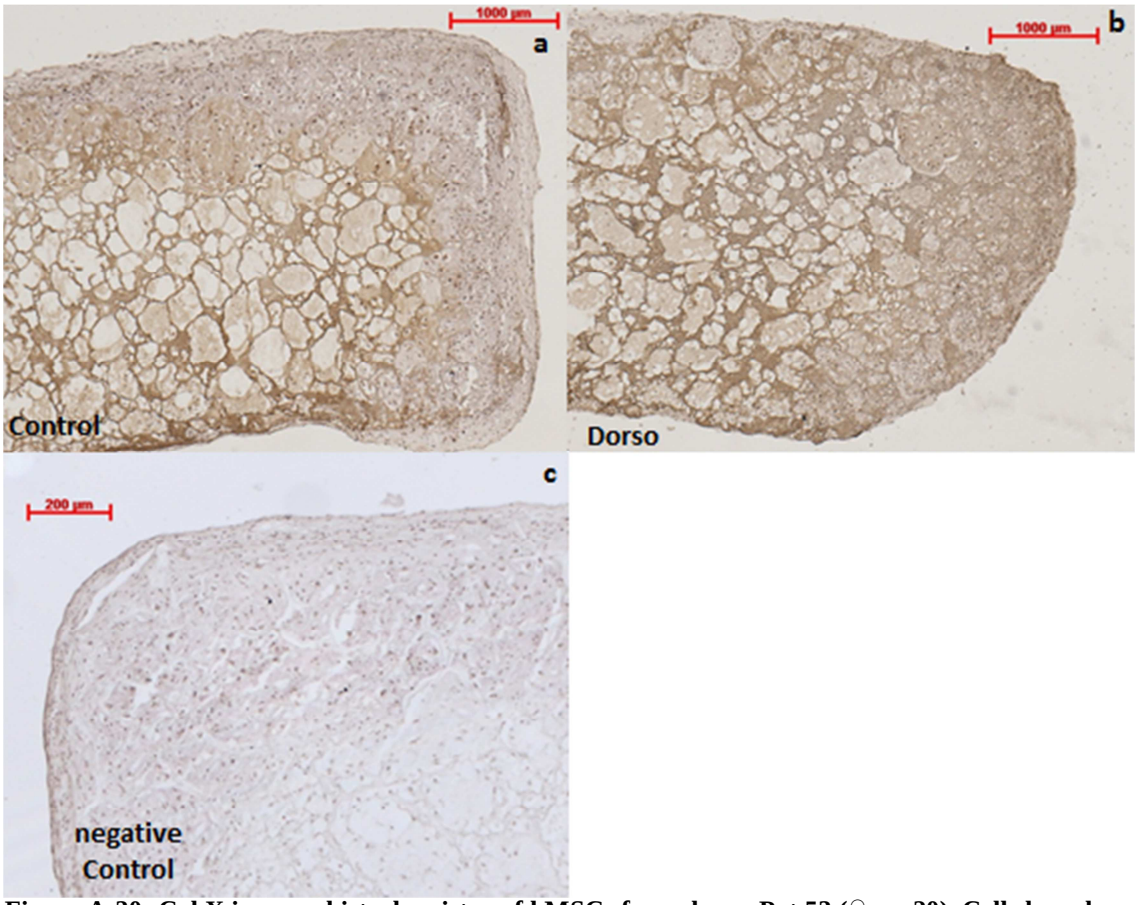

Figure A-30: Col X immunohistochemistry of hMSCs from donor Pat 53 (♀age 20). Cells have been cultured in fibrin-PU composite scaffolds for 28 days. During the first two weeks of culture, both groups were cultured in chondrogenic medium. Subsequently, they were cultured for another two weeks in either chondrogenic medium (control) or in chondrogenic medium supplemented with an additional 10µM of dorsomorphin (Dorso). Scaffolds were fixed in 99.5% methanol and cryo-sectioned. Col X immunohistochemistry was conducted using the Col-10 antibody as primary antibody. Negative control sections were treated with PBS-T instead of the primary antibody. Representative images (control a, Dorso b and negative control c) are shown. Images of the negative control sections were taken with a higher magnification.

A6. Publications and conference abstracts, associated with the PhD work.

Publications:

- Neumann,A.J., Alini,M., Archer,C.W., and Stoddart,M.J. (2013). Chondrogenesis of Human Bone Marrow-Derived Mesenchymal Stem Cells Is Modulated by Complex Mechanical Stimulation and Adenoviral-Mediated Overexpression of Bone Morphogenetic Protein 2. Tissue Eng Part A.
- Neumann,A.J., Alini,M., Archer,C.W., and Stoddart,M.J. (2013). Retroviral-mediated overexpression of human bone morphogenetic protein 2 affects human mesenchymal stem cells during monolayer proliferation: A cautionary note. Electron J Biotechnol 16.
- Neumann,A.J., Schroeder,J., Alini,M., Archer,C.W., and Stoddart,M.J. (2013). Enhanced Adenovirus Transduction of hMSCs Using 3D Hydrogel Cell Carriers. Mol. Biotechnol. 53, 207-216.
- Neumann A.J, Gardner O.F.W, Alini M., Archer C.W. and Stoddart M.J. "The effect of complex mechanical stimulation and adenoviral-mediated overexpression of bone morphogenetic protein 2 on the chondrogenesis of human articular cartilage progenitor cells". (manuscript in preparation).

Conference abstracts:

- "Enhanced gene delivery using adenoviral vectors" (poster). ORS 2011: 2011 Annual Meeting of the Orthopaedic Research Society, January 13th-16th , 2011, Long Beach, California, United States of America.
- "The effect of retroviral-mediated overexpression of BMP-2 on hMSCs during monolayer proliferation" (poster). ICRS 2012: 10th World Congress of the International Cartilage Repair Society, May 12th-15th, 2012 Montreal, Canada.
- "Combined effect of mechanical load and BMP-2 overexpression on the chondrogenesis of human bone marrow derived stem cells" (poster). ICRS 2012: 10th World Congress of the International Cartilage Repair Society, May 12th -15th , 2012 Montreal, Canada.
- "Combined effect of mechanical load and BMP-2 overexpression on the chondrogenesis of human bone marrow derived stem cells" (poster). 2013 Annual Meeting of the Orthopaedic Research Society, January 26th-29th San Antonio, Texas, United States of America.