# Differentiation and extracellular matrix interactions of chondrocytes in response to signaling molecules and biomaterials for cartilage repair

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# Differenzierung und Interaktionen von Chondrozyten mit der extrazellulären Matrix als Reaktion auf Signalmoleküle und Biomaterialen für die Wiederinstandsetzung von Knorpel

Dissertation

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## Acknowledgements

## Abbreviations

| | |
|---|---|
| $[M\text{-}PFB]^-$ | pentafluorobenzyl ion |
| $[P]^-$ | Precursor ion |
| $[2(CH_3)_3SiOH]^-$ | 2-Trimethylsilanol ion |
| 3-D | Three-dimensional |
| ACI | Autologous chondrocyte implantation |
| ALP | Alkaline phosphatase |
| APS | Ammonium persulfate |
| BMP | Bone morphogenetic protein |
| bp | Base pair |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium ion |
| $CaCl_2$ | Calcium chloride |
| Calcein-AM | Acetomethoxy derivative of calcein |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complementary DNA |
| $CH_3COOH$ | Acetic acid |
| cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| Col I | Collagen type I |
| Col II | Collagen type II |
| Col X | Collagen type X |
| COMP | Cartilage oligomeric matrix protein |
| Ct | Cycle threshold |

| | |
|---|---|
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| *e.g.* | *exempli gratia* |
| ECM | Extracellular matrix |
| EDTA | Ethylenediamine-N,N,N',N'-tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EP | Prostaglandin E receptor |
| et al. | *et alii* |
| etc. | etcetera |
| EtOH | Ethanol |
| FCS | Fetal calf serum |
| $FeCl_2$ | Ferrous chloride |
| FGF | Fibroblast growth factor |
| Fig. | Figure |
| g | Gram |
| GAG | Glycosaminoglycans |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GC/MS/MS | Gas chromatography-tandem mass spectrometry |
| GDF | Growth differentiation factor |
| GFP | Green fluorescence protein |
| h | Hour |
| H&E | Haematoxylin & Eosin |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |

| | |
|---|---|
| $H_2SO_4$ | Sulfuric acid |
| HAC | Human articular chondrocytes |
| HCl | Hydrochloric acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HeLa | Henrietta Lacks |
| HRP | Horseradish peroxidase |
| *i.e.* | *id est* |
| Ig | Immunoglobulin |
| IGF | Insulin-like growth factor |
| Ihh | Indian hedgehog |
| IL | Interleukin |
| IRES | Internal ribosome entry site |
| IS | Internal standard |
| kb | Kilobase |
| kDa | Kilodalton |
| l | Liter |
| LB | Lysogeny broth |
| M | Molar |
| *m/z* | mass-to-charge ratio of the fragment |
| mA | Milliamper |
| MEM | Minimal essential medium |
| MetOH | Methanol |
| mg | Milligram |
| $MgCl_2$ | Magnesium chloride |
| $MgSO_4$ | Magnesium sulfate |
| min | Minute |

| | |
|---|---|
| ml | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| MMP | Metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| MWM | Molecular weight marker |
| N | Normal concentration |
| $Na_2EDTA$ | Disodium ethylenediamine tetraacetate |
| $Na_2HPO_4$ | Sodium hydrogen phosphate |
| NaCl | Sodium chloride |
| $NaHCO_3$ | Sodium bicarbonate |
| NaOH | Sodium hydroxide |
| N-CAM | Neural cell adhesion molecule |
| ng | Nanogram |
| nm | Nanometer |
| nM | Nanomolar |
| OA | Osteoarthritis |
| $^oC$ | Celsius degree |
| OCD | Osteochondritis dissecans |
| PAA | Polyacrylamid |
| PAGE | Polyacrylamid gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCNA | Proliferating cell nuclear antigen |

| | |
|---|---|
| PCP | Procollagen-C proteinase |
| PFA | Paraformaaldehyde |
| pg | Picogram |
| PGA | Polyglycolic acid |
| $PGE_2$ | Prostaglandin $E_2$ |
| PLA | Polylactic acid |
| PLGA | Poly(lactic-co-glycocolic) acid |
| pmol | Picomol |
| PTHLH/PTHrH | Parathyroid hormone related protein |
| qrt-PCR | Qualitative real time reverse transcription polymerase chain reaction |
| RA | Rheumatoid arthritis |
| RAGE | Receptors for advanced glycation end products |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RT | Room temperature |
| RT-PCR | Reverse transcription polymerase chain reaction |
| s | Second |
| SD | Standard deviation |
| SDS | Sodium dodecylsulfate |
| SLRP | Small leucine-rich proteoglycans |
| SOB | Super optimal broth |
| SOC | Super optimal broth with catabolite repression |
| Sox | Sex determing region box |
| TBE | Tris-borate-EDTA |
| TE | Tris-EDTA buffer |

| | |
|---|---|
| TEMED | N,N,N’,N’-Tetramethylethylendiamine |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| Tris | Tris-(hydroxymethyl)-aminoethane |
| U | Unit |
| UV | Ultraviolet |
| V | Volt |
| Wnt/β | Wingless int/β |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |

## Summary

Chondrocytes live isolated in the voluminous extracellular matrix of cartilage, which they secrete and is neither vascularized nor innervated. Nutrient and waste exchanges occur through diffusion leading to low oxygen tension around the cells. Consequently even normal cartilage under normal physiological conditions suffers from a poor reparative potential that predisposes to degenerative conditions, such as osteoarthritis of the joints, with significant clinical effects.

One of the key challenges in medicine is the structural and functional replacement of lost or damaged tissues. Current therapeutical approaches are to transplant cells, implant bioartificial tissues, and chemically induce regeneration at the site of the injury. None of them reproduces well the biological and biomechanical properties of hyaline cartilage.

This thesis investigates the re-differentiation of chondrocytes and the repair of cartilage mediated by signaling molecules, biomaterials, and factors provided in mixed cellular cultures (co-culture systems). As signaling molecules we have applied prostaglandin $E_2$ ($PGE_2$) and bone morphogenetic protein 1 (BMP-1) and we have transfected chondrocytes with BMP-1 expressing vectors. Our biomaterials have been hydrogels of type-I collagen and gelatin-based scaffolds designed to mimic the architecture and biochemistry of native cartilage and provide a suitable three-dimensional environment for the cells. We have brought chondrocytes to interact with osteosarcoma Cal 72 cells or with murine preosteoblastic KS483 cells, either in a cell-to-cell or in a paracrine manner.

Exogenous stimulation with $PGE_2$ or BMP-1 did not improve the differentiation or the proliferation of human articular chondrocytes. BMP-1 induced chondrocytic de-differentiation in a dose-dependent manner. Prostaglandin stimulation from gelatin-based scaffolds (three-dimensional culture) showed a certain degree of chondrocyte re-differentiaton. Murine preosteoblastic KS483 cells had no beneficial effect on human articular chondrocytes jointly cultivated with them in hydrogels of type I collagen. Although the hydrogels provided the chondrocytes with a proper matrix in which the cells adopted their native morphology; additionally, the expression of chondrocytic proteoglycan increased in the co-cultures after two weeks. The co-culture of chondrocytes with osteoblast-like cells (in transwell systems) resulted in suppression of the regular de-differentiation program that passaged chondrocytes undergo when cultured in monolayers. Under these conditions, the

extracellular matrix of the chondrocytes, rich in type-II collagen and aggrecan, was not transformed into the extracellular matrix characteristic of de-differentiated human articular chondrocytes, which is rich in type-I collagen and versican.

This thesis suggests novel strategies of tissue engineering for clinical attempts to improve cartilage repair. Since implants are prepared *in vitro* (*ex-vivo*) by expanding human articular chondrocytes (autologous or allogeneic), we conclude that it will be convenient to provide a proper three-dimensional support to the chondrocytes in culture, to supplement the culture medium with $PGE_2$, and to stimulate chondrocytes with osteoblastic factors by cultivating them with osteoblasts.

# 1. Introduction

## 1.1. Cartilage

Cartilage is a dense connective tissue and major constituent of the embryonic and young vertebrate skeleton; it is converted largely to bone with maturation and it is composed of a unique cell type called chondrocyte. Unlike other connective tissues, cartilage does not contain blood vessels. The chondrocytes are fed by diffusion, helped by the pumping action generated by compression or flexion. Thus, compared to other connective tissues, cartilage grows and repairs more slowly. Because of its avascularity, cartilage has no contact with the immune system. Chondrocytes carry transplantation antigens at their surface and are immunogenic (Langer and Gross, 1974; Langer et al., 1972). The macromolecules of the extracellular matrix (ECM) provoke only a weak immunological response. These properties are due to an abundant ECM with a high content of water synthesized by the chondrocytes. Depending on the properties of this ECM there are three different types of cartilage: (i) hyaline cartilage is found in the joints, rib cartilage, nose, trachea and larynx, (ii) elastic cartilage is found in the ear, epiglottis and larynx, and (iii) fibrous cartilage is found in the invertebral discs (Keller-Peck, 2008). The focus of this study is the hyaline articular cartilage. Besides being involved in skeletal development through the process of endochondral ossification, hyaline cartilage also provides the joint with a low friction surface, resilience and compressive stiffness, and in normal conditions this unique tissue is wear-resistant (Cohen et al., 1998). The different cell numbers, organization and distribution, as well as differences in water, and other major components across the tissue are responsible for the existence of four different zones with respect to depth from the articular surface, *i.e.* superficial, transitional, radial and calcified (Poole et al., 2001) (Fig. 1.1). These structural and functional zones will be thoroughly described in the following sections.

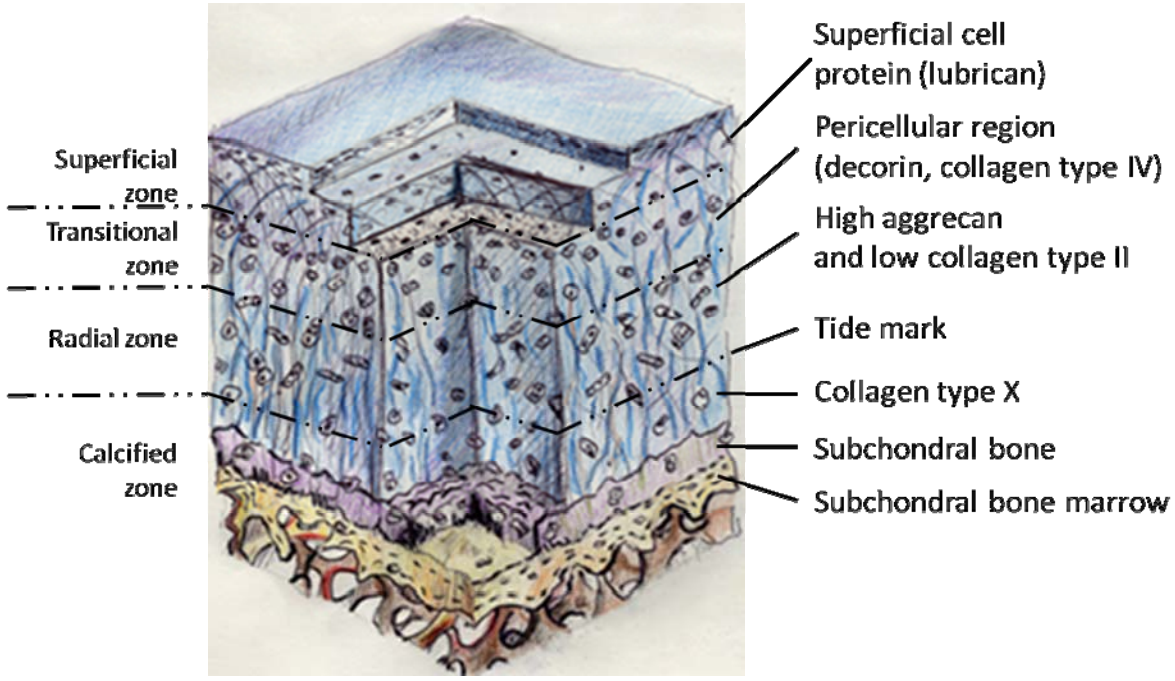



**Fig.1.1. Structural and functional zones of articular cartilage.** Adapted from Poole, A.R. *et al.* (Poole et al., 2001).

### 1.1.1 Cartilage Embryology

The embryonic connective tissue develops from cells migrating from the mesoderm. These cells aggregate and proliferate forming the mesenchyme, a tissue of undifferentiated cells with a multipotency to develop into different tissues, i.e. cartilage, muscle, adipose and so on. Cartilage formed by these mesenchymal stem cells appears in human embryos at five weeks of age. Continued growth of the condensations and differentiation of the mesenchymal stem cells into chondrocytes results in the formation of cartilage templates, or anlagen, which prefigure the future skeleton (Cameron et al., 2009). The embryonic cartilage appears at this time as a plate consisting of cells scattered in a strongly basophilic matrix and surrounded by what is called a perichondrium. The cells in this periphery are still immature chondrocytes called chondroblasts, which start to increase in size and produce an ECM. The increased amount of matrix separates the cells individually or as a couple of cells now called chondrocytes (Gibson-Gamble, 1988). The chondrocytes located in the centre of the anlage proceed through a series of discrete developmental stages that include proliferation, maturation and hypertrophy (Hall and Miyake, 2000). The hypertrophic cartilage is first

calcified and then, following vascular invasion, it is replaced by primary bone that is subsequently remodeled to form secondary bone (DeLise et al., 2000; Roach, 1997).

There are several biological differences between the cartilage of the growth plate and the adult hyaline human articular cartilage. In the embryo there is initially a high cell to matrix ratio, whereas adult cartilage consists only of 2% chondrocytes. The cartilage formed in the embryo is gradually changed during development to approach the needs and demands of the adult organism. The exact composition of the cartilage matrix and the biomechanical properties are also dependent on age (Guo et al., 2007; Poole et al., 2001).

### 1.1.2. Cartilage Biochemistry and Morphology

Cartilage with a matrix up to 90% of the dry weight of the tissue, is particularly rich in ECM (Hardingham and Fosang, 1992). The major part of the matrix consists of water (65 – 80%) and the rest of solid material, of which about 60% is collagen, 30% proteoglycans and 10% other proteins.

According to the structural zones mentioned above (Fig. 1.1), the major component of the superficial zone which lines the synovial joint is lubricin and superficial zonal proteins. These proteins function as lubricant and are normally only found in this area of the articular cartilage (Swann et al., 1985). The elongated and flat cells in this area synthesize mainly type I collagen. In the transitional zone cells start to become more rounded and pericellular collagen type IV can be found. The major proteoglycan is decorin. The collagen fibers in these two first regions are horizontally oriented while in the radial zone, where mostly collagen type II is found, the fibers are vertically oriented (ap Gwynn et al., 2002) (Fig. 1.1). In the radial zone chondrocytes are more sparsely distributed, and the major matrix proteins are collagen type II and the proteoglycan aggrecan. Next to the subchondral bone the cartilage becomes mineralized and the matrix is rich in collagen type X (Poole, 1997).

The mechanical function of proteoglycans is to act as shock absorbers, whereas the collagens are resistant to shear stress. The main functions of the collagen fibrils are to give articular cartilage its shape, strength and tensile stiffness. The fibrils are composed of aggregates of five tropocollagens (three identical $\alpha_{II}$ polypeptide chains assembled in a superhelix) that have a diameter of 20 nm. This unique structure of the fibrils makes collagen insoluble and resistant to attack by degrading enzymes (Lehninger A.L., 1993). The main type of collagen in articular

cartilage is type II (80-90% of the collagen content), but small amounts of types I, V, VI, IX, X and XI can be detected (Blitterswijk, 2008) of which collagen type I is particularly abundant in degenerated fibrocartilage. Collagen type II is highly crosslinked via collagen type IX and XI, forming a fibrous framework (Responte et al., 2007; van der Rest and Garrone, 1991). The turnover of collagens in adult normal cartilage is very slow (Eyre, 2002).

Structurally, proteoglycan monomers contain a core protein to which molecules of glycosaminoglycans (GAGs) are covalently bound. A GAG is an unbranched polysaccharide made up of repeating disaccharides, one component of which is an aminosugar (i.e. keratan sulfate, chondroitin sulfate or dermatan sulfate), while the other is uronic acid. The chains are long, linear carbohydrate polymers that are negatively charged under physiological conditions, due to the occurrence of sulfate and uronic acid groups. Large aggregates are formed with several proteoglycan monomers via a link protein connecting the central protein cores to a chain of hyaluronic acid, which is further bound to the chondrocytes through the surface receptor CD44 (Alberts B., 2003). The main proteoglycan in articular cartilage is aggrecan (Dudhia, 2005; Kiani et al., 2002), which serves the role of providing the osmotic resistance necessary for cartilage to resist compressive loads and control movements. Thus CD44, hyaluronic acid and aggrecan comprise important elements for pericellular matrix assembly and maintenance of matrix homeostasis. (Knudson and Knudson, 2001). An additional component of the ECM and member of the hyaluronan-binding proteoglycans is versican; present in cartilage at low levels, it may play specific roles in the articular surface (Matsumoto et al., 2006). It also has a significant role in regulating cell phenotype (Wight, 2002), being a typical marker for degenerated fibrocartilage (Grover and Roughley, 1993). There are other minor proteoglycans like the small leucine-rich proteoglycans (SLRPs) which include decorin, biglycan, fibromodulin, lumican, epihycan and perlecan; and the cell surface proteoglycans syndecan, lubricin and glypican.

Chondrocytes are responsible for the growth and homeostasis of articular cartilage and continually replace matrix macromolecules lost during normal turnover. Normal cartilage metabolism is characterized by a highly regulated balance between the synthesis and degradation of matrix components governed by a significant number of humoral factors. Critical factors include ions, low molecular weight components such steroid hormones, isolated portions of ECM molecules, peptide hormones and full sized proteins. Some of these components are delivered from outside the cartilage (paracrine) but many are autocrine factors, generated by chondrocytes themselves and deposited in their immediate vicinity for storage and eventual release on demand, or continuous availability (Gaissmaier et al., 2008).

## 1.2.Chondrocyte

### 1.2.1 Chondrogenesis

Chondrogenesis is the earliest phase of skeletal development, involving mesenchymal stem cell recruitment and migration, condensation of progenitors, and chondrocyte differentiation, and maturation, resulting in the formation of cartilage and bone during endochondral ossification (Hickok et al., 1998; Olsen et al., 2000). This process consists of a highly coordinated and orchestrated series of events controlled by cellular interactions with surrounding matrix, growth and differentiation factors, and other environmental factors that initiate or suppress cellular signaling pathways and transcription of specific genes in a specific temporo-spatial manner. However, the specific mechanisms regulating these processes remain unclear (Archer and Francis-West, 2003). Vertebrate limb development is controlled by interacting patterning systems involving prominently the fibroblast growth factor (FGF), the transforming growth factor (TGF)-β family, cartilage-derived morphogenetic proteins, bone morphogenetic protein (BMP), insulin-like growth factor (IGF)-1, and hedgehog pathways (Kronenberg, 2003; Phornphutkul et al., 2006). Both positive and negative signaling kinases and transcription factors, such as Sox9 (Hardingham et al., 2006; Healy et al., 1999) and Runx2 (Yoshida et al., 2004), and interactions among them determine whether the differentiated chondrocytes endure as cartilage elements in articular joints or undergo hypertrophic maturation prior to ossification (Tickle, 2002; Tickle, 2003).

#### *1.2.1.1. Mesenchymal Stem Cell Condensation and Determination of Chondroprogenitors*

The process of chondrogenesis occurs in stages beginning with mesenchymal stem cell recruitment and migration, proliferation (Fig 1.2.) and like many connective tissues, the first manifest sign of expression of the differentiated state is the formation of a cellular condensation at the site where the skeletal elements will form (Hall and Miyake, 2000). This process depends on signals initiated by cell-cell and cell-matrix interactions and is associated with increased cell adhesion and formation of gap junctions and changes in the cytoskeletal architecture. TGF-β, which is among the earliest signals in chondrogenic condensation, stimulates the synthesis of fibronectin, which in turn regulates neural cell adhesion molecule

(N-CAM). Syndecan binds to fibronectin and downregulates N-CAM, thereby setting the condensation boundaries. The ECM molecules, which also include tenascins and thrombospondins, including cartilage oligomeric protein (COMP), interact with the cell adhesion molecules to activate intracellular signaling pathways and initiate the transition from chondroprogenitor cells to a fully committed chondrocyte (DeLise et al., 2000).

BMPs set the stage for bone morphogenesis by initiating chondroprogenitor cell determination and differentiation, but also regulate the later stages of chondrocyte maturation and terminal differentiation to the hypertrophic phenotype (Pizette and Niswander, 2000; Tickle, 2003).

#### *1.2.1.2. Chondrocyte Differentiation*

The differentiation of chondrocytes from the condensated mesenchymal stem cells can be divided into various stages that are characterized by the expression of typical markers (de Crombrugghe et al., 2000). The transcription factor Sox9 (BMP dependent) is a major factor in the commitment and development of chondrogenic cells, indispensable for the early stages of chondrocyte differentiation by regulating cell condensation and the production of early chondrogenic markers like collagen type II (Archer and Francis-West, 2003; Healy et al., 1999). Further differentiation of chondrocytes requires the expression of two other members of the Sox-family, L-Sox5 and Sox6 that are coexpressed with Sox9 during chondrogenesis. However, in contrast to Sox9 these molecules do not have any activity as transcription factors (Ikeda et al., 2004; Lefebvre et al., 2001). The fates of chondrocytes vary greatly, depending on their origin and location. The cells of articular cartilage persist and survive. Those that comprise the epiphyseal growth plate proceed through a differentiation program leading to cell hypertrophy, the terminally differentiated state that facilitates endochondral ossification whereby bone is laid down on the calcified cartilaginous matrix of hypertrophic chondrocyte. At this late stage, the chondrocyte seems to have two possible fates. Either the cell dies by apoptosis or it can transdifferentiate into an osteoblast, thus converting its surrounding matrix form cartilage to bone (Zerega et al., 1999) (Fig. 1.2.).

Above all, to enable hypertrophic differentiation, the expression of Sox9 must be downregulated in late proliferative chondrocytes. The transcription factor is involved in hypertrophic differentiation. The subsequent stages of chondrocyte differentiation are characterized by the expression of typical marker genes (Fig. 1.2.) (Blitterswijk, 2008; Goldring et al., 2006).

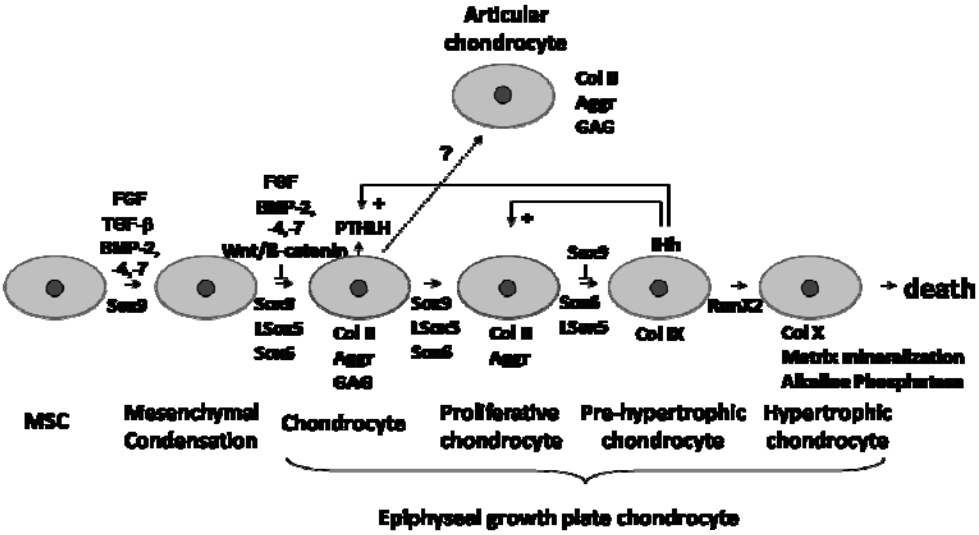



**Fig 1.2.: Chondrogenesis.** The differentiation of chondrocytes from MSCs can be divided into various stages that are characterized by the expression of typical markers. The transcription factor Sox9 is a positive regulator and Wnt/β-catenin is a potent negative regulator of chondrogenesis. To enable hypertrophic differentiation, Sox9 needs to be downregulated. Other important transcription factors are L-Sox5 and Sox6 that are particularly important in early differentiation of chondrocytes while RunX2 is involved in hypertrophic differentiation. Indian hedgehog (Ihh) is expressed by prehypertrophic chondrocytes and stimulates chondrocyte proliferation. It also induces parathyroid hormone related protein (PTHLH) expression in early stage chondrocytes. The molecular mechanism that results in the formation of articular chondrocytes instead epiphyseal growth plate chondrocytes is presently unknown (Blitterswijk, 2008).

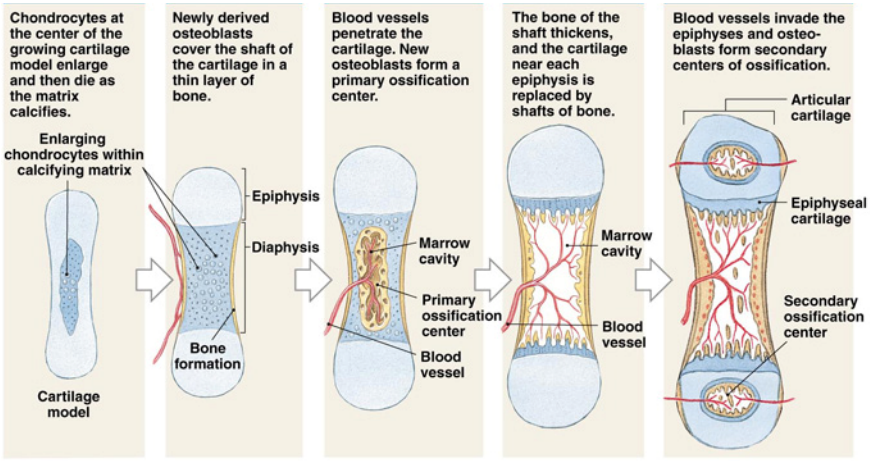
### *1.2.1.3. Chondrocyte Proliferation and Hypertrophy*

Chondrocyte proliferation and hypertrophic differentiation is regulated by a variety of growth factors. Throughout chondrogenesis, the balance of signaling by BMPs and FGFs determines the rate of proliferation, thereby adjusting the pace of differentiation (Minina et al., 2002). Most notable is the Indian Hedgehog/Parathyroid hormone related peptide (PTHrP) negative feedback loop in which PTHrP inhibits hypertrophic differentiation by keeping the chondrocytes in a proliferation competent stage (Kronenberg, 2006) (Fig. 1.2.). Besides its role in the regulation of the onset of chondrocyte hypertrophy, Ihh is a potent

regulator of chondrocyte proliferation (Lai and Mitchell, 2005). Thus, Ihh and PTHrP, by transiently inducing proliferation markers and repressing differentiation markers, function in a temporo-spatial manner to determine the number of cells that remain in the chondrogenic lineage versus those that enter on endochondral ossification pathway. TGFβ is another important growth factor involved in chondrogenesis by stimulating the production of cartilage ECM molecules such as GAGs (Grimaud et al., 2002) and by inhibiting terminal chondrocyte differentiation, blocking therefore endochondral ossification (Aigner et al., 2004; Yang et al., 2001).

The development of long bones from the cartilage anlagen occurs by endochondral ossification, which besides the terminal differentiation of chondrocytes to the hypertrophic phenotype involves cartilage matrix calcification, vascular invasion, and ossification (Ballock and O'Keefe, 2003; Colnot and Helms, 2001; Ferguson et al., 1998) (Fig. 1.3.). This process is initiated when the cells in the central region of the anlage begin to hypertrophy, increasing cellular fluid volume by almost 20 times. Wnt/β-catenin, which is a potent negative regulator of chondrogenesis, Ihh, which is required for endochondral bone formation and synchronizes skeletal angiogenesis with perichondrial maturation (St-Jacques et al., 1999), and BMPs are expressed in prehypertrophic chondrocytes as they exit the proliferative phase, enter the hypertrophic phase, and begin to express the hypertrophic chondrocyte markers collagen type X and alkaline phosphatase. The latter two mediate the initiation of osteoblast differentiation from uncommitted precursors. RunX2, which serves as a positive regulatory factor in chondrocyte maturation to the hypertrophic phenotype (Enomoto et al., 2000), is expressed in the adjacent perichondrium and in prehypertrophic chondrocytes but less in late hypertrophic chondrocytes (Kim et al., 1999), overlapping with Ihh, collagen type X, and BMP-6 (Colnot, 2005).

Other ECM proteins, including osteocalcin and osteopontin, are known to play functional roles in cell-matrix interactions during endochondral ossification. The ECM remodeling that accompanies chondrocyte terminal differentiation is thought to induce an alteration in the environmental stress experienced by hypertrophic chondrocytes, which eventually undergo apoptosis (Ferguson et al., 1998). This indicates that ECM remodeling is the dominant rate-limiting process for chondrocyte hypertrophy, angiogenesis, and osteoblast recruitment during endochondral ossification (Ortega et al., 2004).




Source: Pearson Education, Inc. 2007

**Fig. 1.3.: Endochondral bone formation.** Schematic presentation of subsequent stages of endochondral ossification.

### 1.2.2. Morphology and Functions

Mature human chondrocytes are predominantly round cells located in matrix cavities called *lacunae*. With a mean diameter of 13 µm, the chondrocyte represents the sole constituent cell of cartilage. It is cytoplasmically isolated from neighboring cells, it has no ready access to the vascular system and the tissue is not innervated (Hunziker et al., 2002). The chondrocyte and its pericellular microenvironment together represent the chondron, historically considered the primary structural, functional and metabolic unit of hyaline cartilage (Poole, 1997). Morphologically, each chondron consists of chondrocyte(s) linked at its surface to a transparent pericellular glycocalyx which is confined and enclosed by a fibrillar pericellular capsule (Fig. 1.4.) (Poole et al., 1988).

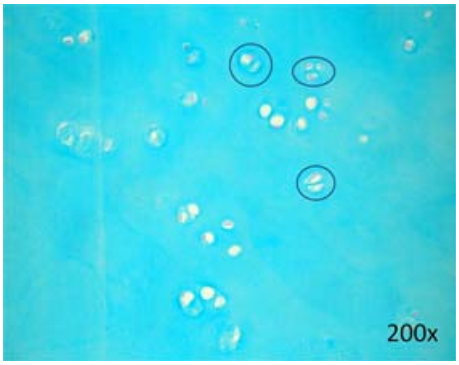



**Fig. 1.4.: Representative image of a cartilage section stained with Alcian blue.** The bright blue is characteristic of an ECM rich in GAGs, in which the chondrons (in circles) are embedded. Remarkable is the lack of intercellular contacts and the low cell to matrix ratio. (Courtesy of C. Brochhausen.)

The prime function of chondrocytes that occupy supporting structures such as articular cartilage, tracheal cartilage and nasal cartilage is to synthesize and maintain an ECM which provides an environment of nutrition diffusion for chondrocytes and provides a structure with biomechanical competence. Chondrocytes have a high endogenous production of ECM in comparison with other cells and are therefore less dependent on extracellular influences than other cell types. The other major function of chondrocytes is in growth, particularly that associated with epiphyseal plates (Archer and Francis-West, 2003).

Because articular cartilage is not vascularized, it must rely on diffusion from the articular surface for nutrient and metabolite exchange. Consequently, the entire metabolism of the cell is geared towards operating at a low oxygen tension (ranging from 10% at the surface to <1% in the deep layers, relative to metabolically active cells) with the majority of the cell's energy requirements coming from glycolysis. As a result, chondrocytes normally do not contain abundant mitochondria and have a very low mitotic activity. Even so, the cells can be remarkably active synthetically (Grimshaw and Mason, 2001; Rajpurohit et al., 1996).

## 1.3. Cartilage Diseases

Diseases of articular joints present a major medical, social and economic burden on the societies of the western world and this will inevitably increase as the proportion of the elderly in the population increases, since ageing is the main risk factor. There are several diseases that can affect cartilage, leading to a disturbance of growth and subsequent ossification of cartilage. Among others, osteochondritis dissecans, rheumatoid arthritis, and osteoarthritis are of major concern, causing a very frustrating baseline for clinicians but also a very motivating one for researchers.

Osteochondritis dissecans (OCD) is a joint condition in which a piece of cartilage, along with a thin layer of the bone beneath it, comes loose from the end of a bone. It is caused by blood deprivation in the subcondral bone. This loss of blood supply causes the subchondral bone to die in a process called avascular necrosis. The bone is then reabsorbed by the body and the articular cartilage is damaged. OCD occurs most often in adolescents and young adults, particularly after an injury to a joint. The knee is most commonly affected, although OCD can occur in other joints (Williams et al., 1998).

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints. The inflammation causes swelling, pain, stiffness and redness; it can also cause inflammation of the tissue around the joints, (tendons, ligaments and muscles) as well as in other organs in the body (Young and Koduri, 2007). It is believed that the tendency to develop rheumatoid arthritis may be genetically inherited. It is also suspected that certain infections or factors in the environment might trigger the activation of the immune system in susceptible individuals (Grassi et al., 1998).

Osteoarthritis (OA) or degenerative arthritis is a type of arthritis that is caused by the breakdown and eventual loss of the cartilage of one or more joints. Among the over 100 different types of arthritis conditions, OA is the most common. It is age-related and occurs more frequently in females (Buckwalter and Mankin, 1998). Most cases of OA have no known cause and are referred to as primary osteoarthritis. When the cause of the OA is known, the condition is referred to as secondary osteoarthritis (Kuettner and Cole, 2005). Primary osteoarthritis is the disease most strongly correlated with ageing. With ageing, the water content of the cartilage increases, and the protein makeup of cartilage degenerates.

In OA, cartilage eventually begins to degenerate by flaking or forming tiny crevasses. In advanced cases, there is a total loss of the cartilage cushion between the bones of the joints (Fig. 1.5.).

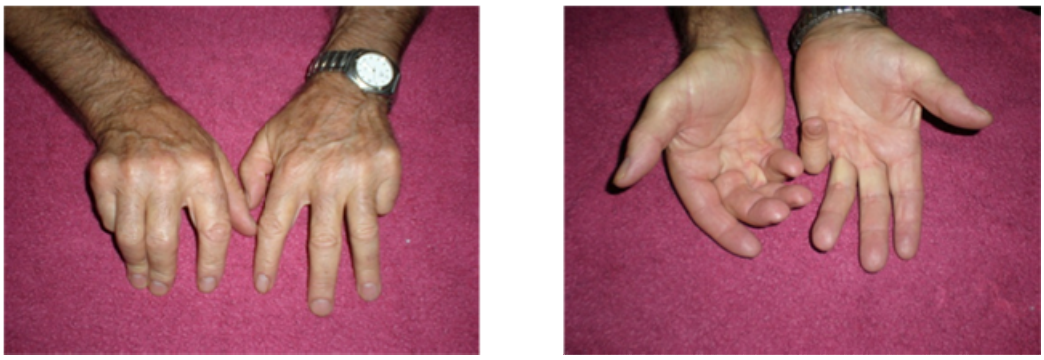


**Fig. 1.5.: Osteoarthritis patient in its late stage.** Swelling and stiffness of the joints are patent in the hands of this patient.

Repetitive use of the worn joints over the years can irritate and inflame the cartilage, causing joint pain, swelling and limitation of joint mobility (van der Kraan and van den Berg, 2008). Inflammation of the cartilage can also stimulate new bone outgrowths (spurs, also

referred to as osteophytes) to form around the joints. Primary OA has a characteristic cellular, molecular and mechanical pattern (Aigner and McKenna, 2002). Excesive catabolism of articular cartilage, while not directly leading to symptoms, results in the release into synovial fluid of matrix breakdown products including chondroitin sulfate and keratan sulfate peptides, proteoglycan fragments, collagen type II peptides, chondrocyte membranes, etc., all of which are antigenic and can elicit an inflammatory response in the synovial membrane. The activated synovial macrophages in the membrane recruit further neutrophils from the circulation, establishing a synovitis and releasing cytokines (IL-1, TNF-α, etc.), $PGE_2$, proteinases (Ghosh, 1999) and reactive oxygen species (superoxide, nitric oxide) (Evans et al., 1996b) into adjacent tissues and the synovial fluid. These mediators in turn can act on chondrocytes and synovial fibroblasts, modifying their biosynthesis of proteoglycans, collagen and hyaluronan as well as promoting release of catabolic mediators (Fig 1.6.) (Aigner and McKenna, 2002; Ghosh and Smith, 2002; Kerin et al., 2002).

Much attention has been given to the accumulation of glycation end products in cartilage (DeGroot, 2004). These are non-enzymatic protein modifications that accumulate with time. Accumulation of glycation products results in increased stiffness of the matrix. It has been suggested that the increased stiffness makes the matrix more brittle and more vulnerable to mechanical stress-induced damage. An additional, non-mechanical, consequence of the accumulation of glycation end products is the activation of receptors for advanced glycation end products (RAGE) on chondrocytes (Loeser et al., 2005).

Formation of glycation end products is associated with reduced chondrocyte function. Activation of RAGEs on chondrocytes results in activation of catabolic pathways and formation of reactive oxygen species (ROS) (DeGroot et al., 2001). Oxidative stress caused by ROS has been suggested to be involved in ageing and OA (Henrotin et al., 2005). Oxidative stress has been demonstrated to accelerate chondrocyte senescence as measured by telomere shortening. ROS have been shown among others to reduce signaling via the IGF-1 receptor and to inhibit mitochondrial function (Studer et al., 2000). The source of the ROS can be the chondrocytes themselves or continuing low grade inflammation in the synovial lining (Zhai et al., 2006). The exposure to ROS can cause reduced responsiveness to IGF-1 in aged chondrocytes (Fortier and Miller, 2006). Loss of responsiveness to IGF-1 results in decreased anabolic function of chondrocytes.

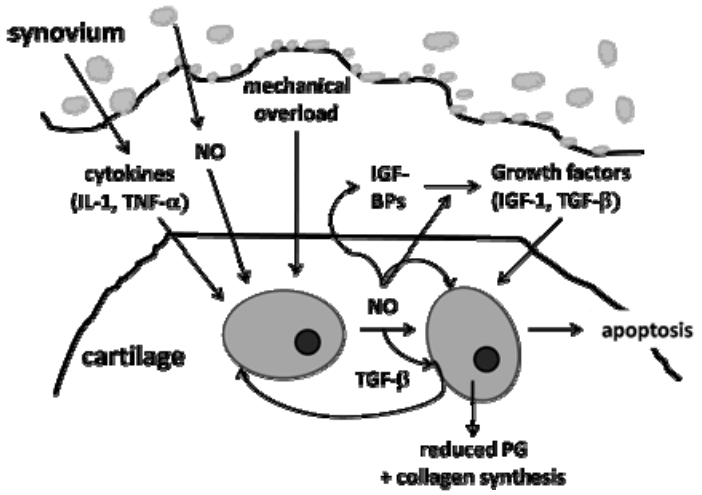



**Fig. 1.6.:** Summary of some of the metabolic pathway responsible for cartilage degradation in OA joints. Several studies have indicated that cytokines (e.g. IL-1, TNF-α) produced by activated synoviocytes act, both directly and indirectly on chondrocytes modifying their gene expression (Studer et al., 2000).

## 1.4. Cartilage Injuries

Cartilage is a very organized structure. This and its limited capacity of repair make it a particularly difficult tissue to restore once it is damaged or lost. Injury to any part of this complex system can disrupt the functional properties of cartilage (Brittberg and Winalski, 2003). Due to the fact that the biomechanical properties surrounding the defect are altered, the remaining cells will be subjected to new mechanical forces that may result in cell death or apoptosis, and the loss of cartilage function may lead to a painful joint with decreased mobility.

Cartilage injuries can be divided into three categories. The repair response of the cartilage tissue distinguishes between these three types of injuries (Brittberg, 1996):

1. The integrity of the articular surface is preserved, but the macromolecular matrix and/or the population of cells and/or the subchondral bone show sign of damage.
2. Partial thickness defect: Chondral fractures or ruptures, sparing the subchondral bone (Fig. 1.7.).
3. Full thickness defect: Osteochondral fractures, breaking through both the cartilage and the subchondral bone.

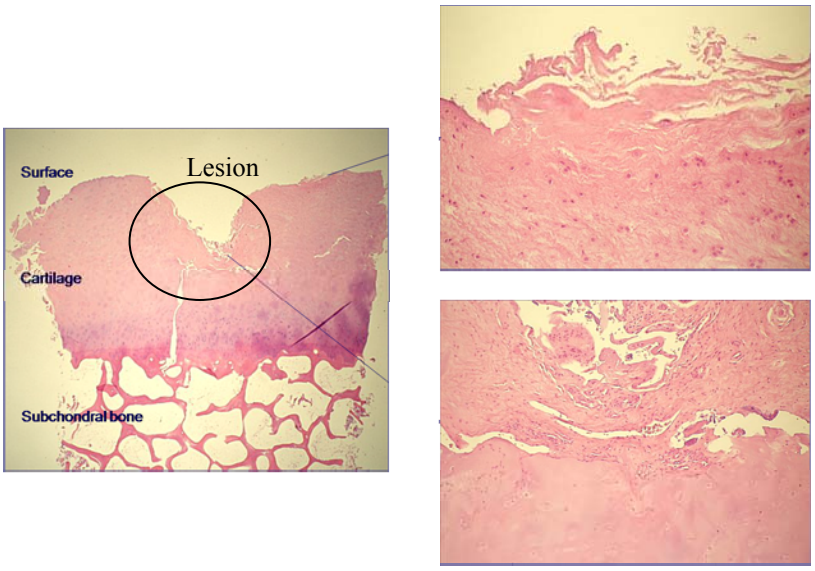



**Fig 1.7.: Histology section of partial thickness defect.** The images show major evidence of deterioration of the superficial tissue sparing the subchondral bone. (Courtesy of C. Brochhausen.)

The typical vascularized tissue response is divided into three phases: necrosis, inflammation and repair. Since cartilage tissue is avascular and cannot respond to an injury with inflammation, which is dependent on the vascular system, no fibrin clot is created and no inflammatory or pluripotent cells can invade the defect area to clean it and subsequently differentiate into cells with a reparative capacity. The reparative task is entirely left to the chondrocytes in the matrix surrounding the defect area (Buckwalter, 1998; Mankin, 1982).

The chondrocytes are thought to be able to repair damage to the macromolecular framework to a certain extent, but it is not evident at what point the amount of damage goes beyond their restorational capability. Larger chondral injuries, including fractures and fissures, cause cell death in the cartilage areas at the margin of the injury. There is a short period of mitotic activity and increased matrix synthesis of the chondrocytes nearest the defect, but they are unable to fill the defect. Injuries that break through both the articular cartilage and subchondral bone reach the vascular system, giving rise to bleeding and a subsequent fibrin clot. The inflammatory and repair phases can now take place and the fibrin clot is remodeled by invading cells from the blood and marrow. The repair tissue formed by the pluripotent cells from the bone-marrow is an intermediate tissue between hyaline cartilage and fibrocartilage, and it has inferior mechanical properties due to its slightly different biochemical content compared to the surrounding hyaline cartilage (Buckwalter, 1998).

Many factors, i.e. epidemiological, biochemical and morphological, are associated with cartilage destruction. Little is known about which chondral lesions will progress to osteoarthritis and which will not (Buckwalter and Mankin, 1998; O'Connor and Brandt, 1993). However, the causes of post-traumatic OA (secondary OA) are probably multifactorial and include the age of the patient, obesity, the site and depth of the lesion, joint laxity and activity grade (Messner and Maletius, 1996).

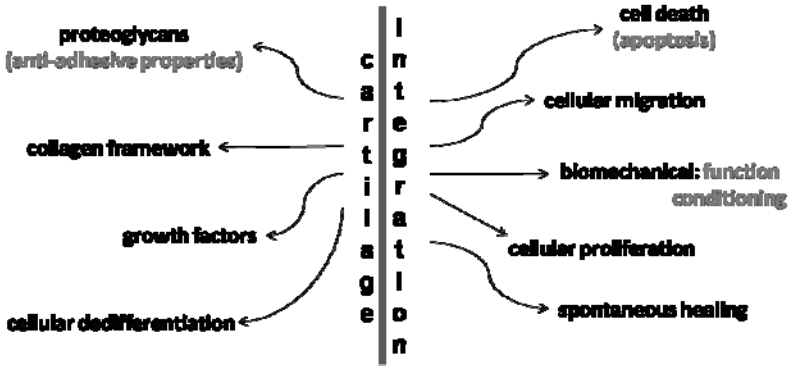



**Fig 1.8.:** Factors known to directly or indirectly affect lateral integrative cartilage repair (Khan et al., 2008)

While there are several techniques for the treatment of cartilage injuries, not enough is known about which lesions need treatment or about the proper treatment modality for a particular lesion (Brittberg and Winalski, 2003; Hunziker, 2002). There are numerous factors that directly and indirectly affect tissue integration (Steinert et al., 2007), *e.g.* the presence of proteoglycans provides an intrinsic barrier for chondrocyte migration to the site of wounding (Hunziker and Kapfinger, 1998); collagen forms the structural framework underpinning the functional capabilities of the tissue, and therefore integration of collagen fibrils is probably a major factor in influencing the success or failure of integrative cartilage repair (DiMicco and Sah, 2001); (Fig. 1.8.). Although the weight of each factor on impeding cartilage fusion may vary, better understanding of their effects may lead to improved strategies to enhance lateral integration following cartilage repair. It is therefore of the utmost importance that we increase our knowledge of cartilage biology, the tissue reaction to trauma and the intrinsic attempts to repair the defects as well as extrinsic methods (Khan et al., 2008).

## 1.5. Treatment of Cartilage Injuries

### 1.5.1. Total Knee Arthoplasty

Osteochondral defects are typically derived from congenital diseases or traumatic events in young patients and from osteoarthritis in old individuals. In both cases the result may be associated pain, joint stiffness and instability, often leading to the replacement of joint functionality with suitable prosthesis.

Knee replacement, or knee arthroplasty, is a surgical procedure to replace the weight-bearing surfaces of the knee joint to relieve the pain and disability of osteoarthritis (Barmada, 1974). The surgery involves exposure of the front of the knee, with detachment of part of the quadriceps muscle from the patella. The patella is displaced to one side of the joint allowing exposure of the distal end of the femur and the proximal end of the tibia. The ends of these bones are then accurately cut to shape using cutting guides oriented to the long axis of the bones. The entire cartilage and anterior cruciate ligament are removed; the posterior cruciate ligament may also be removed but the tibial and fibular collateral ligaments are preserved. Metal components are then impacted onto the bone or fixed using polymethylmethacrylate cement. A round ended implant is used for the femur, mimicking the natural shape of the bone. On the tibia the component is flat, although it often has a stem which goes down inside the bone for further stability. A flattened or slightly dished high density polyethylene disc is then inserted onto the tibial component so that the weight is transferred from metal to plastic, not metal to metal (Fig 1.9.).

The history of knee replacement is the story of continued innovation trying to limit the problems of wear, loosening and loss of range of motion. Nevertheless, it should be considered only when repair strategies have been exhausted (Leopold, 2009).

**Fig 1.9.: Metal knee prosthesis.** These prosthesis are the result of combined efforts of surgeons and engineers combining materials and techniques. Materials are designed to enable movements similar to those of the native joint. Prosthesis components usually consist of two metal components that fit into a plastic component on both sides. Several metals are used; stainless steel, cobalt alloys, chromium and titanium. The plastic component is made of extremely resistant and long lasting polyethylene.

### 1.5.2. Repair Strategies

The goal of the treatment of a cartilage defect is to obtain an outcome that will provide pain relief for a prolonged time and will prevent further deterioration of the joint. It is, however, important to define what we mean by repair of the injured tissue (Redman et al., 2005). Healing a cartilage defect means restoring the structural integrity and function of the damaged tissue. It could entail full restoration of the original tissue or scar formation. Repairing means replacing damaged or lost cells and matrix with new cells and matrix, but the tissue is not necessarily restored to its original function and structure (Brittberg, 1996).

There are two major problems that need to be addressed in the repair of articular cartilage. The first is to fill the defect void with a tissue that has the same mechanical properties as articular cartilage. The second is to promote successful integration between the repair tissue and the native articular cartilage. The different types of treatment that are mentioned below ultimately produce and/or deliver different types of repair cells that are thought to appear as mature chondrocytes producing a more or less differentiated hyaline articular cartilage at the end of their differentiation lineage (Hunziker, 2002; Tyyni and Karlsson, 2000).

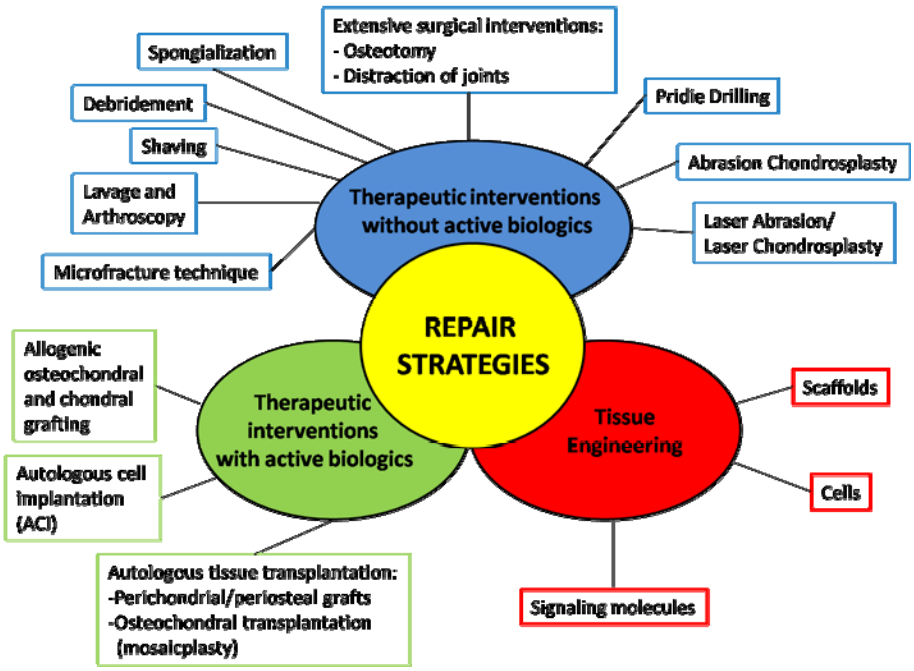



**Fig 1.10: Articular Cartilage Repair Strategies.** The aim of a cartilage repair treatment is to restore the surface of an articular joint's hyaline cartilage. Over the last decades surgeons and researchers have endeavoured to refine surgical cartilage repair interventions. Though these solutions do not perfectly *restore* articular cartilage, some of the latest technologies are beginning to bring very promising results in *repairing* cartilage from traumatic injuries and chondropathies.

The following methods deal mostly with articular cartilage injuries/damage to the femoral, patellar and tibial surfaces of the knee, although articular cartilage damage is found in many other joints.

Clinical efforts to treat articular cartilage injuries can be divided into surgical interventions undertaken in the absence of active biologics, those involving them and tissue engineering (Hunziker, 2002) (Fig. 1.10.):

1. Therapeutic intervention without active biologics:

- Joint lavage was introduced as an empirical approach to treating painful joint conditions involving structural lesions (Ike and Arnold, 1992). In short it is a lavage of the joint with different solutions, using the closed-needle-hole procedure. There exists no solid evidence of any biological or repair activity stimulated by lavage, but it does have beneficial effects for limited periods of time.

- The concept of shaving, abrasion or debridement is to remove the irregularities of the lesion to produce a smooth, stable articular surface (Brittberg, 1996). A post-operative temporary relief in pain is often seen, but no signs of cartilage repair. This procedure was first described by Haggart (1941). Laser abrasion may be used as an alternative to the surgical removal of tissue by mechanical cutting. One of the advantages of employing laser light is that it coagulates the tissue, and it can be used in an aqueous environment (England et al., 1997; Iliev et al., 1997).

- Abrasion chondroplasty (Friedman et al., 1984), as well as Pridie drilling; conceived by Pridie in 1959 (Insall, 1974) and the microfracture technique (Steadman et al., 1999) involve surgical access to the bone-marrow spaces, which together with other vicinal compartments (such as the vascular and perivascular spaces, the bone tissue itself and adipose tissue) are consequently stimulated (Buckwalter and Lohmander, 1994). These interventions essentially lead to a spontaneous repair response, which is based upon therapeutically-induced bleeding from subchondral bone spaces and subsequent blood-clot formation (Bert, 1993; Moseley et al., 1996; Shapiro et al., 1993). A different way of achieving resurfacing via an opening in the subchondral bone marrow cavity is spongialization (Ficat et al., 1979), which is applied chiefly in patellar surgery to treat very localized defects.

- Osteotomy and distraction of joints are both palliative surgical procedures most frequently adopted for the treatment of painful OA. Osteotomy is undertaken with the intention not to induce articular cartilage repair but to relieve pain and to improve alignment as well as biomechanical load transfer in knee joints (Coventry, 1987). On the other hand, joint distraction has been postulated to promote an articular cartilage repair response within the treated joint (van Valburg et al., 2000).

2. Therapeutic intervention with active biologics:

- In the 1970s perichondrial or periosteal tissue was first utilized as an autologous transplantation material for repair induction in articular cartilage defects (Skoog et al., 1972), although the chondrogenic potential and repair-promoting properties of the tissue were recognized as early as 1925 by Haebler *et al*. The biological basis of this transplantation principle lies in the observation that the cambial (*i.e.* germinative) layer of perichondrial or periosteal tissue manifests continuous, life-long, chondrogenic (or osteogenic) activity (Nakahara et al., 1991). Likewise the idea of implanting chondral or osteochondral tissue itself within articular cartilage defects also dates back to the beginning of the last century and this concept still forms the basis of clinical strategies involving both autografts and allografts (Girdler, 1993). In osteochondral transplantation (or mosaicplasty), osteochondral plugs are transferred from an undiseased and non-weight bearing region to a debrided lesion site (Matsusue et al., 1993). The advantages of the technique are that rapid bone healing provides good vertical fixation of osteochondral plugs into the implant site and that relatively large defects can be filled using this technique. However mosaicplasty is associated with much potential collateral damage to joint tissue *i.e.* donor site morbidity and extensive cell death of chondrocytes in the superficial aspect and margins of the osteochondral plugs (Huntley et al., 2005).

- One option to treat focal lesions is autologous chondrocyte implantation (ACI) (Grande et al., 1989). A small cartilage tissue biopsy is taken arthroscopically, chondrocytes are then enzymatically isolated and culture expanded *in vitro* under conditions that maintain the chondrogenic potential of the cells. Subsequently the

expanded cells are harvested and transplanted with fibrin beneath a periosteal flap sutured around the defect (Brittberg, 1999; Brittberg et al., 1994; Brittberg et al., 1996)

- Allogeneic osteochondral grafts have been used for several decades to fill articular cartilage defects. The approach does not attempt to induce a cartilage repair response, but represents a means of substituting failed or lost tissue with healthy articular cartilage, usually derived from deceased donors (Paccola et al., 1979). Although cartilage is generally considered to occupy an immunologically privileged position within the joint, immunological problems are inevitably listed among the disadvantages related to this technique (Langer and Gross, 1974; Langer et al., 1972; Langer et al., 1978; Stevenson, 1987).

The tissue engineering approach to cartilage repair, in which not only cells and scaffolds but also signaling substances are employed for the re-establishment of the tissue, will be treated more extensively in the following section.

### 1.5.3. Tissue Engineering

Hunziker describes tissue engineering as the "art of reconstituting mammalian tissues, both structurally and functionally" (Hunziker, 2002). Cells and/or bioactive molecules are delivered to a defect via a biomaterial scaffold to achieve tissue regeneration. Tissue engineering is an interdisciplinary field that involves cell biology, material science, reactor engineering, and clinical research with the goal of creating new tissues and organs; in most cases, it attempts to recapitulate certain aspects of normal development to stimulate cell differentiation and organization into functional tissue assembly (Lavik and Langer, 2004). Much progress has been made in tissue engineering over the past two decades; these advances have led to clinical successes for simple tissues such as skin and cartilage. However, despite such successes there are clear challenges that remain (Langer, 2007).

Since the aim of the present study is focused on tissue engineering of articular cartilage, the following will detail the key concepts and state of the art in each area of this field *i.e.* scaffolds, cells and signaling molecules (Fig 1.10.).

### *1.5.3.1. Scaffolds*

The purpose of the scaffold is to provide the repair cells with a three-dimensional (3-D) support and protection, and it should also act as a template for the developing repair tissue, thus contributing to the phenotypic stability of the chondrocytes (Rotter et al., 2007). Scaffolds follow basic principles: The surface should permit cell adhesion and growth; they must be biocompatible; structurally and mechanically stable; must support the loading of an appropriate cell source to allow successful infiltration and attachment in conjuction with appropriate bioactive molecules in order to promote cellular differentiation and maturation; the porosity must be at least 90% in order to provide a high surface area for cell-scaffold interactions; there must be sufficient space for matrix regeneration and minimal diffusional constraints during *in vitro* culture; the scaffold must resorb once it has served its purpose of providing a template for the regenerating tissue and the resorption rate must be adjustable to match the rate of tissue regeneration (Freed et al., 1994; Redman et al., 2005).

| **Table I: Classes of scaffolds** (Stoop, 2008; Tuli et al., 2003) | |
|---|---|
| **Natural materials** | Fibrin<br>Fibroin<br>Collagen<br>Gelatin<br>Hyaluronic acid<br>Chondroitin sulfate<br>Agarose<br>Alginate<br>Chitosan<br>Cellulose |
| **Synthetic materials** | Polylactic acid (PLA)<br>Polyglycolic acid (PGA)<br>Poly(lactic-co-glycocolic) acid (PLGA)<br>Dacron (polyethylene terephthalates)<br>Teflon (polytetrafluoroethylene)<br>Carbon fibers<br>Polyesterurethane<br>Polybutiric acid<br>Polyethylmethacrylate<br>Hydroxyapatite |

Scaffolds are likely to be advantegous in that they assume the following functions: They deliver the repair materials to the site of the injury; remain in place long enough to effect repair; provide an even distribution of implanted cells; and allow local delivery of polypeptides or chemical molecules that stimulate repair (Martin et al., 2007). It has been demonstrated that chondrocytes seeded in collagen 3-D matrices, in either gel form or porous sponge form, could maintain their phenotype and synthesize cartilage-specific matrix components such as collagen type II and aggrecan (Lu et al., 2001; Vinatier et al., 2009).

Scaffolds can be broadly categorized according to their chemical nature into natural and artificial materials and different combinations of these (Table I).

The mentioned materials have been used to design and produce scaffolds in a rich variety of configurations including woven and non-woven meshes, sponges, foams, hydrogels, glues, composite bilayer and trilayer hybrid solutions, and electrospun nanofibres (Li et al., 2006; Tuli et al., 2003). The assumption that natural substances provide superior templates for cartilage repair of defects is unfounded; they can also cause inflammatory or degradative responses, although the use of autologous biomaterials such as plasma-derived fibrin can prevent this effect (Munirah et al., 2007).

Hydrogels are an appealing scaffold material because they are structurally similar to the ECM of articular cartilage, can often be processed under relatively mild conditions, and may be delivered in a minimally invasive manner (Noth et al., 2007). Synthetic hydrogels have been extensively used since their chemistry and properties are controllable and reproducible. The above material requirements in turn determine gel formation dynamics, crosslinking density, and material mechanical and degradation properties (Drury and Mooney, 2003). Naturally derived hydrogels forming polymers have frequently been used in tissue engineering applications because they are either components of or have macromolecular properties similar to the natural ECM (Lee et al., 2007; Wu et al., 2007). Hydrogels have a wide application range within the field of both soft and hard tissue engineering (Dubruel et al., 2007; Yamaoka et al., 2006). Some also have been shown to interact in a favorable manner *in vivo* (Lee and Mooney, 2001).

Hyaluronic acid and collagen-based scaffolds are among the most popular natural scaffolds as they offer a substrate that is naturally present in the structure of native articular cartilage. Hyaluronic acid is found in nearly every mammalian tissue and fluid. It is especially prevalent during wound healing and in the synovial fluid of joints. Its natural degradation by hyaluronidase allows the cells present in the engineered tissue to regulate the clearance of the material in a localized manner (Alberts B., 2003). Because of its abundance, ubiquity and biocompatibility, collagen type I is the most attractive material for biomedical applications (Glowacki and Mizuno, 2008; Zehbe et al., 2005). Collagen is naturally degraded by metalloproteases, specifically collagenase and serine proteases (Alberts B., 2003), again allowing a locally controlled degradation.

General acceptance of tissue engineering in the treatment of patients requires more research work due to several persisting problems, such as application-specific scaffold design, complexities of cell isolation and delivery, and complex implantation constraints. The progress made in material science, cell biology and mechanobiology will be key to supporting advances in this challenging field (Rotter et al., 2007; Swieszkowski et al., 2007).

#### *1.5.3.2. Cells*

It is unclear which cell type is optimal for articular cartilage tissue engineering. Chondrocytes are the predominant cells but their difficult isolation and growth outside their matrix housing has been researched for decades in hope of better understanding of this resilient cell and exploiting its function for therapeutic gain (Vinatier et al., 2009). Monolayer culture of chondrocytes is employed extensively, but fails to maintain the chondrogenic phenotype during long-term culture (Bonaventure et al., 1994; Lin et al., 2006). Other chondrocyte culture techniques, such as 3-D culture, pellet culture, and bioreactor culture, have been created to obtain large amounts of cultured chondrocytes with a well-maintained phenotype; enabling therefore ACI applications.

Stem cells have the "capacity for self-renewal or unlimited self renewal under controlled conditions and they retain the potential to differentiate into a variety of more specialized cell types" (Ryan et al., 2005). Therefore, these are cells with multipotent differentiation capacity. There are a number of stem cell and stem cell-like sources, of which embryonic stem cells and induced pluripotent stem cells have recently gained most attention. However, it is the adult mesenchymal stem cell (MSC) that is of most interest in articular cartilage repair (Awad et al., 2004; Guilak et al., 2004; Heng et al., 2004; Koelling and Miosge, 2009). Application of MSCs in cartilage tissue engineering requires well defined and efficient protocols for directing the differentiation of the stem cells into the chondrogenic lineage (Heymer et al., 2008; Oliveira et al., 2006; Yang et al., 2009), followed by their selective purification and proliferation *in vitro*.

Regenerating tissues comprising multiple cell types with specific and complex organization is a major goal of tissue-engineering studies. It has been reported that tissues composed of multiple different cell types of cells can be regenerated by transplanting the various cell population together on polymer scaffolds (Humes et al., 1999; Oberpenning et al.,

1999). Several studies on osteoblast and chondrocyte co-cultures have been carried out revealing cell-cell contact and paracrine interactions (Jiang et al., 2005; Mo et al., 2009; Nakaoka et al., 2006) and how these modulate cell phenotypes. Furthermore, co-cultures with stem cells have also revealed strategies for enhancing chondrocytic phenotype (Hildner et al., 2009).

#### *1.5.3.3. Signaling Molecules*

Growth and homeostasis of cartilage tissue during embryogenesis, postnatal development and adulthood are governed by a significant number of humoral factors. These factors may also be used during intrinsic or artificial repair and induced regeneration. Paracrine components can be delivered through typical nutrient supply mechanisms (Gaissmaier et al., 2008). With regard to cell-based therapies for articular cartilage regeneration, it is widely thought that exposure of chondrogenic cells (chondrocytes, periosteal/perichondrial cells, MSCs) to specific stimuli that promote chondrogenic differentiation and maintenance of the chondrocyte phenotype can significantly enhance the repair potential of this type of procedure and improve the clinical outcome (Steinert et al., 2007).

The ability to combine growth factors/signaling molecules with cells and scaffolds to produce more phenotypically suitable tissue-engineered constructs, is an exciting prospect (Pei et al., 2002; Stoop, 2008). A number of different growth factors and signaling molecules have been demonstrated to have an impact on articular cartilage repair (Table II), but it is how these are used that holds the key for tissue regeneration (Liang et al., 2006; Martin and Buckwalter, 2000; Noth et al., 2007; Yun and Moon, 2008).

Prostaglandins are naturally occurring eicosanoids that are derived from arachidonic acid metabolism. They act as important regulators in a number of tissues, including cartilage and bone. $PGE_2$ is the most abundantly produced prostaglandin and is of utmost importance in skeletal tissues (O'Keefe et al., 1992). Numerous studies have implicated $PGE_2$ in limb cartilage formation (Biddulph et al., 2000; Kosher and Walker, 1983) and chondrocyte differentiation (Miyamoto et al., 2003). However, the precise action and signal transduction pathways of $PGE_2$ are largely unknown. $PGE_2$ exerts its effects through its interactions with specific cell surface receptors, which are members of the G-protein-coupled receptor family.

To date four subtypes of the prostaglandin E receptor (EP) are known, *i.e.* EP1, EP2, EP3 and EP4. EP1 is coupled to intracellular $Ca^{2+}$ mobilization (Katoh et al., 1995), EP2 and EP4 increase intracellular the cAMP concentration (an essential second messenger for the action of $PGE_2$ in chondrocyte differentiation), (Honda et al., 1993; Regan et al., 1994), and EP3 inhibits intracellular cAMP accumulation (Sugimoto et al., 1992). It is conceivable that this diversity in signal transduction through receptors is one reason why effects of $PGE_2$ are not well understood. Many studies have been published on the effects of $PGE_2$ on arthritic cartilage; several studies have reported that $PGE_2$ also makes an important contribution to cartilage formation and matrix synthesis in growth plate chondrocytes (O'Keefe et al., 1992; Schwartz et al., 1998). Sylvia et al. have shown that the EP1 receptor mediates various $PGE_2$-induced cellular responses in chondrocytes derived from rat costochondral cartilage leading to decreased proliferation and enhanced differentiation (Sylvia et al., 2001). Furthermore, EP2 and EP4 receptors are shown to be required for $PGE_2$-dependent chondrocyte differentiation (Miyamoto et al., 2003).

| **Table II: Factors that promote chondrogenesis** (Heng et al., 2004) | |
|---|---|
| **Cytokines/Growth Factors** | Various isoforms of TGF-β<br>Various isoforms of BMP<br>Activin<br>Osteogenic protein-1<br>Growth differentiation factor-5 (GDF-5)<br>Fibroblast growth factor-2<br>Insulin-like growth factor-1<br>Prolactin<br>Interleukin-1β<br>Growth hormone |
| **Nonproteinaceous Chemical Factors** | Prostaglandin E2<br>Thyroid hormone<br>1,25-dihydroxy vitamin D<br>Ascorbic acid<br>Dexamethasone<br>Ethanol<br>Staurosporine<br>Dibutryl cAMP<br>Concanavalin A |

An additional factor used in the attempt of promoting chondrogenesis in the present study was bone morphogenetic protein-1. BMP-1 was isolated in the latter half of the 1980s from osteogenic fragments of bone and was shown to induce bone and cartilage development *in vivo* (Wozney et al., 1988). Unlike other BMPs, BMP-1 does not belong to the TGFβ superfamily. It is a zinc protease of the astacin family of proteins (Bond and Beynon, 1995) and is also known as procollagen C-proteinase (PCP). BMP-1/PCP cleaves the C-terminal propeptides of procollagen I, II, and III, and its activity is increased by the

procollagen C endopeptidase enhancer protein, playing therefore a key role in formation of ECM by converting precursor proteins into their mature and functional forms (Suzuki et al., 1996). The precursor proteins identified as substrates for BMP-1/PCP include collagens, biglycan, laminin 5, dentin matrix protein-1, and lysyl oxidase (Steiglitz et al., 2004). Hence, the C-proteinase seems to orchestrate the assembly of the ECM and certain patterning events. BMP-1-like proteinases also affect morphogenesis by activating BMP-2 and -4 via cleavage of the extracellular antagonist Chordin and thus regulate patterning (Pappano et al., 2003; Piccolo et al., 1997).

Because BMP-1 is potently induced by TGFβ-1, its role in TGFβ-1 activation completes an amplification loop in vertebrate tissue remodeling (Ge and Greenspan, 2006; Lee et al., 1997). This loop, illustrated in Fig. 1.11., is likely to feature in various morphogenetic events in which both BMP-1-like proteinases and TGFβ have been implicated as key players.

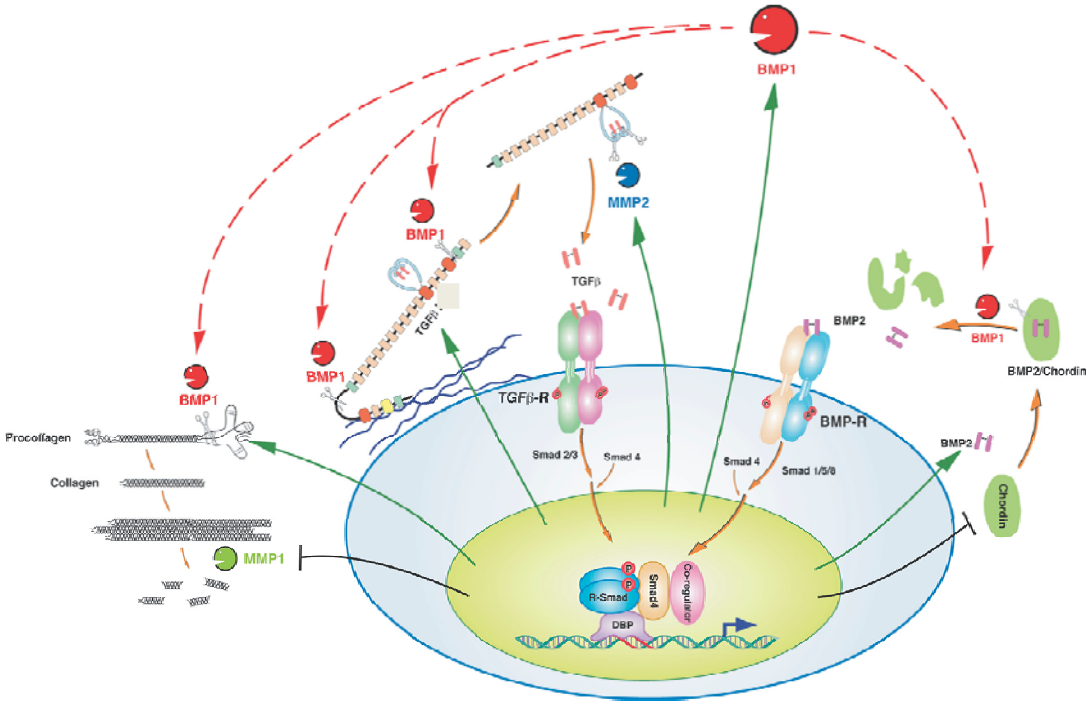



**Fig. 1.11.: Manifold roles for BMP-1-like proteinases and the BMP-1/TGFβ feed-forward loop for tissue remodeling from. Ge, G. and D. S. Greenspan (2006).** BMP-1-like proteinases biosynthetically process ECM precursors (*e.g.*, procollagen) to mature functional ECM components. They also activate TGFβ by MMP-2; activated TGFβ induces activation of R-Smad2 and -3, which combine with Smad4 for translocation to the nucleus and up-regulation (vertical arrows) of BMP-1, ECM precursors, MMP-2 and TGFβ itself. TGFβ also down-regulates some MMPs that degrade ECM (*e.g.*, MMP-1). BMP-1-like proteinases also activate BMP-2/-4 by cleaving antagonist Chordin, thus inducing activation of R-Smad1, -5, and -8.

MMP-2 and various other MMPs are capable of playing key roles in the tissue remodeling associated with growth and angiogenesis (Sternlicht and Werb, 2001; Yu and Stamenkovic, 2000). Thus, the fast-forward loop involving activation of TGFβ by TGFβ-inducible BMP-1, with subsequent roles played by MMPs, is of potential importance to tissue remodeling.

Finally, as mentioned above, BMP-1-like proteinases are also responsible for activating BMP-2 and -4 via cleavage of Chordin (Pappano et al., 2003; Piccolo et al., 1997). Thus they may serve to orchestrate signaling by these two morphogenetic TGFβ superfamily ligands and perhaps contribute to coordination between R-Smad2/3 and R-Smad1/5/8 signaling pathways, used by TGFβ and BMP-2/-4, respectively (Massague et al., 2005).

#### *1.5.3.4. Future Perspectives*

As described above, a number of factors have been identified as having positive effects on cartilage regeneration. Gene transfer offers an alternative approach to protein delivery that may satisfactorily overcome limitations of conventional methods such as exogenous addition or scaffold-mediated delivery (Trippel et al., 2004). The concept of gene therapy was first described by Evans *et al*. in 1996 (Evans et al., 1996a). It involves the delivery of complementary DNA (cDNA) encoding a growth factor or signaling molecule, into target cells which then produce that factor *in vivo*. A vector is used to carry the cDNA. Direct application of the vectors involves, for example, injection into the joint and transfecting synovial cells or chondrocytes *in vivo*. This is fairly easy and inexpensive, but it is more difficult to control transfection and there are potential safety issues (Steinert et al., 2008). Indirect transfer involves transfection *ex vivo*, then re-implantation of tissue-engineered grafts containing gene-enhanced cells (Madry et al., 2005). A variety of cDNAs have been cloned which may be used to stimulate biological processes that could improve cartilage healing by (i) inducing mitosis and the synthesis and deposition of cartilage ECM components by chondrocytes (ii) induction of chondrogenensis by MSCs or (iii) inhibiting cellular responses to inflammatory stimuli. The challenge is to adapt this technology into a useful clinical treatment (Trippel et al., 2004).

Since chondrocytes are the native cell of articular cartilage, they would seem a logical target for genetic modification. However, because of the dense ECM that surrounds these

cells, they have typically been unavailable for genetic modification by direct intra-articular injection of most recombinant vectors (Ghivizzani et al., 2001). Because articular cartilage is not innervated and does not signal distress, damage, or deterioration in the early phases of disease, an additional complication to accomplish genetic modification is that there is often considerable articular cartilage substance loss by the time a problem is recognized; therefore pharmaceutical agents delivered by gene transfer will have difficulty in activating the remaining cells to restore the lost tissue. Thus, in many cases, successful cartilage repair will likely require administration of supplemental cells. This, coupled with the inability to specifically target gene delivery to chondrocytes *in situ*, has caused many laboratories to focus attention to *ex vivo* approaches. Although laborious, this approach satisfies important criteria; it targets transgenic expression specifically to the site of repair and it enables analysis of cells post-modification for levels of transgene expression and for potential safety issues prior to delivery (Trippel et al., 2004).

## 1.6. Aim of the Study

Cartilage degeneration due to primary OA or from trauma, resulting in cartilage loss is a major healthcare problem affecting an increasing number of people. Attempts to repair or regenerate cartilage and reduce the debilitating effects of this condition have, to date, been limited. Thus the socio-economic consequences remain considerable.

The objective of the present investigation was to study different methods to promote chondrogenic differentiation *in vitro*; with emphasis on cell-based stimulation of human articular chondrocytes (HACs) with the use of specific signaling molecules with or without scaffolds.

The following aspects were examined:

1. Characterization of the composition of hyaline cartilage with emphasis on cells, types of collagen and proteoglycans (ECM components).
2. The role of exogenous stimulation with signaling molecules – $PGE_2$ and BMP-1 – within HAC differentiation.
3. The effects of collagen-based biomaterials on HAC proliferation and differentiation.
4. The responses of HACs in co-culture systems with stem cell-like cells (murine preosteoblastic KS483 cells) or osteosarcoma Cal 72 cell line; closely related to normal osteoblasts.

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Stains

| **Weigert's Haematoxylin** | | |
|---|---|---|
| Weigert A | 10 g Haematoxylin in 96% EtOH | 50 ml |
| Weigert B | 40 ml 29% $FeCl_2$ in 250 ml $H_2O$ + 10 ml HCl | 50 ml |

| **Alcian blue pH 1.0** | Alcian blue | 1 g |
|---|---|---|
| | $H_2O$ | 90 ml |
| | HCl 1 N | 10 ml |

| **Neutral red stain** | Neutral red | 1 g |
|---|---|---|
| | $H_2O$ | 100 ml |
| | $CH_3COOH$ | 0.1 ml |

### 2.1.2. Buffers and Solutions

| **Tris/EDTA Stock Buffer** | 0.372 g EDTA |
|---|---|
| | 950 ml 0.05 M Tris –HCl pH 7.6 |
| | Adjusted to 1000 ml with $H_2O$ |

| **Collagen I Gel Reconstitution Buffer** | $H_2O$ | 1065 µl |
|---|---|---|
| | 7,5% Stock $NaHCO_3$ | 735 µl |
| | Hepes 200 mM | 500 µl |
| | NaOH 2 M | 200 µl |

| SDS-Resolving gel | | 12.5% |
|---|---|---|
| | $H_2O$ | 5.1 ml |
| | 1.5 M Tris-HCl pH 8.8 | 3 ml |
| | 20% SDS | 60 µl |
| | Acrylamide/Bisacrylamide | 3.6 ml |
| | TEMED | 6 µl |
| | 10% APS | 60 µl |

| SDS-Stacking gel | $H_2O$ | 3.3 ml |
|---|---|---|
| | 1 M Tris-HCl pH 6.8 | 0.57 ml |
| | 20% SDS | 22.5 µl |
| | Acrylamide/Bisacrylamide | 0.57 ml |
| | TEMED | 4.5 µl |
| | 10% APS | 45 µl |

| Laemmli Stock for PAGE | 30 g Tris |
|---|---|
| | 144 g Glycine |
| | Adjusted to 1 l with $H_2O$ |

| PAGE Running buffer | 200 ml Laemmli Stock Solution |
|---|---|
| | 5 ml 20% SDS |
| | Adjusted to 1 l with $H_2O$ |

| PAGE Transfer buffer | 100 ml Laemmli Stock |
|---|---|
| | 250 ml MetOH |
| | Adjusted to 1 l with $H_2O$ |

### 2.1.3. Media

| SOB Medium | Reagents (per liter) | |
|---|---|---|
| | Tryptone | 20 g |
| | Yeast extract | 5 g |
| | NaCl | 0.5 g |
| | Adjusted to 1 l with $H_2O$ and autoclaved | |
| | $MgCl_2$ 1 M (filter-sterilized) | 10 ml |
| | $MgSO_4$ 1 M (filter-sterilized) | 10 ml |

| SOC Medium | Reagents (per 100 ml) | |
|---|---|---|
| | Glucose 2 M (filter-sterilized) | 1 ml |
| | SOB medium | 99 ml |

| LB Medium | Reagents (per liter) | |
|---|---|---|
| | Tryptone | 10 g |
| | Yeast extract | 5 g |
| | NaCl | 10 g |
| | Adjusted to 1 l with $H_2O$ and autoclaved | |
| | Cooled to 55 $^{o}$C | |
| | Kanamycin 10 mg/ml (filter-sterilized) | |

| LB-Kanamycin Agar | Reagents (per liter) | |
|---|---|---|
| | Tryptone | 10 g |
| | Yeast extract | 5 g |
| | NaCl | 10 g |
| | Agar | 20 g |
| | Adjusted to 1 l with $H_2O$ and autoclaved | |
| | Cooled to 55 $^{o}$C | |
| | Kanamycin 10 mg/ml (filter-sterilized) | 10 ml |

### 2.1.4. Instruments

| Instrument | Model | Supplier |
|---|---|---|
| Analytical balance | A120S | Sartorius, Goettingen, Germany |
| Autoclave oven | Vapoklav | Thermo Scientific, Portsmouth, UK |
| Balance | LC420 | Sartorius, Goettingen, Germany |
| Camera | DC 300 F | Leica, Wetzlar, Germany |
| Camera | CAMEDIA C-740 | Olympus, Hamburg, Germany |
| Centrifuge | Varifuge 3.2 RS | Heraeus, Hanau, Germany |
| $CO_2$ incubator | $CO_2$-Auto-Zero | Heraeus, Hanau, Germany |
| Confocal microscope | DM RE | Leica, Wetzlar, Germany |
| Cooling centrifuge | 5403 | Eppendorf, Hamburg, Germany |
| Deep freezer | Hera freeze | Heraeus, Hanau, Germany |

| | | |
|---|---|---|
| Electrophoresis and transfer apparatus | Mini-PROTEAN | Bio-Rad, Hercules, US-CA |
| Electroporator | Multiporator | Eppendorf, Hamburg, Germany |
| Fluorescent microplate reader | GENios Plus | TECAN, Crailsheim, Germany |
| Fluorescence microscope | DM RX | Leica, Wetzlar, Germany |
| Freezing container | Cryo 1°C | Nalgene, Rochester, US-NY |
| Gas chromatograph/tandem mass spectrometer | TSQ 700 | Thermo Finnigan MAT GmbH, Bremen, Germany |
| Haematocytometer | Neubauer | Marienfeld, Lauda Koenigshofen, Germany |
| Heating block | Dri-Block DB-20 | Techne, Burlington, US-VT |
| Heating block | Thermomixer 5436 | Eppendorf, Hamburg, Germany |
| Inverted fluorescent microscope | DM IRBE | Leica, Wetzlar, Germany |
| Inverted microscope | DM IRB | Leica, Wetzlar, Germany |
| Laminar flow hood | HeraSafe | Heraeus, Hanau, Germany |
| Liquid nitrogen tank | MVE Cryosystem 6000 | German-Cryo, Jüchen, Germany |
| Magnet | MPC-1 | Dynal, Hamburg |
| Magnetic stirrer | IKAMAG RET-GS | IKA-Werke, Staufen |
| Microcentrifuge | SD | Roth, Karlsruhe, Germany |
| Microcentrifuge | Biofuge Pico | Heraeus, Hanau, Germany |
| Microplate reader | Multiscan Plus MK II | Titerscan, Huntsville, US-AL |
| Microtome | HM 355 S | Microm, Walldorf, Germany |
| NanoDrop | ND-1000 | NanoDrop, Wilmington, US-DE |
| pH meter | InoLab 730 | WTW, Weilheim, Germany |
| Plate centrifuge | Biofuge Stratos | Heraeus, Hanau, Germany |
| Power supply | PowerPac HC | Bio-Rad, Hercules, US-CA |
| Real Time PCR Cycler | 7300 | Applied Biosystems, Foster City, US-CA |
| Roll-mixer | Assistent RM5 | Assistent, Sondheim, Germany |
| Shaker | Reax3 | Heidolph, Schwabach, Germany |
| Shaker with regulated temperature | Unimax 1010 with Incubator 1000 | Heidolph, Schwabach, Germany |
| Slide warmer | SW85 | Adamas Instrumenten, Rhenen, The Netherlands |
| Thermocycler | GenAmp PCR System 9700 | Applied Biosystems, Foster City, US-CA |

| | | |
|---|---|---|
| Transfection System | Neon | Invitrogen, Carlsbad, US-CA |
| UV light box | 254 nm | Bachofer, Reutlingen, Germany |
| Vortex | | VWR International, Darmstadt, Germany |
| Water bath | SW-20C | Julabo, Seelbach, Germany |

### 2.1.5. Consumables

| Consumable | Supplier |
|---|---|
| 1,5 ml, 2 ml centrifuge tubes | Eppendorf, Hamburg, Germany |
| 12-well cell culture plates | Greiner bio-one, Kremsmünster, Austria |
| 15 ml, 50 ml centrifuge tubes | BD Falcon, San Jose, US-CA |
| 24-transwell cell culture plates | Corning, Lowell, US-MA |
| 25 $cm^2$, 75 $cm^2$ cell culture flasks | TPP, Trasadingen, Switzerland |
| 48, 24, 6-well cell culture plates | TPP, Trasadingen, Switzerland |
| 96-well cell culture plates | Nunc, Roskilde, Denmark |
| Adhesive seal applicator | Applied Biosystems, Foster City, US-CA |
| Cell scraper | BD Falcon, San Jose, US-CA |
| Cell Strainer 40 µm and 100 µm Nylon | BD Falcon, San Jose, US-CA |
| Cover slips Ø15 mm, 18 mm, 24x60 mm | Roth, Karlsruhe, Germany |
| Cryovials | Nalgene, Rochester, US-NY |
| Electroporation cuvettes | Eppendorf, Hamburg, Germany |
| Electroporation cuvettes | Invitrogene, Carlsbad, US-CA |
| Film development cassette | Appligene, Heidelberg, Germany |
| Gelmount mounting media | Biomedia, Foster City, US-CA |
| Hyperfilm, ECL | Amersham Pharmacia Biotech, Freiburg, Germany |
| Lab-Tek II CC2 Glass Chamber Slide | Nunc, Roskilde, Denmark |
| Microscope slides | Mentel, Braunschweig, Germany |
| Ø 3.5 cm, Ø6 cm, Ø10 cm cell culture dishes | Greiner bio-one, Kremsmünster, Austria |
| Optical adhesive film | Applied Biosystems, Foster City, US-CA |
| Protran Nitrocellulose Transfer Membrane | Schleicher & Schuell, Dassel, Germany |
| Real-time PCR optical 96-well reaction plates | Applied Biosystems, Foster City, US-CA |
| Scalpels | Braun, Tuttlingen, Germany |
| Syringe filters | Nalgene, Rochester, US-NY |

### 2.1.6. Kits

| Kit | Supplier |
|---|---|
| CyQUANT Cell Proliferation Assay | Invitrogen, Carlsbad, US-CA |
| EndoFree Plasmid Maxi Kit | Qiagen, Hilden, Germany |
| GeneJET Plasmid Miniprep Kit | Fermentas, Glen Burnie, US-MD |
| Human TGF-β1 DuoSet ELISA | R&D Systems, Wiesbaden, Germany |
| iScript cDNA Synthesis Kit | Biorad, Hercules, US-CA |
| MinElute Reaction Cleanup Kit | Qiagen, Hilden, Germany |
| QIAquick Gel Extraction Kit | Qiagen, Hilden, Germany |
| RNeasy Micro Kit | Qiagen, Hilden, Germany |

### 2.1.7. Antibodies

| Antibody | Source | Dilution | Supplier |
|---|---|---|---|
| AlexaFluor 546 anti-mouse IgG | goat | 1:1000 | MoBiTec, Goettingen, Germany |
| Anti-PCNA | mouse | 1:100 | DakoCytomation, Glostrup, Denmark |
| Anti-BMP-1 | rat | 1:500 | R&D Systems, Wiesbaden, Germany |
| Anti-GFP | rabbit | 1:200 | Santa Cruz Biotechnology, Santa Cruz, US-CA |
| Anti-rat IgG, HRP linked | donkey | 1:3000 | Amersham Pharmacia Biotech, Freiburg, Germany |
| Anti-rabbit, IgG HRP linked | goat | 1:5000 | Jackson ImmunoResearch, Suffolk, US-NY |

### 2.1.8. Primers

#### *2.1.8.1. RT-PCR Primers*

| Primer | Sequence |
|---|---|
| Aggrecan 3' | 5'-TGAGGAGGGCTGGAACAAGTACC-3' |
| Aggrecan 5' | 5'-GGAGGTGGTAATTGCAGGGAACA-3' |
| BMP-1 3' | 5'-CTGTGAGTGGGTCATTGTGG-3' |
| BMP-1 5' | 5'-GGTGTCATCCGAGTGGGAACT-3' |
| Col I 3' | 5'-TGACGAGACCAAGAACTG-3' |
| Col I 5' | 5'-CCATCCAAACCAGTGAAACC-3' |
| Col II 3' | 5'-CAACACTGCCAACGTCCAGAT-3' |

| | |
|---|---|
| Col II 5' | 5'-CTGCTTCGTCCAGATAGGCAAT-3' |
| Col X 3' | 5'-AATGCCCACAGGCATAAAAG-3' |
| Col X 5' | 5'-AGGACTTCCGTAGCCTGGTT-3' |
| Nhe I – BMP-1 3' | 5'-GACTACTGATACTAATCAG**GCTAGC**ATGCCCG-3' |
| BMP-1 – Sal I 5' | 5'-GACTACTGATACTAATCAG**GTCGAC**AGCTTAGTGAT-3' |
| Sox 5 3' | 5'-AGGGACTCCCGAGAGCTTAG-3' |
| Sox 5 5' | 5'-CTGTTGCTGGAGCAAATTGA-3' |
| Sox 6 3' | 5'-CCAATTGTGTTTGCCTGTTG-3' |
| Sox 6 5' | 5'-GCAAGTGGACCCTTGACATT-3' |
| Sox9 3' | 5'-TACGACTACACCGACCACCA-3' |
| Sox9 5' | 5'-CTCCTCAAGGTCGAGTGAGC-3' |
| Neomycin 5' | 5'-TGAATGAACTGCAGGACGAG-3' |
| Neomycin 3' | 5'-ATACTTTCTCGGCAGGAGCA-3' |

### *2.1.8.2. Quantitative real-time RT-PCR*

| **Primer** | **Sequence** |
|---|---|
| Aggrecan 3' | 5'-TCGAGGACAGCGAGGCC -3' |
| Aggrecan 5' | 5-'TCGAGGGTGTAGCGTGTAGAGA-3' |
| ALP 3' | 5'-CCGTGGCAACTCTATCTTTGG -3' |
| ALP 5' | 5'-CATGCTGAGTGACACAGACAAGAAGCCC -3' |
| Col I 3' | 5'-GTCACCCACCGACCAAGAAACC-3' |
| Col I 5' | 5'-AAGTCCAGGCTGTCCAGGGATG-3' |
| Col II 3' | 5'-CGTCCAGATGACCTTCCTACG-3' |
| Col II 5' | 5'-TGAGCAGGGCCTTCTTGAG-3' |
| Col X 3' | 5'-GCAACTAAGGGCCTCAATGG-3' |
| Col X 5' | 5'-CTCAGGCATGACTGCTTGAC-3' |
| GAPDH 3' | 5'-CGCTCTCTGCTCCTCCTGTT-3' |
| GAPDH 5' | 5'-CCATGGTGTCTGAGCGATGT-3' |
| Osteocalcin 3' | 5'-GAAGCCCAGCGGTGCA-3' |
| Osteocalcin 5' | 5'-CACTACCTCGCTGCCCTCC-3' |
| Versican 3' | 5'-TGGAATGATGTTCCCTGCAA -3' |
| Versican 5' | 5'-AAGGTCTTGGCATTTTCTACAACAG -3' |

### 2.1.9. Vectors

| Vector | Size (Kb) | Supplier |
|---|---|---|
| pIRES2-AcGFP1 | 5.3 | Clontech, Mountain View, US-CA |
| pIRES2-AcGFP1-Nuc | 5.4 | Clontech, Mountain View, US-CA |
| pIRESneo2-BMP1 | 7.4 | Prof. Stoecker, JGU, Mainz, Germany |
| pGEM-T | 3.0 | Clontech, Mountain View, US-CA |

### 2.1.10 Competent Cells

| Cell | Supplier |
|---|---|
| Competent lyophilised *E. coli* | InvivoGen,San Diego, US-CA |
| MAX Efficiency DH5α | Invitrogen, Carlsbad, US-CA |
| XL1-Blue | Stratagene, La Jolla, US-CA |

### 2.1.11. Restriction Enzymes

| Enzyme | Supplier |
|---|---|
| Dra I | Invitrogen, Carlsbad, US-CA |
| Nhe I | Invitrogen, Carlsbad, US-CA |
| Sal I | Invitrogen, Carlsbad, US-CA |
| Ssp I | Fermentas, Glen Burnie, US-MD |

### 2.1.12. Biomaterials

#### *2.1.12.1. Gelatin Sponges*

Oriented porous gelatin sponges with a diameter of 5 mm and a thickness of 1.5 mm thick; a pore size (Feret's diameter) ranging from 300 µm to 400 µm and a porosity of approximately 95%, with unidirectionally aligned fiber coated with collagen type I were used in this study (Gabler et al., 2007; Zehbe et al.). Briefly, either 20 mg/ml or 25 mg/ml of gelatin were dissolved in sterile deionized water to which 0.04 ml/ml of 1 M hydrochloric acid was added. The solution was kept at 50 °C until the gelatin was completely dissolved.

The difference in gelatin content was intended to make the scaffolds slightly more stable in later experiments but from our experience did not have any impact on cellular behavior, as the change in pore size was on a scale not relevant for the cellular response. These gelatin solutions were cast into silicone molds (Ø: 12 mm) situated on top of a voltage-regulated peltier element at -10 °C. After complete freezing, the samples were demoulded and lyophilized in the cold trap of a freeze-drier at -40 °C and 0.1 mbar for one week. The lyophilized samples were cut at the top and the bottom to open up the pore-channel network of the scaffolds. To achieve stability in the liquid cell culture environment, the specimens were chemically cross-linked for 6 h in a desiccator filled with dry polyoxymethylene, resulting in a formaldehyde-saturated atmosphere.

#### *2.1.12.2. Collagen type I Hydrogels*

For a hydrogel with the desired collagen type I end-concentration of 1.5 mg/ml; 0.8 parts of rat tail collagen type I (4.59 mg/ml), 0.1 parts of a 10x reconstitution buffer (1.6 mM NaOH concentration in gel) and 0.1 parts of 10x medium M199 were mixed thoroughly by gently pipetting up and down. The buffer was freshly made and sterile filtered when required and the whole process was carried out on ice to avoid the gelling of the collagen that remains liquid at 4 ºC.

### 2.1.13. Softwares

| Software | Developer |
|---|---|
| LinRegPCR | (Ramakers et al., 2003) |
| SDS Software | Applied Biosystems |

## 2.2. Methods

### 2.2.1. Primary Human Articular Chondrocyte Isolation and Expansion

Human cartilage samples from knee joints were obtained with the written consent from patients undergoing total knee arthoplasty. The experiments were conducted with the approval of and according to the directives of the Ethics Commission, Landesärztekammer Rheinland-Pfalz, Germany.

Articular cartilage was harvested from human knee joints of patients with OA and other joint defects (29 to 82 years old). Cartilage fragments were carefully cut off from the bone specimens to avoid synovial tissue, and the chondrocytic ECM was then digested in 20 ml 0.1% collagenase I-HEPES (273 U/mg) plus 1% penicillin/streptomycin and 10% Dulbecco's modified Eagle's medium (DMEM). The digestion took place overnight at 37 °C with vigorous shaking. After 17-20 h, the cells were filtered successivily through sterile nylon filters with pore sizes of 100 µm and then 40 µm in order to remove matrix debris, centrifuged at 1000 g for 5 min, and the resulting cell pellet was then re-suspended in fresh pre-warmed DMEM with 10% fetal calf serum (FCS), 2% Glutamax I and 1% penicillin/streptomycin. The HACs were plated on tissue-culture polystyrene and cultured in a humidified incubator in an atmosphere of 5% $CO_2$ at 37 °C, changing medium every five days. At confluence, *i.e.* about every three to four weeks, cells were detached with 0.25% trypsin-EDTA (1x), re-suspended in fresh medium and seeded in a concentration of 1:3. The same protocol was applied to all passages.

### 2.2.2. Analysis of Morphology

#### *2.2.2.1. Histology*

Before proceeding to the histological analysis, the samples required fixation either with 4% formalin or with 3.7% PFA-PBS for 30 min at room temperature. Dehydration from aqueous fixatives was initiated in 70% ethanol, progressing through 80%, 96% ethanol, and two or three changes of absolute ethanol before proceeding to the clearing stage. The clearing stage took place overnight at 4 ºC in butanol, which is a substance miscible with the embedding medium (paraffin). Finally, an overnight infiltration with 100% paraffin at 56 – 58 ºC took place, followed by embedding of the samples into paraffin blocks. To optimize

sectioning, the blocks were stored at -20 ºC for 5 – 10 min. 5 µm sections were cut and mounted onto slides placed on a hot plate at 60 ºC for drying. Once the sections had dried, they were deparaffinized by washing the slides twice in xylene, 5 – 10 min each, followed by a reversal of the dehydration process, that is, running the samples through 100% EtOH twice, for 5 min, 96%, 90%, 80% and 70% EtOH for 2 min; and finally rinsing with distilled water for 10 min. A general basophilic-acidophilic staining was carried out with Weigert's Haematoxylin and Eosin (H&E). The sections were first stained with Weigert Haematoxylin for 8 min, washed in warm running tap water for 10 min and rinsed in distilled water and 95% EtOH subsequently (10 dips each), followed by a counterstaining with Eosin for 1 min. Finally, the samples were dehydrated by 95% alcohol, two washes of absolute alcohol, 5 minutes each and cleared in two changes of xylene, 5 min each. When a specific cartilage staining was carried out, a final staining with 0.1% Safranin O (proteoglycan stain) took place during 5 min and the samples were dehydrated through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each and cleared in two changes of xylene, 5 min each. To proceed to the study of the sections, the samples were mounted with GLC mounting medium covered and air dried for 30 min.

The same procedure of fixation and deparaffination was carried out for an acidic proteoglycan staining (sulfated, carboxylic, hyaluronic and chondroitin residues) with Alcian blue. The staining duration was 30 min and the samples were then washed in running tap water for 5 min, followed by a rinse with distilled water. Counterstaining with Neutral red during 1 min was performed and finally the sections were rapidly dehydrated, cleared and mounted, as described above.

#### *2.2.2.2 Calcein-AM Live Staining*

Cells in culture were incubated at 37 ºC for 5 min, with 4 µl/ml of Calcein-AM (1 µg/ml solution in anhydrous DMSO) in DMEM.

### 2.2.3. Cytotoxicity Assays

#### *2.2.3.1. MTS Conversion Assay*

The metabolic cellular activity (an indirect measure of the absence of cytotoxicity) was measured by the conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to formazan, which can be photometrically detected. MTS stock solution was mixed with fresh medium in a ratio of 1:5 and added to the cells cultured at a density of $5x10^4$ per well in flat bottomed 96-well plates for 1.5 h. The cells were then placed into a $CO_2$ incubator at 37 °C. After incubation supernatants were transferred to a microplate and the optical density was measured photometrically at 492 nm against a blank sample (medium with MTS without cells).

#### *2.2.3.2. Crystal violet Staining*

At the end of the MTS conversion assay, the cell culture medium was aspirated, cells were washed once with PBS-0.05% Tween 20 and then fixed for 15 min with 2-propanol. The samples were subsequently washed three times with PBS-0.05% Tween 20 and stained with 0.1% crystal violet solution for 20 min on a shaker. Afterwards the plates were washed under running tap water to remove unbound stain. Crystal violet bound to the cells was extracted by incubation with 33% acetic acid for 20 min on a shaker. The resulting solution was transferred to a new microplate and the optical density was measured at 600 nm against pure 33% acetic acid.

### 2.2.4. Analysis of Gene Expression

#### *2.2.4.1. RNA Isolation*

Before RNA isolation in any of the cell culture experiments, cells had to grow to the point where harvesting was appropriate, *i.e.* to at least 80 – 90% confluence. In some cases some specific adaptations of the procedure were required, so that both the traditional acidic phenol-based (TriZol) extraction and the column-based (RNeasy Micro Kit) purification method could be performed.

#### 2.2.4.1.1. Isolation with TriZol

HACs were disrupted by adding 500 µl TriZol reagent, vortexing and incubating for 5 min on ice. If not processed immediately cell lysates were stored at -80 °C. Afterwards a phase separation was induced by adding 200 µl chloroform to the TriZol cell suspension and centrifuging for 10 min at maximum speed in a cooled (4 ºC) microcentrifuge. The aqueous layer was then carefully harvested and collected in new RNase free Eppendorf tubes. The RNA precipitation was carried out by adding an equal volume of 100% isopropanol and incubating for 10 min on ice. After centrifugation at 4 ºC for 10 min, the supernatants were discarded and 500 µl of 80% ethanol were added to wash the RNA pellets, thereby removing isopropanol, salts, and phenol residues. To recover the washed pellets an additional 10 min centrifugation followed. Afterwards the pellets were air-dried at room temperature. RNase free TE-buffer was used to solubilize the pelleted RNA, (20 µl/pellet).

#### 2.2.4.1.2. Isolation with RNeasy Micro Kit

HACs were lysed by adding RLT Buffer containing 1% β-mercaptoethanol directly onto the growth surface, and attached cells were then suspended with a cell scraper. As mentioned above, if not processed immediately, cell lysates were stored at -80 °C. RNA was precipitated with one volume of 70% EtOH, the mixture was pipetted to an RNeasy spin column and centrifuged for 15 s at 9500 g. 350 µl of Buffer RW1 were added to the column and then centrifuged for 15 s at 9500 g. DNA bound to the column membrane was digested for 15 min with DNase I and then the previous step with RW1 buffer was repeated. The membrane was subsequently washed twice with RPE Buffer (centrifugation at 6000 g for 15s and 2 min) and dried by centrifugation at maximum speed for 1 min. RNA was then eluted in 20 µl of $H_2O$ by centrifugation at 6000 g for 1 min. To increase the concentration of RNA the elution step was repeated with the RNA solution eluted the first time.

For the RNA isolation from biomaterial cultures, a combination of TriZol extraction and RNA affinity column was required. The lysing steps were performed according to the TriZol method, and the RNA precipitation, cleaning, and elution were carried out in the column based method, thus increasing the purity of the RNA eluate.

To determine the concentration of isolated RNA, the absorption at a wavelength of 260 nm ($A_{260}$) of all RNA solutions was measured with a NanoDrop spectrophotometer.

### *2.2.4.2. Reverse Transcription*

From 10 to 1000 ng RNA was reverse transcribed into cDNA depending on starting conditions and/or experimental requirements. The same amount of RNA was taken from different samples of any given experimental set. The volume was adjusted to 15 µl, and 4 µl of 5x iScript reaction mix plus 1 µl iScript reverse transcriptase were added to RNA. The mixture was incubated for 5 min at 25 °C, and then the reaction was allowed to proceed at 42 ºC for 30 min. The inactivation of the reaction occured at 85 ºC, and was complete after 5 min.

### *2.2.4.3. RT-PCR*

The amplification of the cDNA obtained in the reverse transcription was performed via RT-PCR using a Taq DNA polymerase and the appropriate primers. The master mix was prepared according to the following table provided by Qiagen (Hilden, Germany):

| Components | Volume / Reaction | Final Concentration |
|---|---|---|
| **Master Mix** | | |
| 10x PCR Buffer | 2.5 µl | 1x |
| dNTP Mix (10 mM each dNTP) | 0.5 µl | 200 µM each dNTP |
| Primer A (10 pmol / µl) | 0.5 µl | 5 pmol (per 25 µl reaction) |
| Primer B (10 pmol / µl) | 0.5 µl | 5 pmol (per 25 µl reaction) |
| *Taq* DNase Polymerase | 0.125 µl | 1.25 U (per 25 µl reaction) |
| Distilled water | Variable | |
| **Template DNA** | Variable | ≤ 1 µg (per 25 µl reaction) |
| **Total volume** | **25 µl** | |

The thermocycler was programmed according to the following RT-PCR cycling conditions:

| | **Duration** | **Temperature** |
|---|---|---|
| **Initial denaturation:** | 2 min | 94 °C |
| **3-step cycling** | | |
| Denaturation: | 30 s | 94 °C |
| Annealing: | 30 s | 50 – 68 °C |
| Extension: | 30 s | 72 °C |
| **Number of cycles:** | 35 | |
| **Final extension:** | 10 min | 72 °C |

#### *2.2.4.4 Agarose Gel Electrophoresis*

A quality check of the RT-PCR products was carried out by agarose gel electrophoresis. For this purpose, 1% agarose gel in 1xTBE-buffer was prepared by boiling 0.5 g of agarose together with 50 ml of buffer until the agarose was completely dissolved. After cooling to just above melting temperature, 8 µl of ethidium bromide solution were added and the agarose solution was poured into a tray (properly sealed) with a suitable comb placed into it. The solution was allowed to gel at room temperature. Afterwards, the comb was removed, the tray unsealed, and the gel placed into the electrophoresis chamber which was filled up with 1xTBE buffer. Prior to sample loading, 5 µl of 5x loading buffer were added to the RT – PCR product solutions. Then all samples were loaded into the gel alongside, a suitable DNA ladder. Electrophoresis proceeded at 100 V for 45 – 60 min, and the resulting DNA bands were visualized using a UV lightbox.

#### *2.2.4.5 Quantitative RT-PCR*

Quantitative real-time PCR was performed using SYBR Green DNA-binding fluorescent dye. 3.75 ng cDNA were used per reaction. 12.5 pmol of each 3´ and 5´ primer were mixed with cDNA. The volume was adjusted to 12.5 µl with $H_2O$ and 12.5 µl Power SYBR Green Mix was added resulting in a total volume of 25 µl. PCR amplification was carried out in Applied Biosystems' 7300 Real-Time PCR System. Reaction conditions were 10 min at 95°C followed by 40 amplification cycles (15 s at 95°C and 1 min at 60°C). At the end, in order to obtain a DNA melting curve, DNA dissociation was performed by slowly elevating the temperature from 60°C to 95°C. The data were analysed with SDS Software to obtain Ct (cycle threshold) values. Relative gene expression was then determined based on ΔΔCt values using GAPDH as an endogenous control. Briefly, the Ct value of endogenous control was subtracted from the Ct value of the gene studied to obtain ΔCt. Afterwards ΔCt of an experimental reference sample was subtracted from ΔCt of the sample of interest to get individual ΔΔCt values. Relative expression was then calculated with the formula $2^{-\Delta\Delta Ct}$. Data are presented as mean values of relative expression and, error bars indicate the 95% confidence interval. For statistical analysis one-way ANOVA test for independent samples was performed on ΔΔCt values (Yuan et al., 2006). To take into account possible differences in PCR efficiencies the data were additionally analysed with the REST method (Pfaffl et al., 2002) using LinRegPCR Software (Ramakers et al., 2003) to determine efficiencies of single PCR reactions.

### 2.2.5. Analysis of Protein Expression

#### *2.2.5.1. TGF-β1 ELISA*

For detection of TGF-β1 released by HACs into the cell culture medium a DuoSet TGF-β1 ELISA kit was used. Briefly, microplate wells were coated with 100 µl capture antibody (4 µg/ml) overnight and washed afterwards with PBS-0.05% Tween 20. Unspecific binding sites were blocked by incubating for 1 h with 1% BSA, 5% sucrose in PBS, followed by subsequent washes. 100 µl standards or samples were added to the wells and binding to the antibody was allowed to take place for 2 h followed by the incubation with 100 µl of biotinylated detection antibody (20 ng/ml) for 2 h with washes before and after incubation. For the detection of antibody-antigen complexes 100 µl of working dilution of streptavidin-HRP was then added to the wells, incubated for 1 h and after washing 100 µl of the substrate solution (mixture of $H_2O_2$ and tetramethylbenzidine) was applied for 20 min. The reaction was terminated by 50 µl of 2 N $H_2SO_4$ and optical density was measured at 450 nm with wavelength correction at 540 nm. TGF-β1 amount was determined from the standard curve.

### 2.2.6. Statistical Analysis

All experiments were repeated at least three times and the results are presented as means ± standard deviations (SDs). Statistical analysis was carried out with Microsoft Excel's one-way ANOVA test for independent samples.

# 3. Human Articular Chondrocyte Characterization

## 3.1. Experimental Design and Execution

For comparison with the intended studies of chondrocyte re-differentiation strategies, cell characterization of HACs in monolayer culture conditions was first carried out. Cell morphology and gene expression were taken as references for the evaluation of cell differentiation in other systems to be studied.

### 3.1.1. Cell Culture

HACs in passage one were cultured in 12-well plates for an 18-day period. Cells were counted with a haematocytometer and the seeding was adjusted to $1.5x10^5$ cells/well, *i.e.* $4x10^4$ cells/$cm^2$. The same procedure was followed with cells in passages two, three and four.

### 3.1.2. Morphological Analysis

In the HAC isolation process a freshly cut piece of cartilage was fixed in 3.7% PFA-PBS before proceeding with the H&E staining. The morphology of cultured cells was recorded by imaging them by phase contrast microscopy on days three, seven and ten (passage 1). Cells were also live stained with Calcein-AM and fluorescent images were taken at the same time points.

### 3.1.3. Gene Expression Analysis

Cells were harvested every three days (day 3, day 7, day 10, day 13 and day 16). They were first trypsinized, then re-suspended in 350 µl of RLT buffer containing 1% β-mercaptoethanol, and stored at -80 ºC for RNA isolation. After RNA isolation and cDNA reverse transcription, RT-PCRs with the relevant chondrocytic markers were carried out, namely collagen types I, II and X, Sox 5, 6 and 9, and aggrecan. For a complete quantitative study of gene expression during the time span of this experiment, qrt-PCRs were performed

with primers for the three different collagens mentioned above as well as aggrecan and versican, taking gene expression on day 3 as a reference.

## 3.2. Results

### 3.2.1. HACs De-differentitiation Process

Under the supervision of Dr. C. Brochhausen (Universitaetsmedizin, Mainz, Germany) histological sections of freshly cut pieces of cartilage (from different donors) fixed in 3.7% PFA-PBS were stained with H&E. The staining revealed a highly deteriorated tissue, in which the chondrocytes showed a de-differentiated, fibroblastic morphology (Fig. 3.1 A). Alcian blue staining showed areas of a typical light blue homogeneous background staining typical for ECM deficient in proteoglycans and GAGs (Fig. 3.1 B). This obvious down-regulation of cartilage-specific ECM components is consistent with the presence of de-differentiated chondrocytes.

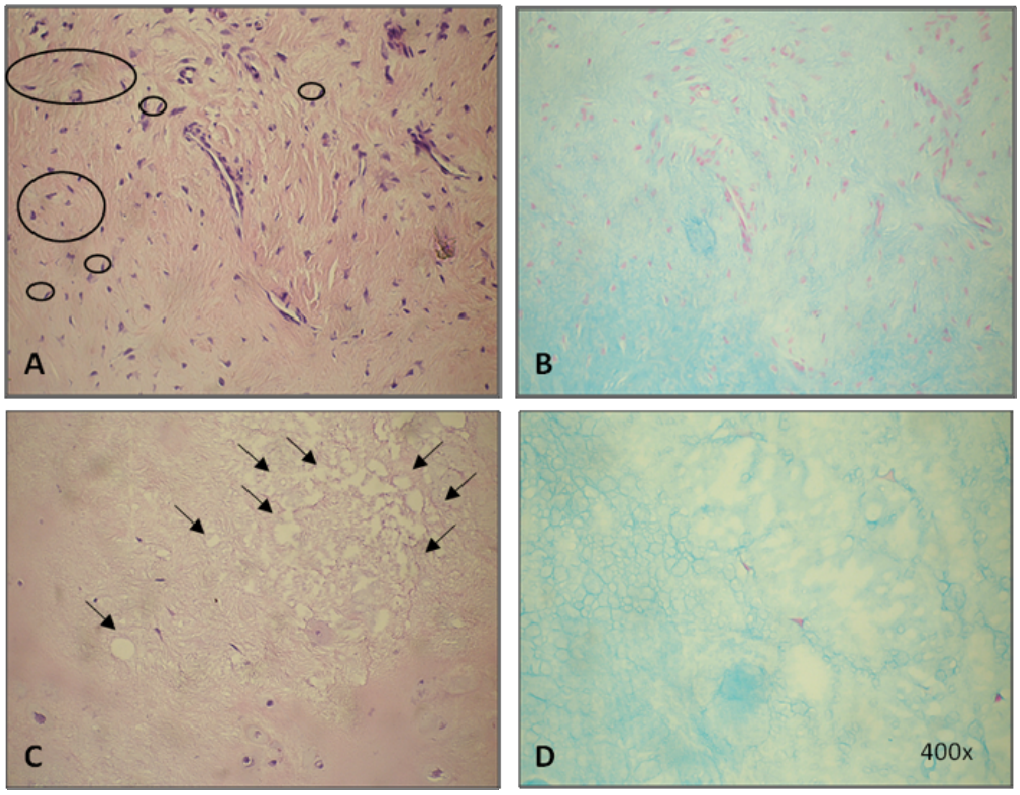



**Fig. 3.1: Representative images taken from different cartilage sections.** The sections were fixed and stained with H&E (A and C). The most relevant characteristics were fibroblastic morphology of the chondrocytes (in circles) and absence of a dense ECM (arrows). Alcian blue (B and D), which stains the major components of the ECM; proteoglycans and GAGs, confirmed the H&E staining, *i.e.* the ECM was in a degradation process.

ECM degradation is the major problem when cartilage is damaged. Cell number, organization and distribution, functional properties, as well as the content of organic water and other major components across the tissue are altered, (Fig. 3.1. C and D) (Lee et al., 2005).

To define the differentiation stage of freshly isolated HACs cultured in monolayer, the morphology was first studied. The appearance of viable round cells during the first days of culture coincided with differentiated chondrocyte morphology found *in vivo*. The low proliferation rate was also characteristic of HACs (Fig 3.2. A and B). Within seven days signs of de-differentiation were observed; with cells becoming elongated and commencement of proliferation (Fig. 3.2 C and D). Thus, chondrocytes changed to a more fibroblastic appearance and behavior (Fig. 3.2. E and F) (Hayman et al., 2006).

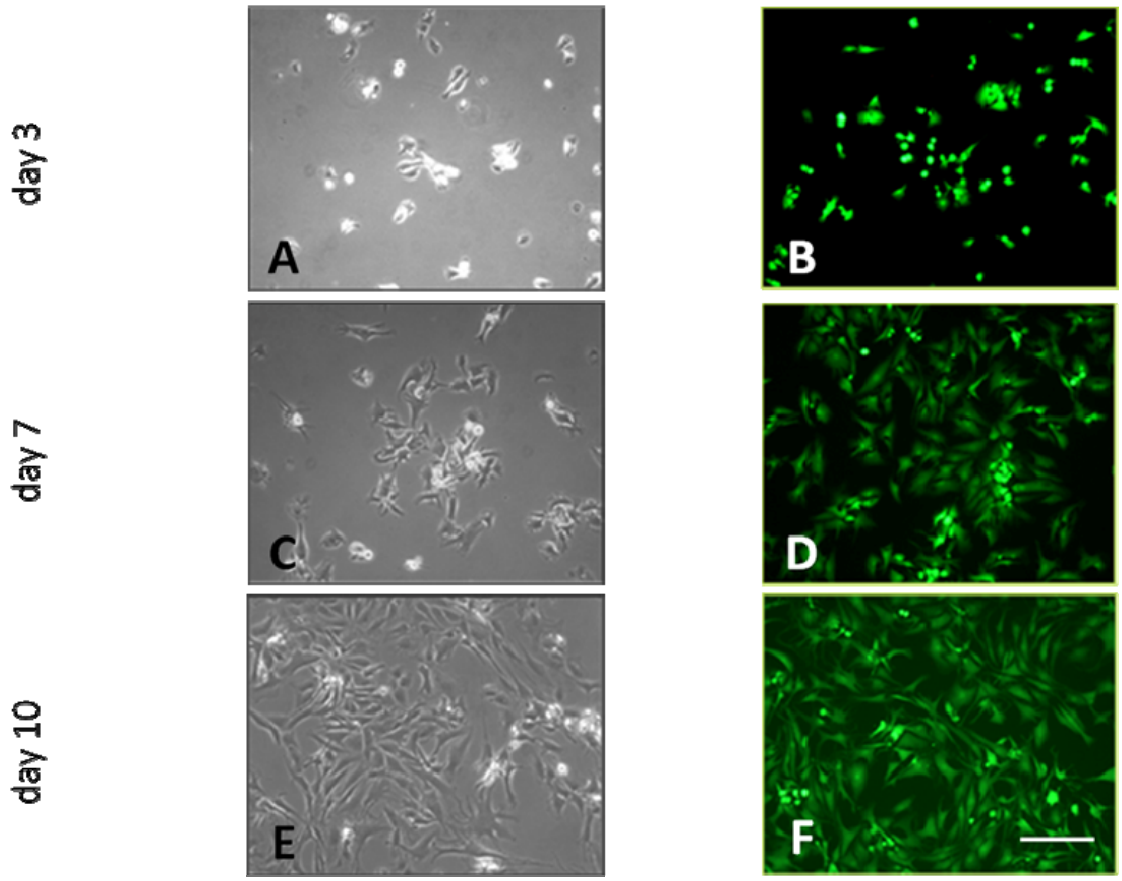



**Fig. 3.2: Alterations in chondrocyte morphology during monolayer culture for up to 10 days.** Phase contrast microscope images (A, C and E) and Calcein-AM live staining (B, D and F). The images taken over a 10-day culture period show a clear evolution of the HACs towards a fibroblastic phenotype (elongated cells) characteristic of de-differentiated HACs, especially remarkable from day 7 on. Scale bar: 200 μm.

### 3.2.2. Gene Expression and De-differentiation

HACs have been found to rapidly de-differentiate and lose their phenotype when cultured in monolayer (Hardingham et al., 2002; Hayman et al., 2006). RT-PCR with cDNA from our cultures of HAC revealed that the earliest change during de-differentiation in monolayer culture was the up-regulation of collagen type I (Fig. 3.3.). Collagen type II, an early and abundant marker of chondrocytes, and aggrecan were expressed for at least 16 days of culture. Likewise, Sox9, which is a transcription factor required for chondrocyte differentiation and for expression of a series of chondrocyte-specific marker genes, including collagen type II and aggrecan (de Crombrugghe et al., 2000), was also continuously expressed. Collagen X, a relevant marker for chondrocyte hypertrophy, was not expressed during the culture period of 16 days. Under the culture conditions mentioned, HACs continued expressing key genes that are also characterisitic of native mature chondrocytes.

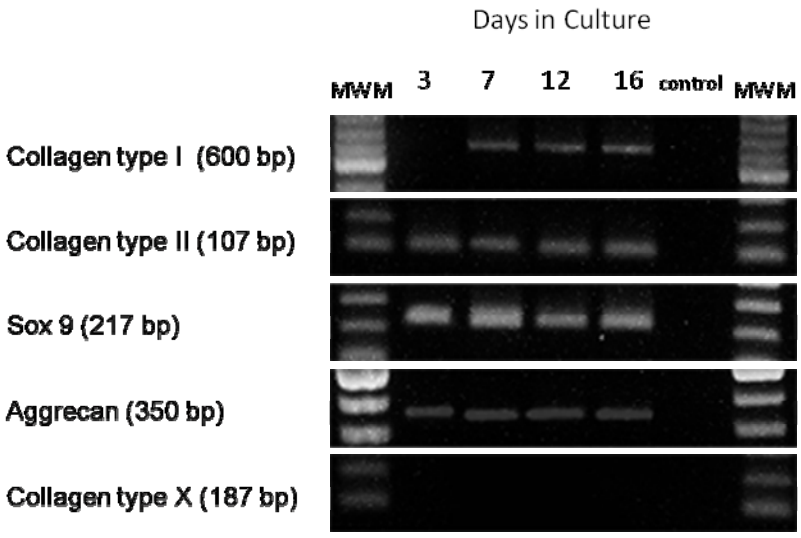



**Fig. 3.3: Time course of typical chondrocytic marker gene expression determined by RT-PCR.** In the time span shown up-regulation of collagen type I expression characteristic of de-differentiated HACs occurred from day 7 on. Nevertheless, the typical markers for differentiated HACs were present during the entire culture period. [Control: PCR negative control, without template. Molecular weight marker (MWM): 100 bp DNA Ladder].

Measurement of collagen types I, II and X and aggrecan expression by qrt-PCR confirmed the qualitative results. In 16 days of monolayer culture the chondrocytic phenotype underwent de-differentiation where collagen type I was up-regulated up to 40-fold (Fig. 3.4. A). Collagen type II and aggrecan mRNA expression was only slightly down-

regulated by day 16 (Fig. 3.4. B). The absence of collagen type X mRNA expression denoted that HACs did not undergo hypertrophy (Fig. 3.4. C).

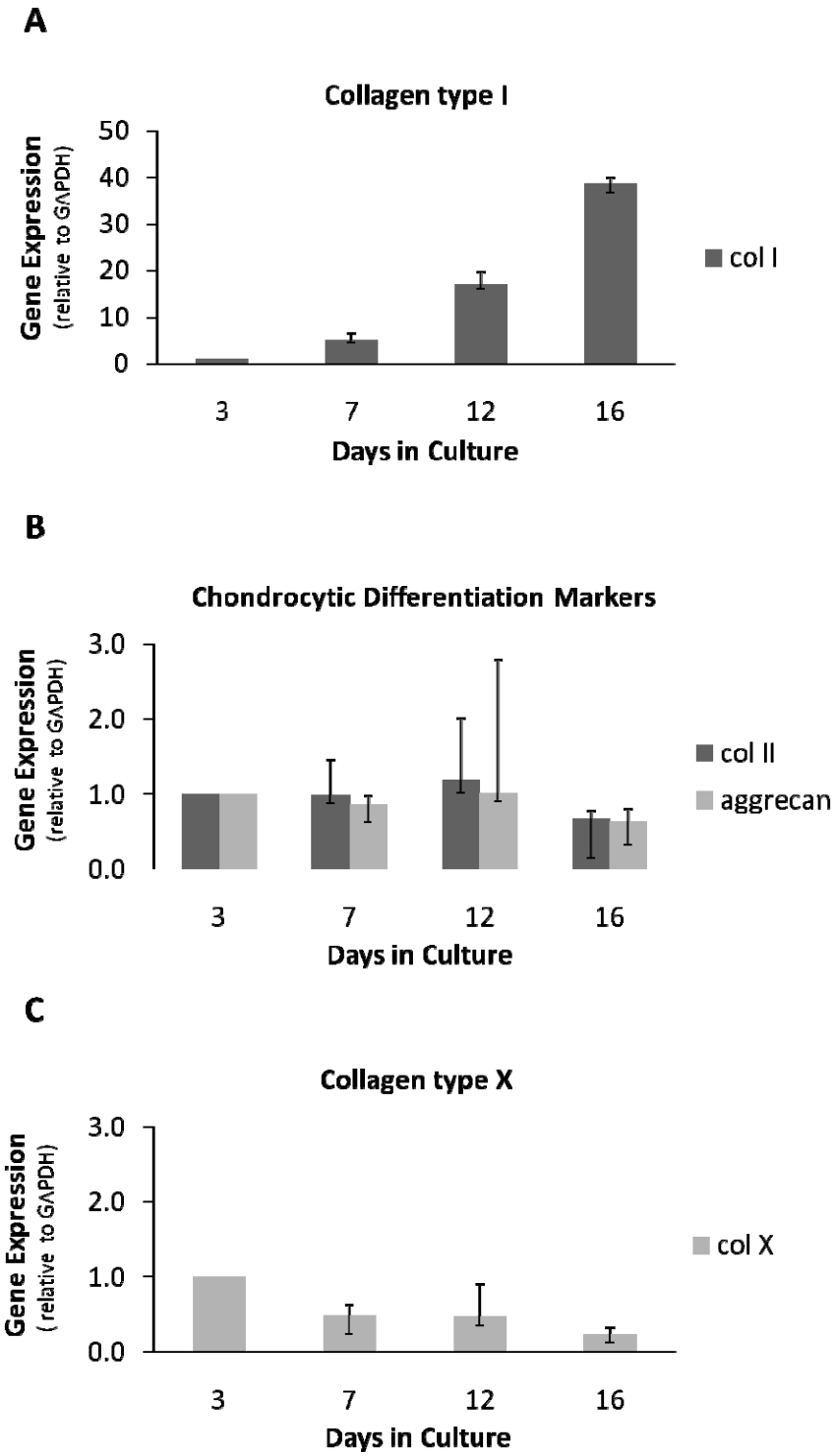



**Fig. 3.4: Relative gene expression of collagen type I (A); collagen type II and aggrecan (B); and collagen type X (C**). Major evidence for chondrocytic de-differentiation is the significant up-regulation of collagen type I despite the fact that the differentiation markers were not as significantly down-regulated, and that collagen type X was not expressed. Results are shown as means of the ratio of target gene expression to expression of GAPDH $\pm$ 95% confidence interval, n=3.

Based on the morphological and molecular data achieved in the present chapter, it is assumed that HACs cultured in monolayer conditions undergo de-differentiation as the up-regulation of collagen type I indicates. However, the fact that collagen type II and aggrecan expression does not decrease and that the cells do not undergo hypertrophy as the absence of collagen type X indicates, points to a preservation of the phenotype. This facilitates the design of the culture models included in this work in which HACs are stimulated towards a potential re-differentiation.

# 4. $PGE_2$ Stimulation of Human Articular Chondrocytes

## 4.1. Experimental Design and Execution

HAC differentiation is mediated by molecular signals such as $PGE_2$. In the present chapter HACs in monolayer culture were stimulated with different concentrations of $PGE_2$. In an attempt to mimick *in vivo* conditions of the ECM, HACs were cultured on gelatin-based scaffolds and additionally stimulated with exogenous $PGE_2$. Under both culture conditions the effects on cell proliferation and differentiation were evaluated.

### 4.1.1. Cell Culture

In the following experiments with serum-containing media, the effects of naturally occurring prostaglandins had to be eliminated from the serum. Therefore, FCS was charcoal-stripped. This procedure included the use of charcoal and dextran to remove prostaglandins as well as other steroids from the FCS; for 200 ml DMEM, 20 ml FCS were treated with 200 mg charcoal and 20 mg dextran in a 50 ml centrifuge tube on a roll-mixer at 4 ºC for 24 h, followed by a sterile filtration.

$PGE_2$ stock solution was made by dissolving 10 mg of $PGE_2$ in 280 ml pre-chilled EtOH, obtaining an end concentration of 0.1 M. The concentrations required for the different stimulation conditions in these experiments were obtained by subsequently diluting the stock solution.

#### *4.1.1.1. $PGE_2$ Stimulation in Monolayer Culture*

HACs in passages 0 – 4 ($3x10^4$ cells/well) were cultured on six 8-well chamber slides in complete DMEM *i.e.* containing 10% FCS, 2% Glutamax I and 1% penicillin/streptomycin. The cultures were stimulated for three days with media containing seven different $PGE_2$ end concentrations ($10^{-7}$ M; $3x10^{-8}$ M; $10^{-8}$ M; $3x10^{-9}$ M; $10^{-9}$ M; $3x10^{-10}$ M and $10^{-10}$ M) plus an untreated control. Medium was changed every 24 h. Before each medium change, two slides were fixed with 3.7% PFA-PBS. An additional monolayer culture was conducted for three

days in 96-well plates ($10^4$ cells/well) and stimulated with two different $PGE_2$ concentrations ($10^{-6}$ M and $10^{-9}$ M) plus an untreated control (in triplicate).

#### *4.1.1.2. $PGE_2$ Stimulation in 3-D Culture*

Cells in passages 0 – 4 were seeded on oriented porous gelatin sponges, glued to the surfaces of the wells of a 48-well plate with 20 µl of fibrin gel (end concentration 3 mg/ml). The seeding was carried out by re-suspending the cells in 3.42 ml of complete DMEM (without FCS) containing 3.6 ml of fibrinogen solution and 180 µl of thrombin ($6x10^4$ cells/ml; 200 µl fibrin gel/sponge) and pipetting the fibrinogen solution onto the sponges before the formation of the fibrin gel. The stimulation consisted of two different $PGE_2$ concentrations ($10^{-6}$ M and $10^{-9}$ M) plus an untreated control, for the duration of two weeks. Media changes took place every three days. The media were collected and stored at -80 ºC for future analysis. On day 7 and day 14, samples were stored in 350 µl RLT buffer containing 1% β-mercaptoethanol at -80 ºC for future RNA isolation.

### 4.1.2. Quantification of $PGE_2$ in Culture Media

Quantification of $PGE_2$ was kindly provided by B. Watzer, (Philipps University of Marburg, Germany). $PGE_2$ concentrations were determined in the medium collected at each medium change by gas chromatography-tandem mass spectrometry (GC/MS/MS). Based on the GC/MS/MS method published by Schweer et al. (Schweer et al., 1994) $PGE_2$ was determined in the medium using this assay with minor modifications.

In short, the method was as follows: 1 ng of tetra-deuterated $PGE_2$ was added to each 500 µl sample as an internal standard (IS). The equilibrated sample was acidified with formic acid. Afterwards; *O*-methylhydroxylamine hydrochloride in sodium acetate solution was added to form methoxime derivatives. After re-acidification with formic acid, the prostanoids were extracted with ethyl acetate-hexane. The solvent was evaporated and acetone, N,N-diisopropylethylamine, and pentafluorobenzyl bromide were added. After incubation, the solvent was evaporated, and the sample purified by thin layer chromatography. The zone corresponding to the prostanoids was scraped off. The analyte was extracted with ethyl acetate. The evaporated sample was derivatized twice with *N*,*O*-

bis(trimethylsilyl)trifluoroacetamide. An aliquot of this solution was injected into the gas chromatograph/tandem mass spectrometer. For quantification, precursor ions ($[P]^-$) were [M-PFB]$^-$ molecules (*m/z* 524 for $PGE_2$ and *m/z* 528 for IS), product ions were $[P\text{-}2(CH_3)_3SiOH]^-$ (*m/z* 344 for $PGE_2$ and *m/z* 348 as IS).

### 4.1.3. Morphological Analysis

#### *4.1.3.1. Immunofluorescence Staining of Chondrocytes in Monolayer*

After fixation of the samples as mentioned in section 4.1.1.1., a permeabilization step using 0.1% Triton X-100 in PBS was carried out. After two washes with PBS the samples were treated with 1% BSA – PBS as blocking agent. The cells were incubated with a mouse PCNA primary antibody (1:100), for 60 min at 37 ºC, followed by detection with secondary antibody: Alexa 546 conjugated goat anti-mouse and Hoechst 33253 dye (1:1000) for an additional hour at 37 ºC. PCNA is an antigen that is expressed in the nuclei of cells during during the DNA synthesis phase of the cell cycle it interacts therefore with proliferating cells. The samples were finally covered with Fluoromount-G and examined under a confocal laser-scanning microscope. The fraction of proliferating cells was determined by dividing the number of PCNA-positive cells (Alexa 546 signal) and cell pairs by the total number of cells (Hoechst signal) in a randomly chosen picture frame. The values from eight different images per condition and donor were averaged to obtain the mean $\pm$ standard deviation.

#### *4.1.3.2. Calcein-AM Live Staining of Chondrocytes in 3-D Cultures*

Every seven days a whole set of scaffolds ($10^{-6}$ M, $10^{-9}$ M and control) was stained with Calcein-AM. The scaffolds were mounted in fluorescence microscopy mounting medium Fluoromount-G and imaged by excitation at 519 nm using a confocal laser scanning microscope.

### 4.1.4. Quantitative Real-time PCR

HAC from both monolayer and 3-D cell cultures were harvested on week 1 and week 2 and gene expression analysis was conducted by qrt-PCR using specific target primers for collagen type II, collagen type I, and collagen type X. As reference points for gene expression the following time points were chosen: week 1 in monolayer cultures and day 1 in 3-D cultures, without $PGE_2$ supplementation (unstimulated).

## 4.2. Results

### 4.2.1. Proliferation and Metabolism

To analyze whether $PGE_2$ supplementation stimulates HAC proliferation, PCNA immunostaining was performed. Over a culture period of three days, the proliferation rate of HACs did not differ significantly in separate cultures with a wide range of $PGE_2$ concentrations (Fig 4.1.). This was in contrast to cultured growth plate chondrocytes from rat where exogenous $PGE_2$ stimulation did indeed have a proliferating-inducing effect in a dose-dependent manner (Brochhausen et al., 2006).

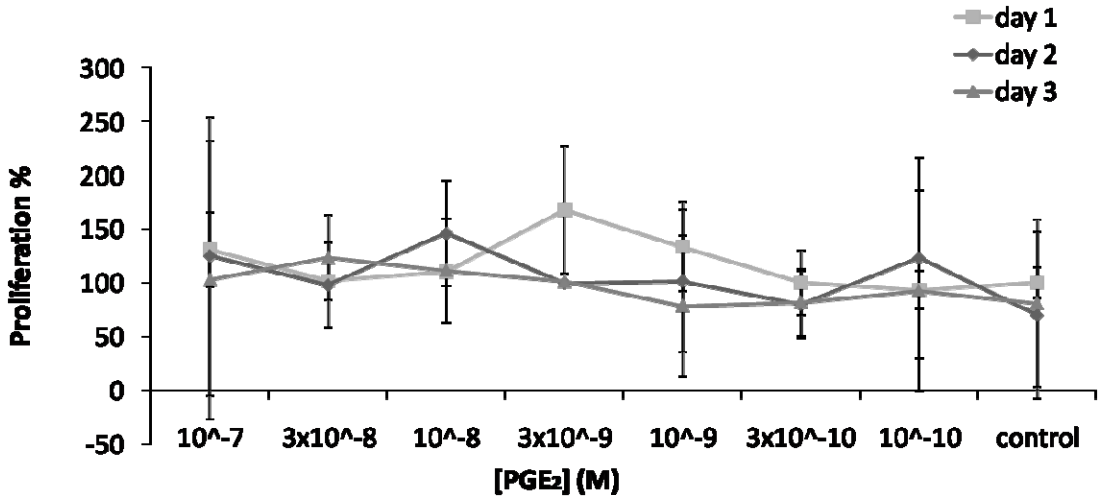
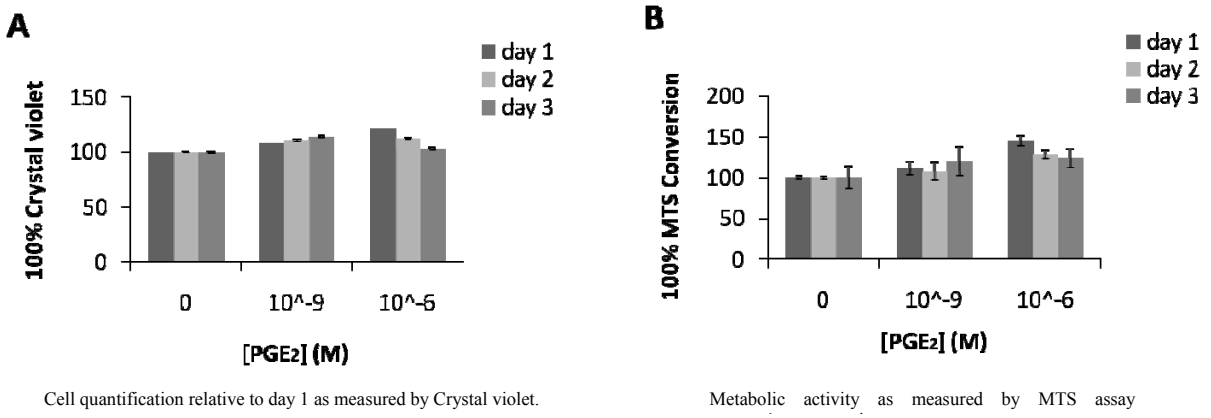



**Fig 4.1.: Effects of different $PGE_2$ concentrations on HACs proliferation rate**. PCNA immunostaining revealed that there was no relevant effect of $PGE_2$ on the proliferation rate of HACs in culture. Data were normalized to the proliferation rate of the untreated control on day 1 (mean $\pm$ SD, n=3).

The fact that $PGE_2$ did not yield a proliferative effect was confirmed by quantification of cell number by DNA staining with crystal violet of cells from three different donors stimulated with a smaller range of concentrations during the same culture period (Fig 4.2. A). Evaluation of cellular metabolic activity with MTS assay in samples with three different donors, that is an indirect measure for viability, showed the effects of $PGE_2$ stimulation on HAC metabolism, where a supplement of $10^{-6}$ M prostaglandin increased 20 to 45% the metabolic activity of the cell, (Fig 4.2. B). This result supported studies with animal cells, where $PGE_2$ stimulated intracellular cAMP production, accumulation of proteoglycan, and up-regulation of collagen type II mRNA expression in a dose-dependent manner in primary rat chondrocytes; giving evidence that $PGE_2$ enhanced chondrocyte differentiation towards hypertrophy and osteoblast/osteoclast formation in monolayer culture (Lowe et al., 1996; Miyamoto et al., 2003).




Cell quantification relative to day 1 as measured by Crystal violet.

Metabolic activity as measured by MTS assay

**Fig 4.2: Crystal violet staining (A) and MTS conversion assay (B) of HACs stimulated with different $PGE_2$ concentrations.** Relative cell number quantification by crystal violet staining did not reveal a trend of cell proliferation due to the effect of $PGE_2$ on HACs (A) and MTS conversion assay suggested stimulation of HAC metabolism under high concentrations of $PGE_2$ (B). Values are means $\pm$ SD of three wells and represent three different donors. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding unstimulated culture on each time point.

### 4.2.2. De-differentiation and Re-differentiation

Several lines of conflicting evidence have been reported on the effects of $PGE_2$ in chondrocyte differentiation. Therefore, the effects of $PGE_2$ on the expression of relevant chondrocytic markers of HACs cultured in monolayer were studied by qrt-PCR (Fig. 4.3.).

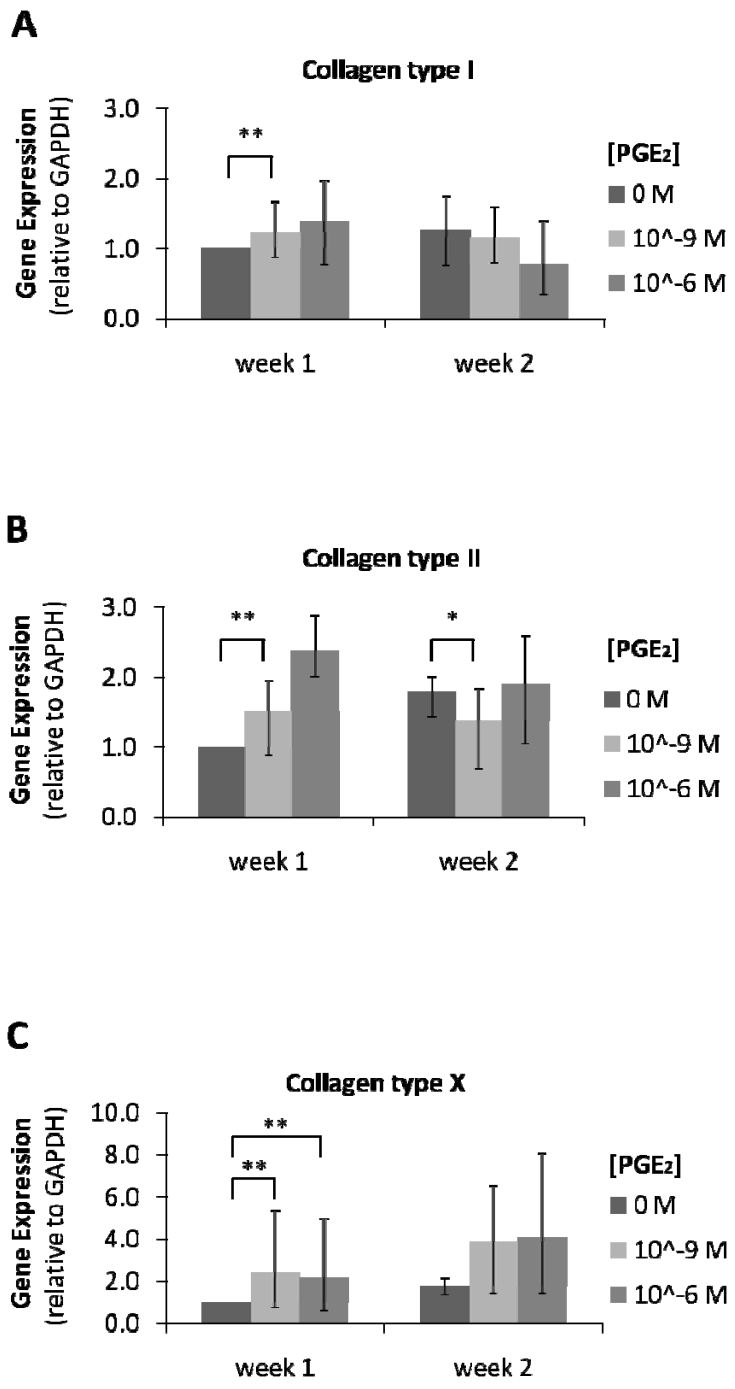



**Fig 4.3.: Normalized relative gene expression of collagen type I (A) collagen type II (B) and collagen type X (C) of HACs cultured in monolayers and stimulated with different concentrations of $PGE_2$.** After two weeks cells exposed to exogenous $PGE_2$ stimulation showed a trend of collagen type X up-regulation and a decrease of collagens type I relative to the non-stimulated control group on each time point. However after two weeks collagen type II tended to increase its expression without stimulation as much as with $10^{-6}$ M $PGE_2$ exogenous stimulation, suggesting that stimulation did not enhance the typical chondrocytic phenotype. Results are shown as the means of target gene expression relative to GAPDH $\pm$ 95% confidence interval, n=3. Values were normalized with respect to gene expression at $[PGE_2] = 0$ on week 1 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding unstimulated culture at each time point.

The stimulation effects on collagen type II expression were remarkable after one week of culture where the expression increased one-fold in the presence of $10^{-6}$ M $PGE_2$; however these effects did not persist after two weeks (Fig. 4.3. B). A certain degree of collagen type I down-regulation in the presence of high $PGE_2$ concentration ($10^{-6}$ M) after week 2 (Fig. 4.3. A) and the corresponding trend of collagen type X up-regulation, (Fig. 4.3. C) suggested hypertrophic behaviour of HACs cultured in monolayer, confirming that stimulation with specific concentrations of $PGE_2$ enhanced specific responses, that could cause the de-differentiation process of HACs cultured in monolayer conditions (Martel-Pelletier et al., 2003).

On the other hand, there are also studies that support the hypothesis that $PGE_2$ may also have positive effects on cartilage by decreasing the expression of pro-inflammatory genes, collagenases and collagen type X (Di Battista et al., 1997; Goldring and Berenbaum, 1999; Li et al., 2004; Tchetina et al., 2006). Based on these positive effects and on the fact that the critical point in chondrocyte monolayer culture appears in the moment in which cells are removed from their ECM, where they adopt a fibroblastic morphology due to the deprivation of a proper 3-D matrix (Lin et al., 2006); in an attempt to decrease this effect, HACs were seeded on gelatin-based scaffolds that were designed to imitate as closely as possible the physical conditions of the ECM (Zehbe et al., 2005).

Prior to seeding, HACs were in confluent monolayer culture where they had taken on the characteristic flattened morphology of fibroblasts. The study revealed that after one week of culture, cells in the absence of $PGE_2$ retained a fibroblast-like, elongated, and flattened morphology indicative of chondrocytic de-differentiation (Fig. 4.4. A and B).

A fibroblast-like morphology also prevailed at low $PGE_2$ concentration of $10^{-9}$ M (Fig. 4.4. C and D). In contrast, cells in the presence of $10^{-6}$ M $PGE_2$ clearly displayed a spherical morphology characteristic of differentiated mature chondrocytes (Fig. 4.4. E and F). This morphology was constant throughout the entire section of the biomaterial and persisted for at least two weeks.

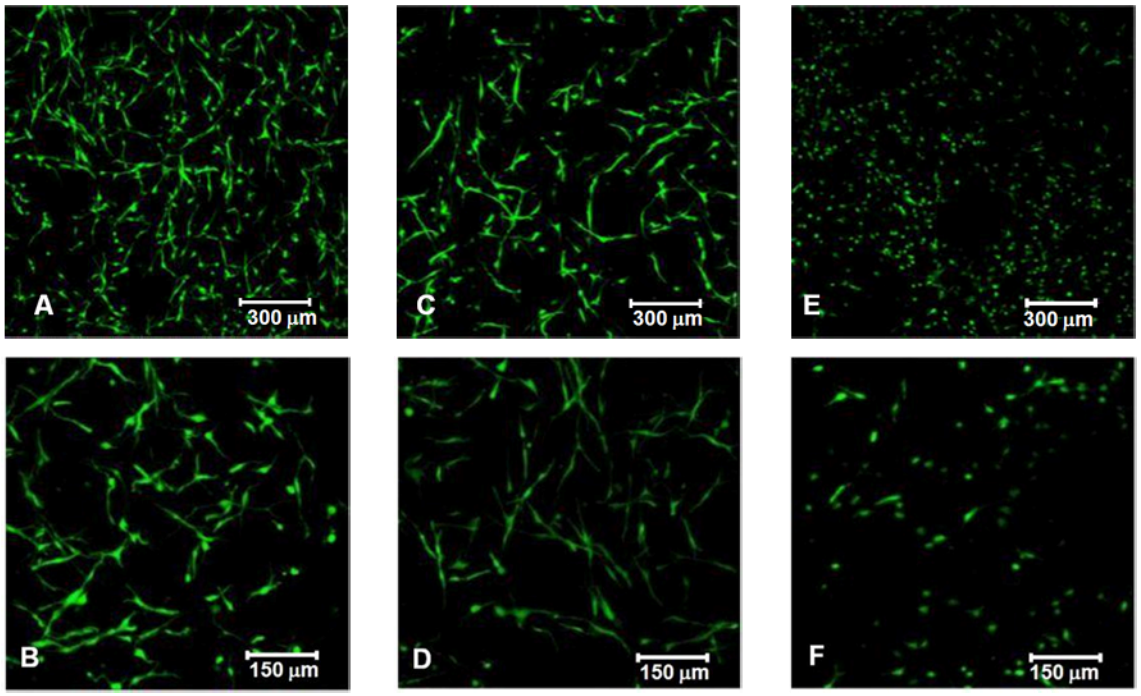



**Fig 4.4.: Representative images of calcein–AM live-stained HACs cultured on gelatin-based scaffolds.** A+B: untreated control (no $PGE_2$); cells assumed a fibroblast-like morphology characteristic of de-differentiated chondrocytes. C+D: with $10^{-9}$ M $PGE_2$ exogenous stimulation; cells retained a de-differentiated appearance and E+F: with $10^{-6}$ M $PGE_2$ exogenous stimulation; at this concentration of $PGE_2$ cells showed a spherical morphology typical of differentiated chondrocytes. Scale bar in A, C and E = 300 µm. Scale bar in B, D and F = 150 µm.

Furthermore, in the presence of $10^{-6}$ M $PGE_2$, cells occurred in clusters of two or three cells, which are also characteristic formations found in native cartilage (Fig. 4.5.).

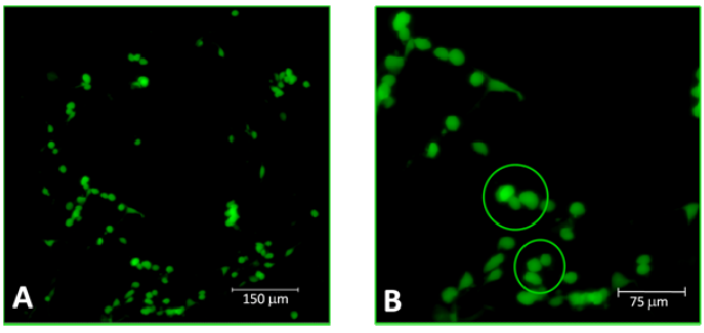



**Fig 4.5.: Calcein-AM stain of HACs cultured on gelatin-based scaffolds.** Cells cultured under exogenous stimulation with $10^{-6}$ M $PGE_2$ showed clear evidence of re-differentiation, namely spherical morphology and a typical chondrocytic structure where cells occured in clusters of two or three cells as in native cartilage. Scale bars: A = 150 µm and B = 75 µm.

To characterize the re-differentiation stage and to confirm the results mentioned above at the molecular level, qrt-PCR was performed with the same chondrocytic markers used for the monolayer culture analysis, *i.e.*, collagens type I, II and X.

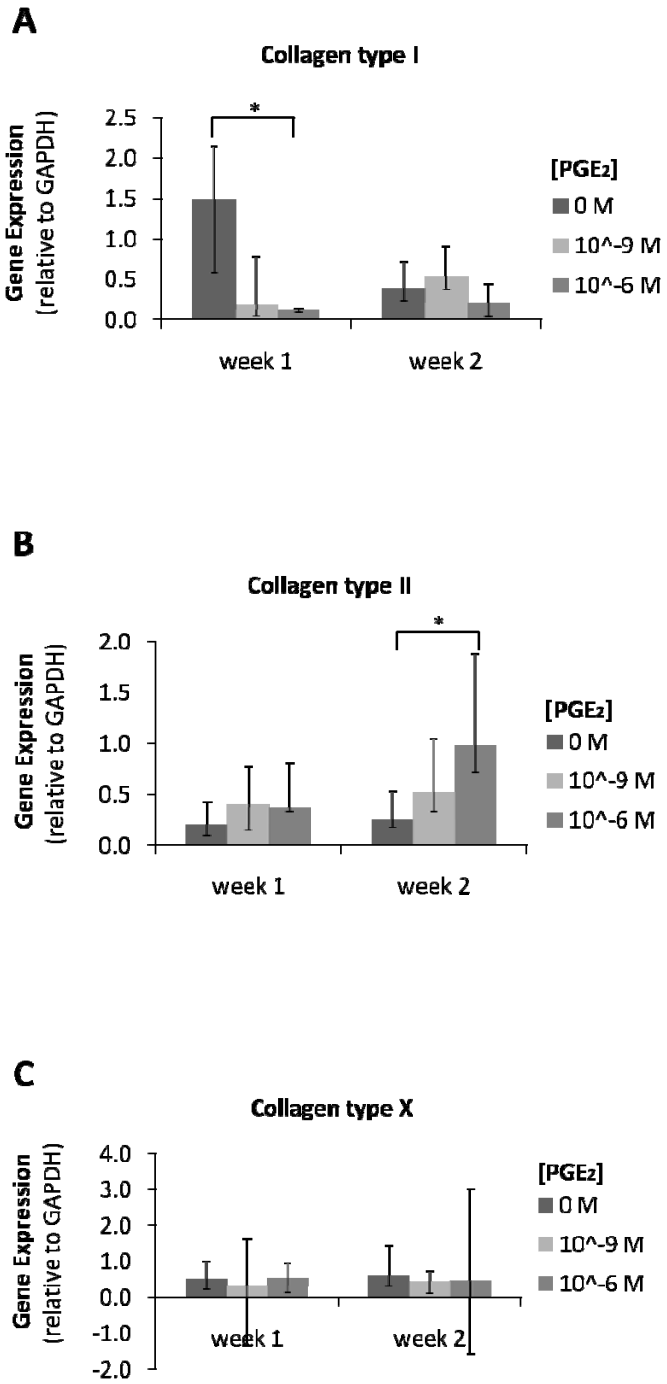
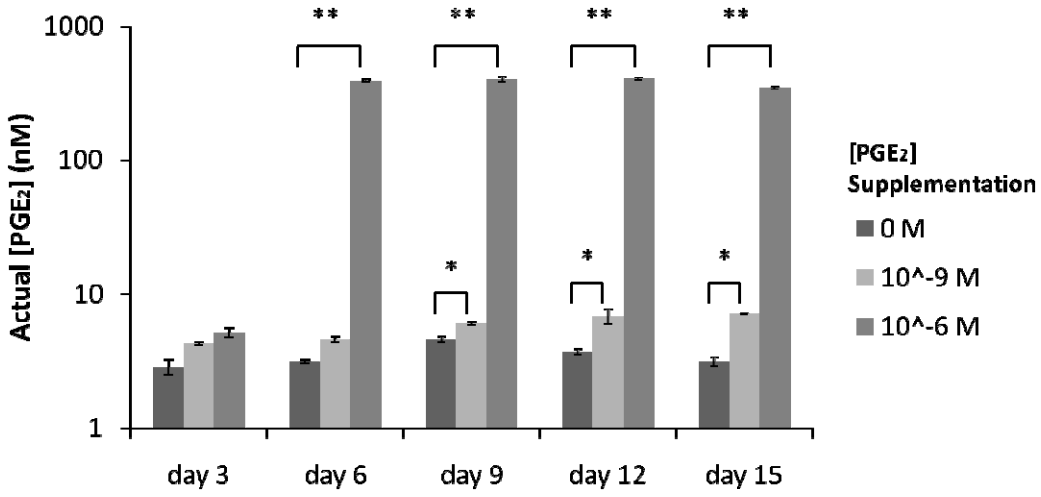



**Fig 4.6.: Relative gene expression of collagen type I (A) collagen type II (B) and collagen type X (C) by HACs cultured on gelatin-based scaffolds and stimulated with different concentrations of $PGE_2$.** Cells stimulated with $10^{-6}$ M $PGE_2$ increased collagen type II mRNA expression after two weeks and decreased collagen type I mRNA expression after week 1, in comparison to unstimulated cells. This was evidence of a re-differentiation process. Moreover, the low expression of collagen type X indicated a trend for cells not to undergo hypertrophy. Results are shown as the means of target gene expression relative to GAPDH $\pm$ 95% confidence interval, n=3. Values were normalized with respect to gene expression at $[PGE_2] = 0$ on t0 set at 1, (not shown). For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding unstimulated culture on each time point.

The qrt-PCR analysis revealed a decrease of collagen type I expression (Fig. 4.6. A) after week 1 and an increase of collagen type II expression (Fig. 4.6. B) after a two week culture period in the presence of $10^{-6}$ M $PGE_2$. Expression levels of collagen type X did not differ significantly from the non-stimulated control at any of the concentrations of exogenous $PGE_2$ (Fig. 4.6. C), indicating that cells did not drift into a hypertrophic state during the two week culture period. In addition, the decrease of collagen type I expression after week 2 also reflected the trend of cells to undergo re-differentiation. This is consistent with the conclusions from the morphological study (Fig. 4.4.), namely that chondrocytic phenotype was enhanced by $PGE_2$ stimulation.

### 4.2.3. Prostaglandin Content in HAC Culture Media

The fact that the above mentioned results were actually due to the exogenous stimulation of the different $PGE_2$ concentrations was confirmed by verifying the concentrations of undegraded $PGE_2$ quantitatively. This was done by harvesting the different cell culture media from the cells seeded on the gelatin scaffolds and measuring the total




content of prostaglandin by gas chromatography-tandem mass spectrometry.

**Fig 4.7.: Gas chromatography-tandem mass spectrometry of the $PGE_2$ contents in the cell culture media from HACs cultured onto gelatin scaffolds.** The contents of $PGE_2$ in the media confirmed that chondrocytes did not secrete $PGE_2$ themselves and that the effects mentioned previously were caused by both the exogenous stimulation of the cells and the 3-D culture environment. Values are means $\pm$ SD, n=3. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding unstimulated culture on each time point. (Measurements carried out by B. Watzer.)

The results showed that over a 15 day culture period, there was no major secretion of endogenous $PGE_2$ and that the exogenous supplementation prevailed as the increase of two to three-fold when stimulated with $10^{-9}$ M of prostaglandin and up to 250-fold when the concentration of prostaglandin supplement was $10^{-6}$ M (Fig 4.7.). This suggested that stimulated cells metabolized partially the exogenous $PGE_2$, – up to a saturation point – and that a large amount remained non-metabolized in the cell medium. These facts suggested that the different effects and reactions that HACs displayed were indeed due to exogenous stimulation by $PGE_2$ in combination with the adequate 3-D environment provided by the gelatin based scaffolds.

# 5. Co-cultures of Human Articular Chondrocytes with Murine Preosteoblastic KS483 Cells and Cal 72 Osteosarcoma Cells

## 5.1. Experimental Design and Execution

Several studies have demonstrated positive effects of different cell types on HACs phenotype and behavior (Hildner et al., 2009; Jiang et al., 2005; Mo et al., 2009; Nakaoka et al., 2006; Yang et al., 2009). By virtue of the MSC and osteoblast co-culture systems additional re-differentiation strategies will be presented within this chapter. In the three different co-culture systems described, the effect of the co-cultured cells on HACs was evaluated.

### 5.1.1. Cell Culture

The murine preostoblastic cell line, KS483, is a well-established model for the study of osteoblast differentiation (Deckers et al., 2000). This cell line possesses mesenchymal characteristics, because KS483 cells can differentiate into either adipocytes or mineralizing osteoblasts, depending on the culture conditions (van der Horst et al., 2002).

Permanent human osteosarcoma cell lines are important tools for the study of bone. As representative of an osteoblastic phenotype, Cal 72 partly reflect their normal osteoblastic counterparts and, thus, represent appropriate models to investigate the mechanism involved in bone remodelling and haematopoetic differentiation (Rochet et al., 1999).

#### *5.1.1.1. Co-culture of HACs with Cal 72 Osteosarcoma Cells and Murine Preosteoblastic KS483 Cells using Transwell Systems*

HACs in passages 4 – 6 were seeded and cultured for two weeks in 24-transwell plates with cells from osteosarcoma cell line Cal 72 cultured routinely in M199 medium, (with 20% FCS, 1% penicillin/streptomycin, and 335 µl Glutamax/100ml as supplements) and KS483 cells cultured in α-MEM (with 10% FCS and 1% penicillin/streptomycin). The inserts

membranes required a prior collagen type I coating. The coating solution consisted of 1 ml of 2.44% collagen I in $CH_3COOH$ 0.1 M. The 24-transwell plates were placed under sterile air flow overnight for proper drying of the coated membranes. To achieve cell seeding, cell density was adjusted to $2.5x10^5$ chondrocytes/cm² –for both mono- and co-cultures– and to $4x10^4$ cells/cm², for the Cal 72 osteosarcoma cells cultured on the bottom of the transwell system –only in co-culture wells–. HACs from two different donors were seeded in two 24-transwell plates (one plate per week). Twelve wells were used for each donor; six for monoculture (membranes seeded with HACs) and six for co-culture (membranes seeded with HACs and bottom surface of the transwell system with Cal 72). Week 1 and week 2 were taken as harvesting time points.

#### *5.1.1.2. Direct Co-culture of HAC with Murine Preosteoblastic KS483 Cells*

KS483 cells (passage 5) and HACs (passages 4 - 6) were cultured in complete α-MEM (containing 10% FCS and 1% penicillin/streptomycin) and complete DMEM respectively. The two cell types were embedded in collagen I hydrogels (250 µl/well) in a ratio of 1:5 and cultured in complete DMEM for 14 days in 48-well plates (harvesting time points: week 1 and week 2). In parallel, HAC and KS483 monocultures were carried out as controls (three wells per culture condition and time point).

### 5.1.2. Quantitative Real-time RT-PCR

After termination of the cell cultures, the transwell membranes and the hydrogels were stored in 500 µl Trizol at -80 ºC for future RNA isolation. A gene expression analysis of the HACs was performed using the relevant chondrocytic markers (col I, II and X, aggrecan and versican). For the transwell cultured HACs additional osteoblast markers like osteocalcin and ALP were also used to complete the study. The time points taken were week 1 and week 2, week 1 being the reference point.

### 5.1.3. Histology

Cells cultured on the membranes and within the hydrogels were fixed with either 4% formalin or with 3.7% PFA-PBS after week 1 and week 2. H&E, Safranin O and Alcian blue staining were carried out. The sections were imaged with an inverted microscope.

### 5.1.4 Proteinase K Digestion

The embedded cells were released from the hydrogel matrix by proteinase K solution digestion, that is, 1 mg proteinase K per ml digestion buffer. The amount of solution was calculated according to the number of cells in the hydrogels (1 ml/$10^6$ cells). Three hydrogels from the same culture conditions were digested in 350 µl proteinase K solution. The digestion took place in a thermomixer at 56 ºC for 16 h. After the digestion the samples were stored at -15 ºC until proceeding with the following assay (5.1.5.).

### 5.1.5. Cell Proliferation Assay

The proliferation rate of the HACs co-cultured with KS483 cells was measured with the CyQUANT proliferation assay using the digested samples mentioned in 5.1.4.. Just prior to running the experiment the CyQUANT-GR stock solution was diluted 400-fold into the 1x cell lysis buffer. First the 1x cell lysis buffer (working solution) was made by mixing 0.5 ml of the 20x stock solution with 9.5 ml of $H_2O$; next 25 µl of the CyQUANT-GR stock solution were added and mixed thoroughly. The working solution was protected from light by covering it with foil to prevent photodegradation of the CyQUANT-GR dye. The DNA standard was prepared by serial dilutions of the 100 µg/ml stock solution of bacteriophage λ-DNA with cell lysis buffer working solution; the standard range was 1 to 500 ng. Proceeding with the assay, 100 µl of each sample and standard curve were pipetted into new Eppendorf tubes and 1x lysis buffer (containing RNase A) was added to a final volume of 500 µl, mixed and incubated at RT for at least 1 h to degrade the cellular RNA. After the RNA degradation, 100 µl of each sample were pipetted into 96-well plates (in duplicate), the same amount of 2x GR-dye solution was added, mixed well and incubated at RT for 10 - 15 min in a shaker protected from light with foil. Fluorescence of the samples was measured with a microplate reader with excitation and emission wavelengths of 480 and 520 nm, respectively. The total

number of cells in the sample was determined by converting the amount of DNA per sample to cell number using the conversion factor of 7.1 pg DNA/cell reported for mammal cells.

### 5.1.6. Extracellular Matrix Quantification

The assortment of images taken from the cell culture sections of the transwell membranes for both HAC monoculture and osteosarcoma cell line Cal 72 /murine preosteoblastic KS483 cell co-cultures, were quantified with respect to the monoculture by manual measurement of the histological section obtained in each culture condition.

## 5.2. Results

### 5.2.1. Direct Co-culture of HACs and Murine Preosteoblastic KS483 Cells

The effects of co-culture were evaluated histologically using H&E and Safranin O, a typical cartilage stain. H&E staining showed that HACs cultured in monoculture retained the typical spherical morphology characteristic of differentiated chondrocytes after two weeks (Fig. 5.1. A); however HACs co-cultured with KS483 clearly assumed an elongated morphology (Fig.5.1. B), indicating a negative influence of the murine cell line on HACs.

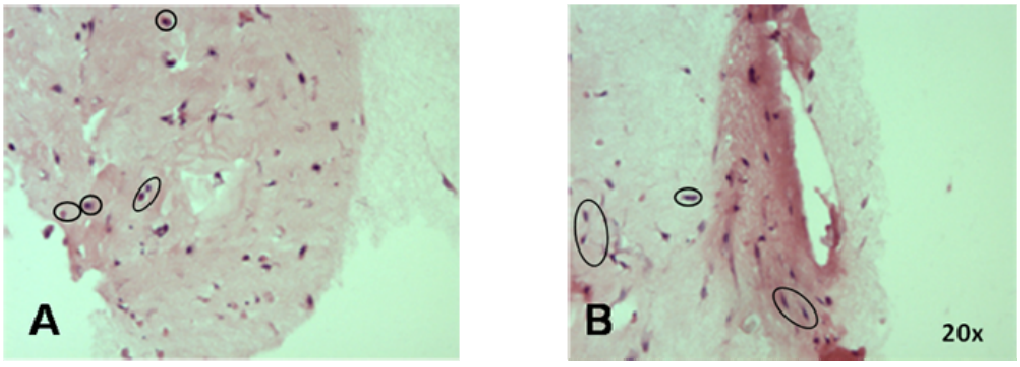



**Fig. 5.1.: Representative images of HACs in monoculture (A) and in co-culture with murine preosteoblastic cell line (B) after 2 weeks in collagen I hydrogels.** The sections were fixed and stained with H&E followed by Safranin O counterstaining. The H&E staining enabled the visualization of the cellular distribution within the hydrogel. HACs in monoculture exhibited a spherical morphology (A) while those in co-culture were elongated (B) (in circles). Safranin O did not reveal data of major interest due to the staining of the hydrogel matrix.

A further remarkable observation was that the spherical chondrocytes embedded in the collagen I matrix were surrounded by *lacunae* as in native cartilage (Fig. 5.1. A, in circle).

Safranin O stained not only GAGs from the ECM but the collagen I matrix from the scaffold as well. This particular circumstance gave confusing results of the effects regarding the co-culture on the ECM composition, necessitating additional and more specific analyses to obtain additional and more reliable results from the effects of the KS483 cells in co-culture with HACs.

To examine whether KS483 cells can contribute to chondrocyte differentiation in co-culture with HACs, quantitative analysis of expression of some representative genes of HAC ECM, *i.e.* collagen types I, II and X, aggrecan and versican (using exclusively human markers), was carried out by qrt-PCR (Fig. 5.2.). The analysis showed a trend for HACs to undergo chondrocytic re-differentiation, as the increase of collagen type II mRNA expression after week 1 under co-culture conditions (1 to 1.5-fold) and the low expression of collagen type X suggested. On the other hand this trend did not correspond with the trend of HACs to increase their collagen type I and versican expression under co-culture conditions with respect to monoculture. These contradictory results suggest that the cell-to-cell contact co-culture system of HACs and KS483 cells within collagen type I hydrogels did not enhance the chondrocytic phenotype consistent with our previous results. They are also inconsistent with the conclusions in several studies with other cell type models (Hildner et al., 2009; Xie et al., 2008; Yang et al., 2009), in which MSCs (or equivalent cell types) induced chondrocytes (and *vice versa)* to express chondrocytic markers and demonstrated a contribution to the chondrogenic potential of co-cultured MSCs.

However, the influence of the collagen I hydrogel matrix within the chondrocytic monoculture revealed an up-regulation of both aggrecan and versican mRNA expression over time (Fig. 5.2.), indicating that the matrix might have enhanced proteoglycan expression.

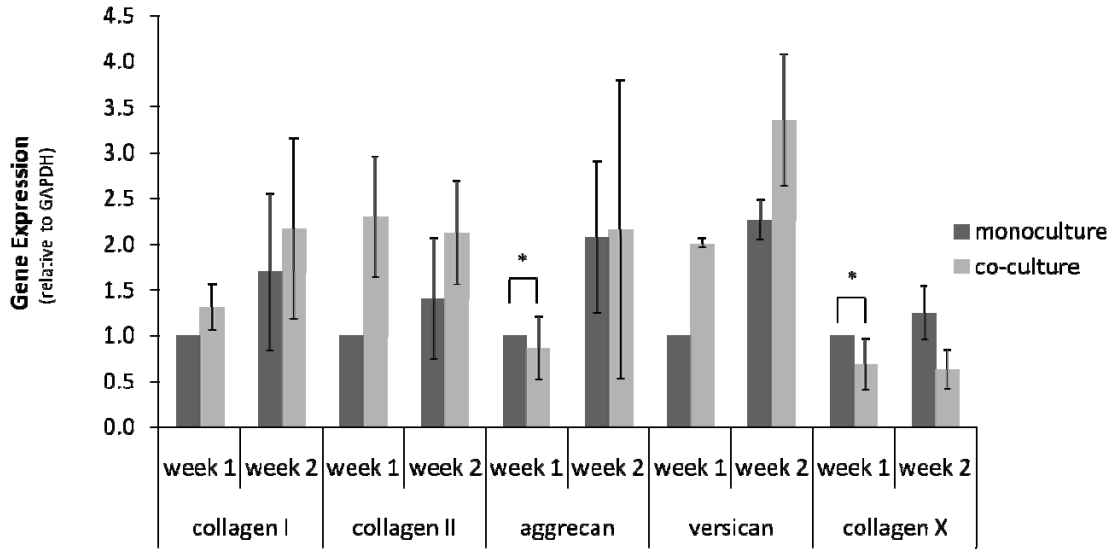



**Fig. 5.2.: Relative gene expression of relevant ECM markers by HACs in co-culture with murine preosteoblastic KS483 cells within collagen type I hydrogels.** Higher collagen type I and versican expression suggested that the effects of the KS483 cells on HACs did not enhance chondrocytic phenotype. Results are shown as the means of the gene expression relative to that of GAPDH $\pm$ SE, n=5. Values were normalized with respect to gene expression under monoculture conditions on week 1 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding monoculture on each time point.

To study the effects of the present co-culture system on the total cell number, a proliferation assay was performed (Fig. 5.3.). No significant effect was observed due to the co-culture. The total cell number within the hydrogel remained relatively constant over time for both the mono- and co-culture samples. No statistical difference was observed between the reference population (monoculture) and the co-culture group.

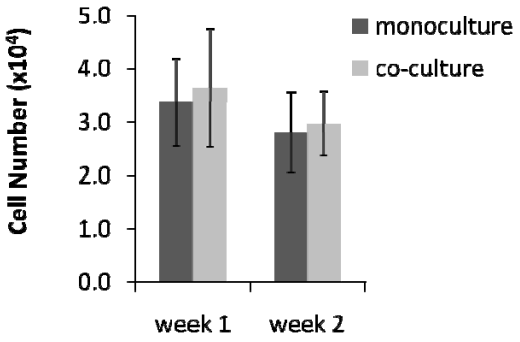



**Fig. 5.3: Cell proliferation in mono- versus co-culture of HACs and KS483 cells.** Cell number calculated based on total DNA content (7.1 pg DNA/cell). The cell number within the collagen hydrogels remained relatively constant over time.

### 5.2.2. Co-culture of HACs and Murine Preosteoblastic KS483 Cells in Transwell Systems

In transwell co-culture experiments cells were allowed to share medium without direct contact. The paracrine signals released from one cell type readily interacted with the other cell type (Nakaoka et al., 2006), thus providing insight into the effects of the co-culture, independent of the effects of the three-dimensional environment that was inherent to the collagen type I hydrogels in our previous experiments.

Histological staining was conducted for a preliminary analysis of the co-culture system. As mentioned above, the staining was performed only on the transwell membranes, that is, exclusively on HACs. H&E staining revealed a homogenous cell distribution under both culture conditions (Fig. 5.4. A). Clear ECM degradation was observed after two weeks under both culture conditions, but was much more notable under co-culture conditions (Fig. 5.4 D) as evident from the swelling of the ECM layer. Alcian blue staining, which was relatively faint in these sections, revealed an ECM deficient in proteoglycans and GAGs and was constant during the two week culture period (Fig 5.5.). The elongated morphology of HACs under both culture conditions also indicated de-differentiation.

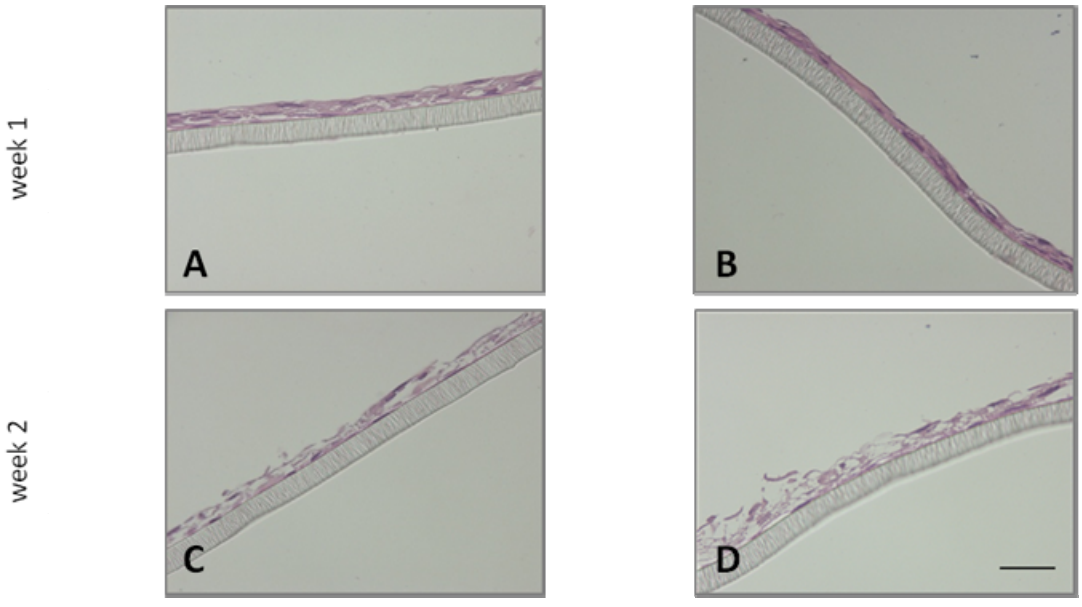



**Fig. 5.4.: Representative images of H&E stained HACs cultured on transwell membranes in monoculture (A and C) and co-cultured with KS483 cells (B and D).** Cells showed a clear trend toward ECM degradation over time. Scale bar = 30 µm.

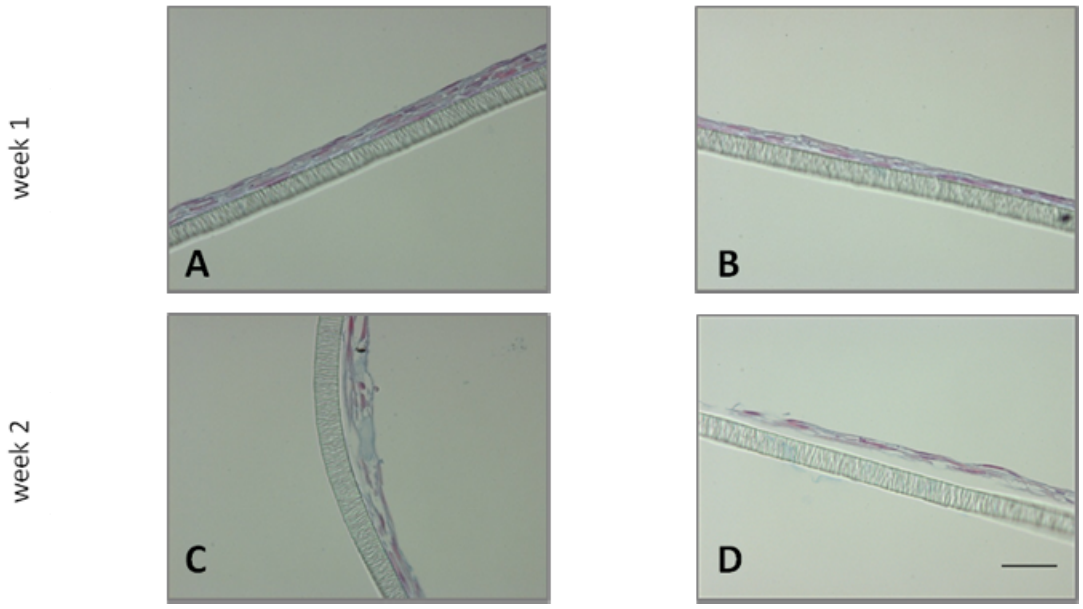



**Fig. 5.5.: Representative images of Alcian blue stained HACs cultured on transwell membranes in monoculture (A and C) and co-cultured with KS483 cells (B and D).** The resulting light blue corresponds to an ECM poor in GAGs and proteoglycans. ECM degradation after two weeks of cell culture has occurred at a significant level. Scale bar = 30 µm.

A quantification of the height of the cell layer was carried out in order to obtain further information about the effects of murine preostoblastic KS483 cells on HACs.

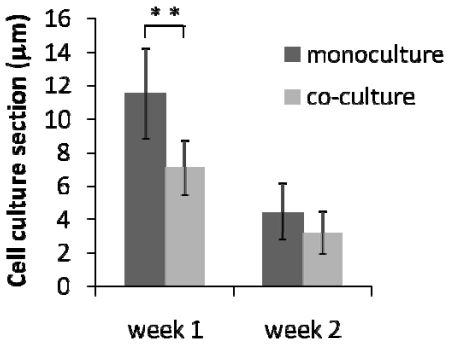



**Fig. 5.6.: Quantitative evaluation of cell culture sections.** Decrease of the height of the sections relative to co-culture conditions and time revealed that the co-culture suffered a greater decrease of ECM production. Values are means $\pm$ SD, n=9. * Statistically significant difference at $p < 0.005$ and ** at $p < 0.001$ from the corresponding reference condition (monoculture).

The ECM height of the cell layers in both culture types was a reflection of how ECM production in HACs decreased relative to their reference culture (monoculture). ECM degradation was evident over time, but under co-culture conditions a much larger extent was observed after week 1 ($p=0.0007$). Co-cultures did not differ much in terms of ECM degradation after week 2, at which time point the height of the sections was nearly the same under both culture conditions ($p=0.0922$) (Fig. 5.6.).

Quantitative analysis of mRNA expression of collagen types I, II and X, aggrecan and versican (using human specific markers) from HACs in both culture conditions was additionally performed by qrt-PCR. (Fig. 5.7.). Persistent up-regulation of collagen type X mRNA would have denoted a hypertrophic behavior of the HACs under these culture conditions. However, the sudden decrease of mRNA expression after week 2 failed to support this hypothesis.

As no relevant differences from the respective reference conditions (monoculture) were found in mRNA expression (excepting versican expression on week 1) and no remarkable changes of chondrocytic phenotype were observed in the present culture model, we concluded that the effects of the co-culture with murine preosteoblastic KS483 cells did not affect HACs expansion potential, morphology, expression of relevant ECM markers nor differentiation abilities.

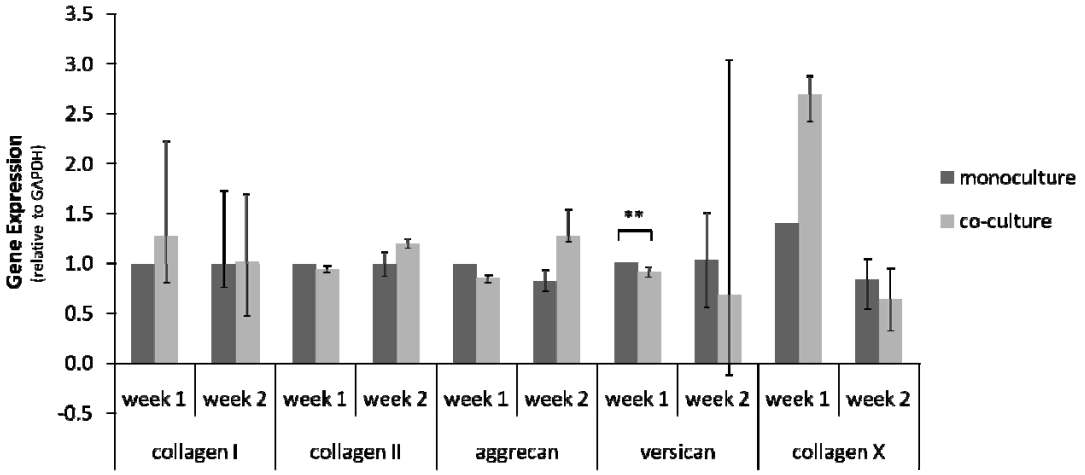



**Fig. 5.7: Relative gene expression of relevant ECM markers of HACs and murine preosteoblastic KS483 cells co-cultured in transwell plates.** No remarkable effects on mRNA expression of any of the markers were observed in this culture model, besides the fact that co-cultured cells seemed to undergo hypertrophy (up-regulation of collagen type X mRNA expression) after week 1, but the down-regulation of the collagen type X after 2 weeks of culture to the level of the expression under monoculture conditions invalidated the hypertrophy hypothesis. Results are shown as the means of target gene expression relative to GAPDH $\pm$ 95% confidence interval, n=3. Values were normalized with respect to gene expression under monoculture conditions on week 1 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding monoculture on each time point.

### 5.2.3. Co-culture of HACs and Cal 72 Osteosarcoma Cells using Transwell Systems

The present culture model was carried out resembling Nakaoka's *et al.* model with animal source cells (Nakaoka et al., 2006), in which the co-culture using transwell system was designed based on the importance of the interaction between osteoblasts and chondrocytes in osteochondral graft regeneration.

Histologically, HACs adopted a spherical morphology (in circle) and produced a wide ECM under co-culture conditions (Fig. 5.8. B and D). In contrast, HACs in monoculture did not secrete as much ECM (Fig. 5.8. A) and after two weeks of culture, cells adopted a fibroblast-like morphology (arrows) and underwent strong ECM degradation (Fig. 5.8. C).

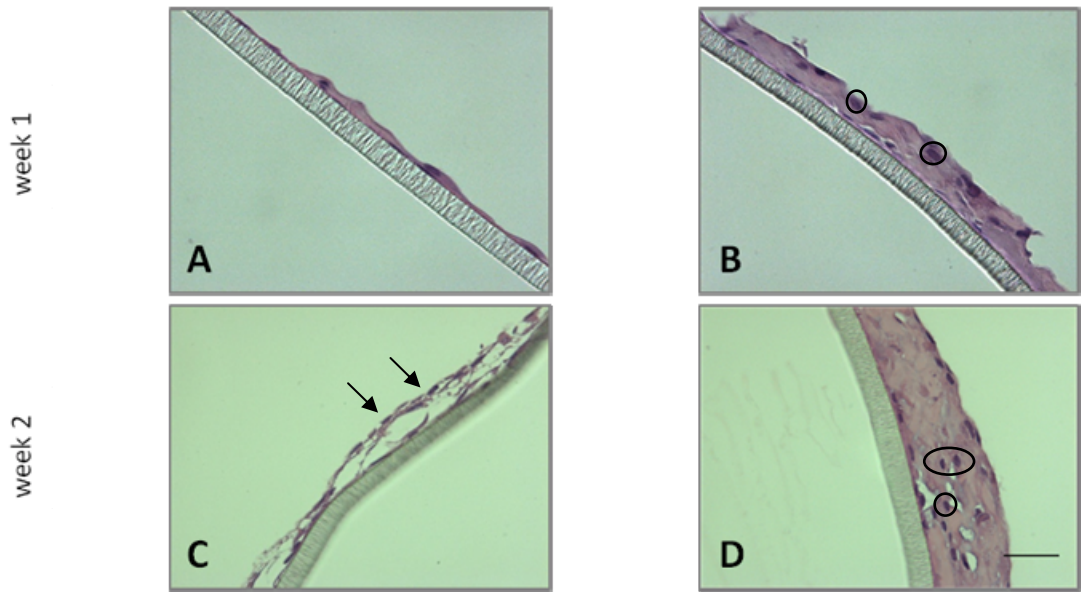



**Fig. 5.8.: Representative images of H&E stained HACs cultured on transwell membranes in monoculture (A and C) and co-culture with CAL 72 osteosarcoma cells (B and D).** A clear increase of ECM on HACs in co-culture with Cal 72 cells was observed. This fact remained consistent during the entire culture period (B and D) contrary to what occurred to HACs in monoculture, where no ECM production was observed after week 1 (A) and where ECM degradation occurred after two weeks in culture (B). Scale bar = 30 μm.

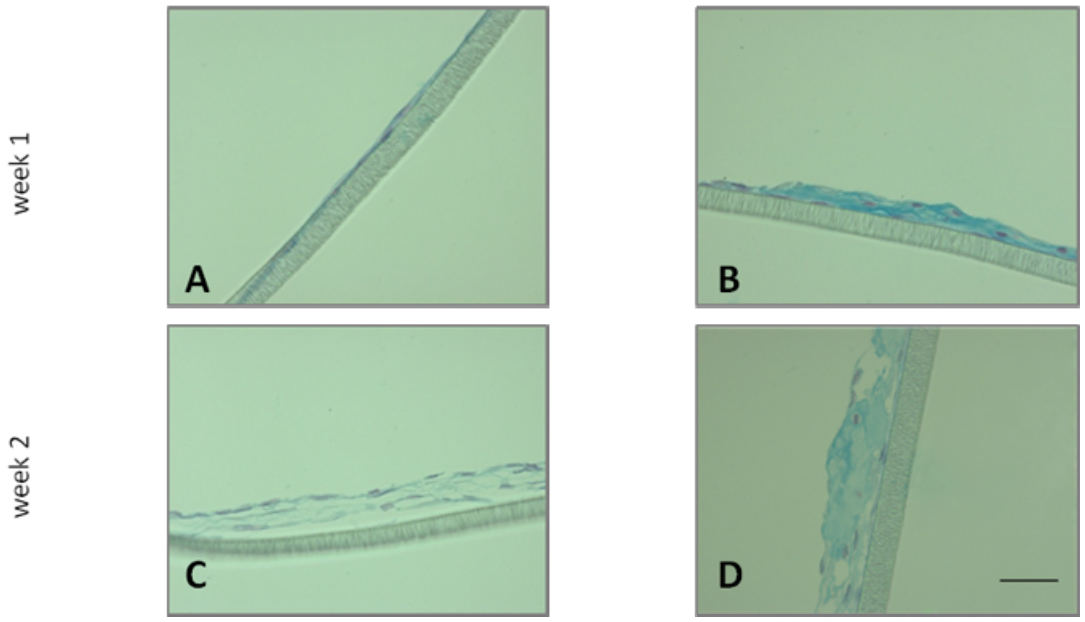



**Fig. 5.9.: Representative images of Alcian blue stained HACs cultured on transwell membranes in monoculture (A and C) and co-culture with CAL 72 osteosarcoma cells (B and D).** The intense staining of the sections corresponding to co-culture conditions (B and C) denoted an up-regulation of GAG expression. In contrast, cells cultured in monoculture after week 2 (C) displayed a less intense blue and showed ECM degradation (C). Scale bar = 30 μm.

Alcian blue staining demonstrated GAG expression by HACs when cultured together with Cal 72 cells. Less pronounced staining was observed in monoculture conditions, indicating lower content of GAG after week 1 (Fig. 5.9. A), and a significant matrix degradation on week 2 (Fig. 5.9. C).

Since an up-regulation of the secretion of ECM components was obvious from the histological studies, and since this denoted an enhancement of chondrocytic phenotype, further analyses based on these results were conducted. Quantification of cell culture sections followed by quantitative analysis of mRNA expression of typical markers from chondrocytes and osteoblasts were of major importance to elucidate some of the effects caused by the interaction of one cell type with the other cell type, in a paracrine manner.

Quantification of cell culture sections revealed that the ECM under co-culture conditions was indeed more extensive and richer in GAGs and other matrix components. The difference was markedly significant on week 2, at which point the ECM was two-fold wider with respect to week 1 and up to four-fold with respect to monoculture ($p = 3.6x10^{-8}$) conditions on week 2. HACs in monoculture conditions suffered a large ECM decrease (Fig. 5.10.).

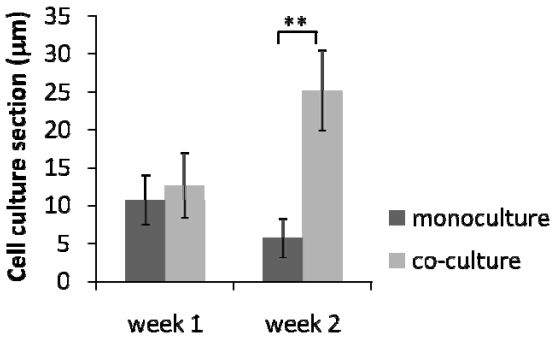
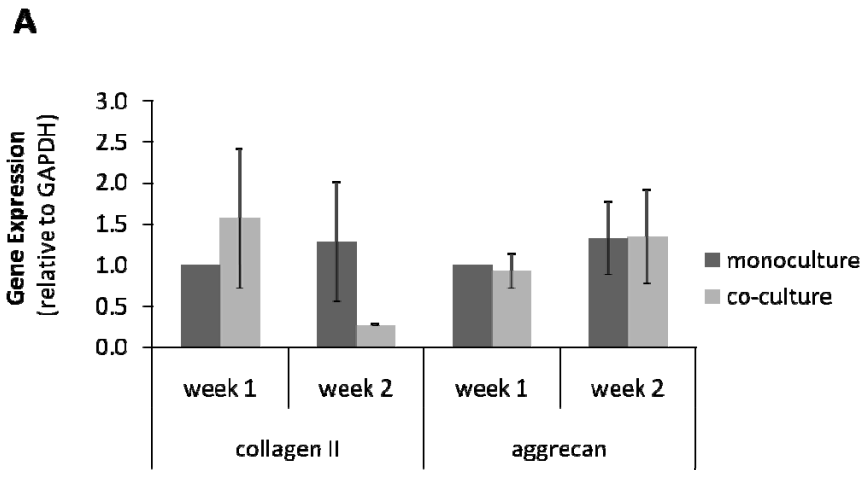



**Fig. 5.10.: Quantitative evaluation of the cell culture sections.** A 2-fold increase of the culture height under co-culture conditions after week 2 with respect to the monoculture gave evidence of an up-regulation of the ECM components secreted by the HACs. Values are means ± SD, n=9. * Statistically significant difference at $p < 10^{-6}$ and ** at $p < 10^{-7}$ from the corresponding reference condition.

Analysis of mRNA expression of collagens type I, II and X, aggrecan, and versican (typical markers for HACs), and osteocalcin and ALP (typical osteoblast markers) revealed additional aspects of the regulation of HAC differentiation process via co-culture with Cal 72 cells (Jiang et al., 2005; Nakaoka et al., 2006).



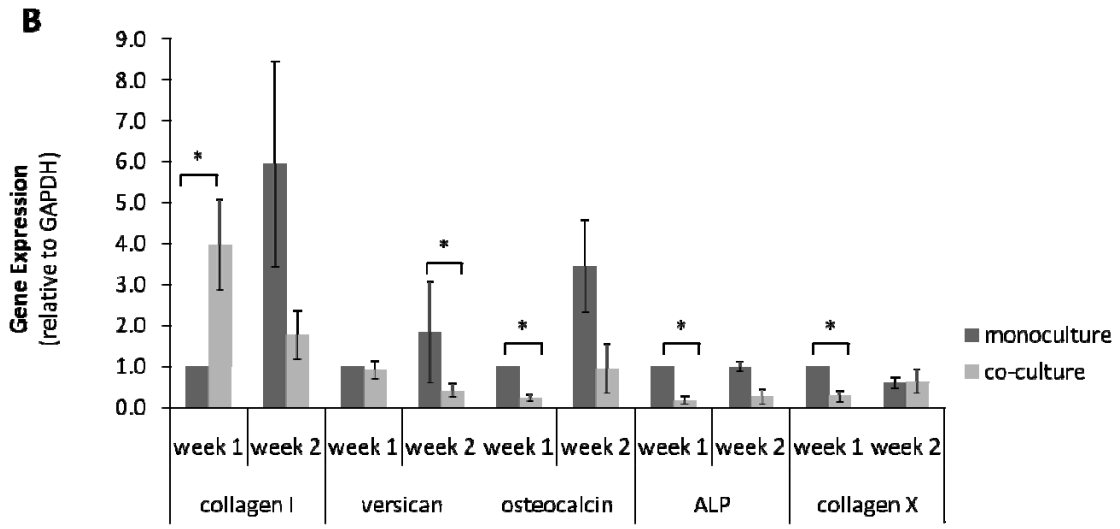



**Fig. 5.11.: Relative gene expression of relevant markers of HACs and osteoblast type cells.** Down-regulation of collagen type II mRNA on week 2 suggested a de-differentiation trend, although an increase in aggrecan expression under both culture conditions was not consistent with a general de-differentiation trend (A). A clear effect of Cal 72 and HAC co-cultures was with respect to reduction of the de-differentiated chondrocytic phenotype, as the decrease in each mRNA expression level of co-cultured HACs on week 2 indicated (B). Results are shown as the means of target gene expression relative to GAPDH ± SE, n=3. Values were normalized with respect to gene expression under monoculture conditions on week 1 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on ΔΔCt values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding monoculture on each time point.

Data for relevant differentiation markers (chondrocyte-specific markers) like collagen type II and aggrecan (Fig. 5.11. A) revealed that passaged chondrocytes on membranes in co-culture with Cal 72 gave no evidence of a chondrocytic phenotype enhancement relative to monocultures. Actually a slight decrease of collagen type II expression occurred, suggesting some de-differentiation. In contrast, quantification of de-differentiation and osteospecific markers, *i.e.* collagen type I, versican, osteocalcin and ALP (Fig 5.11. B) showed a decreasing trend in co-cultured chondrocytes, suggesting that paracrine effects via soluble factors of Cal 72 cells slowed down chondrocytic de-differentiation. The expression of a marker for hypertrophic chondrocytes, collagen type X, remained at insignificant levels during the entire culture period, in both mono- and co-cultures.

Due to the potential of osteoblasts to regulate chondrocyte metabolism (Lacombe-Gleize et al., 1995) and the implication of TGF-β1 in the regulation of many genes and proteins during chondrogenic maturation (Denker et al., 1995; Han et al., 2005) and in order to examine the interrelationship between this growth factor and chondrocytic differentiation an ELISA was carried out for the detection of TGF-β1 release from both cell types in their

respective culture media. The samples harvested to accomplish the assay corresponded to a 14 day cell culture, in which HACs and Cal 72 cells were cultured in monoculture and in co-culture. The media of all three conditions where collected separately every three days.

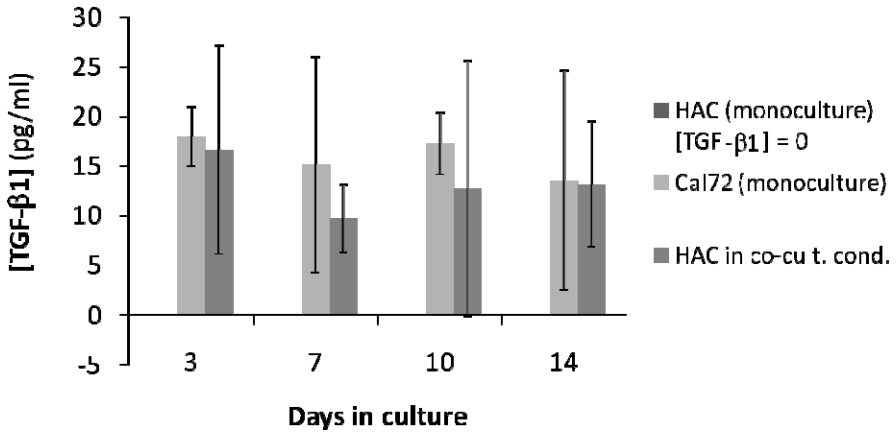



**Fig 5.12.: TGF-β1 released by HACs, Cal 72 and HACs-Cal 72 co-cultures.** HACs under co-culture conditions were under the influence of the TGF-β1 released into the medium by Cal 72 and in contrast to those cultured in monoculture, these HACs showed a clear trend of undergoing chondrocytic re-differentiation. Results are shown as means ± SD, n=3.

The absence of TGF-β1 release in HAC monoculture was indicative of a lack of maturation process. However, Cal 72 did indeed release TGF-β1 into the medium and as the increase of the growth factor up to 12-16 pg/ml (Fig. 5.12) in co-cultured HACs revealed, a diffusion process of TGF-β1 by Cal 72 into the HACs medium took place. The resulting difference between mono- and co-cultured HACs revealed how HACs initiated a maturation/re-differentiation process in the presence of Cal 72 in response to the paracrine effects of TGF-β1 (as well as other growth factors and proteins not included in this study) released in the HACs culture medium. These effects persisted for the 14 days of cell culture.

# 6. Effects of Exogenous BMP-1 Stimulation on Human Articular Chondrocytes

## 6.1. Experimental Design and Execution

In light of the important role of BMP-1 in bone and cartilage development (Steiglitz et al., 2006), we studied the response of HACs, cultured under monolayer conditions, to exogenous BMP-1 stimulation. Proliferation and differentiation parameters were taken as a reference for the evaluation.

### 6.1.1. Cell Culture

Prior to stimulation, recombinant human BMP-1 (10 μg) had to be reconstituted to 100 μg/ml in 100 μl sterile 25 mM HEPES and 0.01% Brij-35 at pH 7.5.

HACs in passages two and three were seeded and cultured in 24-well plates ($4x10^4$ cells/well). The six day stimulation consisted of three different conditions (200, 400, and 1000 ng/ml) and an untreated control. Media changes took place every two days. The media were collected and stored at -20 ºC for future TGF-β1 ELISA.

An additional BMP-1 stimulation was performed in 96-well plates ($10^4$ cells/well) taking the same conditions as mentioned above (in triplicate) and culturing for three days for crystal violet staining and an MTS assay.

### 6.1.2. Quantitative Real-time RT-PCR

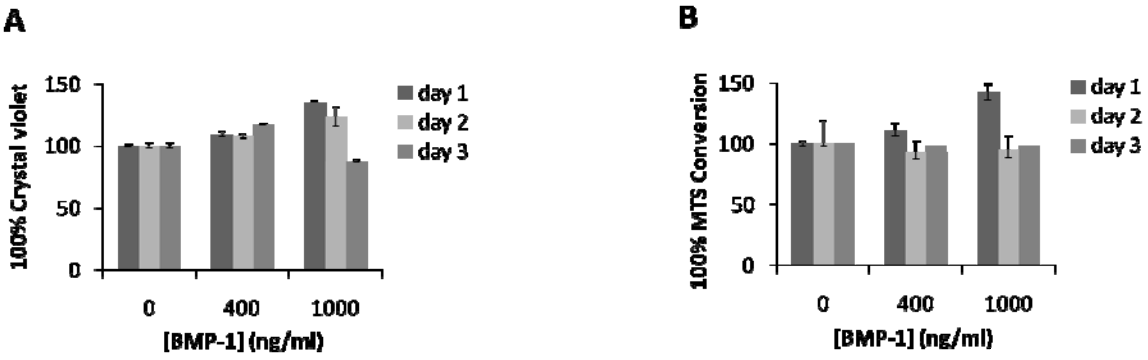
HACs from the 24-well plate culture were harvested on days three and six and gene expression analysis was performed by quantitative real-time RT-PCR using relevant chondrocytic target primers, namely collagen type I, collagen type II, and collagen type X. Gene expression on day three was taken as reference.

## 6.2. Results

### 6.2.1. Proliferation and Metabolism

A preliminary study of the effects of exogenous BMP-1 stimulation of HACs was performed. Cell proliferation was determined with crystal violet staining and an MTS assay revealed the effects of the recombinant protein on cell metabolism.

As Fig. 6.1.A shows, there was a dose-dependent effect on cell proliferation until day two. MTS assay revealed an additional effect of BMP-1 on cell metabolism, in that cells increased their metabolism after day one as a function of increasing BMP-1 concentration. This increase in metabolic activity turned into time-dependent after day two (Fig. 6.1.B), with the effects remaining constant independent of the concentration of the different exogenous stimulation.




Cell quantification relative to day 1 as measured by Crystal violet.

Metabolic activity as measured by MTS conversion.

**Fig 6.1.: Crystal violet staining (A) and MTS Assay (B) of HACs stimulated with different BMP-1 concentrations.** Crystal violet staining show no trend of HAC proliferation due to the effect of BMP-1 (A) and the MTS assay suggested that HAC metabolism was stimulated in a dose-dependent manner on day 1, and in a time-dependent manner on days 2 and 3 (B). Values are means $\pm$ SD of three wells and represent three different donors. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding unstimulated culture on each time point.

### 6.2.2. Influence of Exogenous Stimulation by BMP-1 on HAC De-differentiation

Analysis of mRNA expression of collagen types I, II and X, versican and aggrecan by qrt-PCR (Fig. 6.2.), revealed dose- and time-dependent up-regulation of those genes characterisitic for HACs undergoing de-differentiation, *i.e.* collagen type I and versican. No major differences were observed on day three in collagen type I mRNA expression with

respect to the reference condition (untreated sample on day three) under most stimulation conditions except under the highest BMP-1 concentration (1000 ng/ml) where a two-fold increase in mRNA expression occured. However, after six days in culture and under stimulation conditions mentioned above, there was indeed a dose-dependent up-regulation, and an apparent saturation point of the stimulation could be identified between 400 – 1000 ng/ml, since there was no longer a proportional increase of the mRNA expression under the influence of 1000 ng/ml of exogenous BMP-1 stimulation. Versican underwent a remarkable up-regulation with respect to control under stimulation of 400 ng/ml (2.5-fold), on both day three and day six. These facts revealed no effect of BMP-1 exogenous stimulation towards an enhancement of chondrocytic phenotype since there was no relevant variation of collagen type II and aggrecan (typical differentiation markers). A positive aspect, as collagen type X mRNA expression denoted, is that HACs did not undergo hypertrophy during the six days of cell culture in which the stimulation took place.

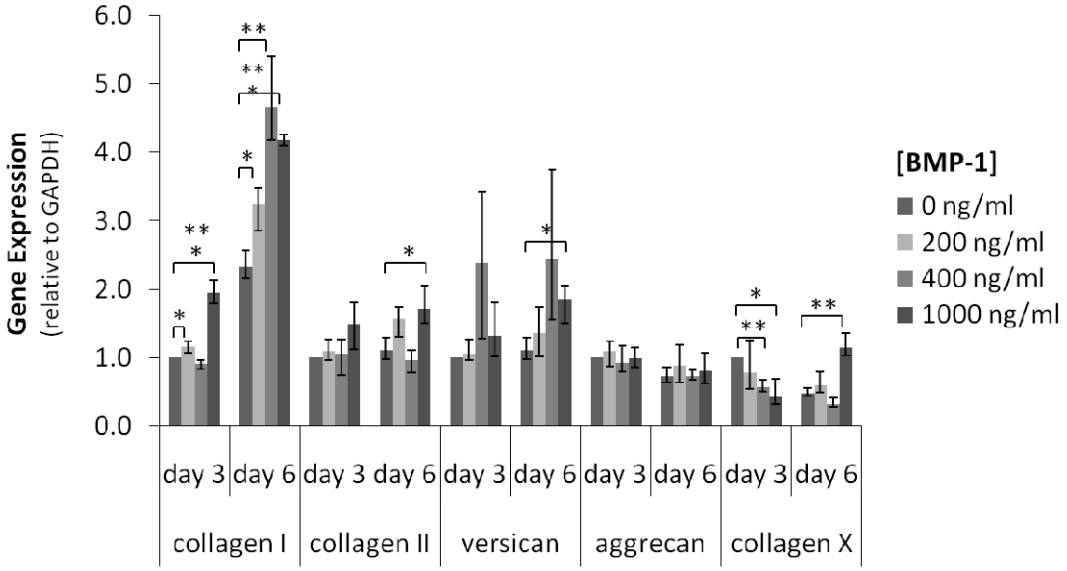



**Fig. 6.2.: Relative gene expression for relevant ECM markers of HACs cultured under monolayer conditions and stimulated with different concentrations of BMP-1.** Data showed an increase of collagen type I mRNA expression with respect to the reference condition (untreated sample day 3). This increase was remarkable on day 3 only under high concentrations of BMP-1; however on day 6 there was a dose-dependent up-regulation. Versican denoted a remarkable increase of its mRNA expression under stimulation with 400 ng/ml. No relevant effects on typical differentiation markers of HACs (collagen type II and aggrecan) were observed. The low expression of collagen type X mRNA indicated that cells did not undergo hypertrophy. Results are shown as the means of target gene expression relative to GAPDH $\pm$ 95% confidence interval, n=3. Values were normalized with respect to gene expression under [BMP-1]=0 on week 1 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ , ** at $p < 0.01$, and *** at $p<0.001$ from the corresponding unstimulated culture on each time point.

### 6.2.3. Effects of BMP-1 Exogenous Stimulation on TGF-β1 Activation

To elucidate whether BMP-1 exogenous stimulation enhanced TGF-β1 activation (Ge and Greenspan, 2006) in HAC monolayer cultures, an ELISA was carried out. The assay revealed that there was indeed an effect of BMP-1 exogenous stimulation on TGF-β1 activation. As Fig. 6.3. shows, BMP-1 concentrations higher than 200 ng/ml exceeded the limits of assimilation of HACs, eliciting at this concentration the highest level of TGF-β1 to a 2.2-fold increase with respect to the reference condition (untreated sample day three). On day six, there was no evidence of TGF-β1 activation in untreated HACs, nor in those stimulated with 1000 ng/ml. Thus, 200 ng/ml of BMP-1 remained the optimal condition for TGF-β1 activation at this time point.

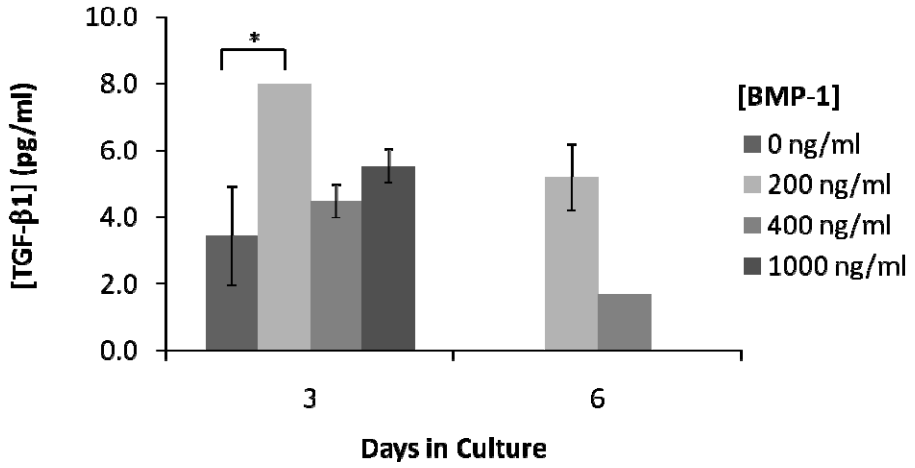



**Fig 6.3.: TGF-β1 released by HACs cultured under monolayer conditions and stimulated with different concentrations of BMP-1.** HACs stimulated with 200 ng/ml BMP-1 showed a 2.2-fold up-regulation of TGF-β1 release with respect to the reference conditions (untreated sample on day three). Higher concentrations of BMP-1 exogenous stimulation led to lower up-regulation, revealing an assimilation limit of HACs. On day six the up-regulation of TGF-β1 was higher with 200 ng/ml of BMP-1 exogenous stimulation, confirming the fact that higher concentrations did not enhance TGF-β1 activation. Results are shown as means $\pm$ SD, n=3. * Statistically significant difference at $p < 0.05$ from the corresponding unstimulated culture on each time point.

# 7. Co-culture of Human Articular Chondrocytes with Transiently Transfected Cal 72 Osteosarcoma Cells

## 7.1. Experimental Design and Execution

In an attempt to apply gene therapy to HACs, BMP-1 was cloned into two different vectors and these vectors were included in different strategies following different lines of transfection and culture models. In short, competent cells were transformed with the pIRES-AcGFP1 and pIRES-AcGFP1-Nuc vectors. The plasmids were purified, the BMP-1 gene was isolated from the pIRESneo2-BMP-1 vector and Nhe I and Sal I restriction sites were attached to the ends of the gene. Finally, the BMP-1 gene was ligated into the pIRES-AcGFP1 and pIRES-AcGFP1-Nuc vectors, and transient transfections of HeLa and Cal 72 cells, as well as HACs were carried out.

### 7.1.1. Transformation of Competent Cells with pIRES-AcGFP1 and pIRES-AcGFP1-Nuc

Prior to the transformation, the stock solutions of both plasmids (50 ng/μl) were diluted to an end concentration of 5 ng/μl and the XL1-Blue competent cells were thawed on ice. Once the cells were thawed, they were gently mixed and 50 μl were transferred into two pre-chilled 2 ml tubes (one for each transformation). 4 μl of each vector were added (separately) to the competent cells. The tubes were swirled gently and incubated on ice for 20 min in order to facilitate the opening of the membranes for the insertion of the exogenous DNA. The tubes were then heat-pulsed in a 42 ºC water bath for 45 s thus allowing the DNA to enter the cells, and immediately cooled down on ice for 2 min to allow the recuperation of the conditions of the competent cells. 900 μl of pre-warmed SOC medium were then added and incubation at 37 ºC with shaking at 250 rpm was carried out for 30 min. In this step the actual transformation took place. After the incubation period, the transformation mixture was plated on LB agar plates containing kanamycin (200 μl/plate) and incubated at 37 ºC overnight.

### 7.1.2. Plasmid Purification: EndoFree Plasmid Maxi Kit

A single colony from the transformed cells grown overnight was picked and inoculated as a starter culture in 5 ml LB-kanamycin medium followed by incubation for 8 h at 37 ºC with vigorous shaking (300 rpm). The starter culture was diluted 1:500 in 250 ml selective LB medium, and once more incubated for 15 h. The cells were harvested by centrifugation at 6000 g for 15 min at 4 ºC, the supernatant was removed and the pellet was resuspended in Buffer P1 for cell lysing. 10 ml of Buffer P2 were added and mixed gently but thoroughly by inverting 4-6 times. After 5 min of incubation at RT, 10 ml of chilled Buffer P3 were added to the lysate and mixed immediately again by inverting 4-6 times thus enhancing DNA precipitation. The lysate was directly poured into the barrel of a QIAfilter Cartridge and incubated at RT for 10 min. A precipitate containing proteins, genomic DNA, and detergent formed a layer on top of the solution. This ensured convenient filtration without clogging. Once the incubation was over, the cap from the QIAfilter outlet nozzle was removed and the plunger was carefully inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into a 50 ml tube. 2.5 ml Buffer ER were added to the filtered lysate, mixed by inverting approximately 10 times and incubated on ice for 30 min. The lysate was then applied to a QIAGEN-tip (previously equilibrated with 10 ml Buffer QBT), and allowed to enter the resin by gravity flow. Thereafter, the tip was washed twice with 30 ml Buffer QC and DNA elution was carried out with Buffer QN. The eluted DNA precipitated by adding 0.7 volumes of isopropanol, mixing and centrifuging at 15000 g for 30 min at 4 ºC. After carefully decanting the supernatant, the DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 15000 g for 10 min. The supernatant was discarded, the pellet was air-dried for 10 min and the DNA was redissolved in Buffer TE.

### 7.1.3. Isolation of the BMP-1 Gene from the pIRESneo2-BMP-1 Vector

The BMP-1 gene was isolated by RT-PCR using specific primers that included the sequences encoding Nhe I and Sal I restriction sites as overhangs, thus obtaining from the RT-PCR the gene with Nhe I and Sal I at 3' and 5' ends respectively (Fig. 7.1.). A high transcription fidelity was achieved with the HotStarTaq Plus DNA polymerase which required an additional activation step of 5 min at 95 ºC. The annealing temperature for the specific primers was 65 ºC and the concentration of the vector sample was 10 pg/µl. Agarose gel

electrophoresis with the PCR product was performed, and the band corresponding to the insert was excised from the gel with a clean scalpel for DNA extraction.

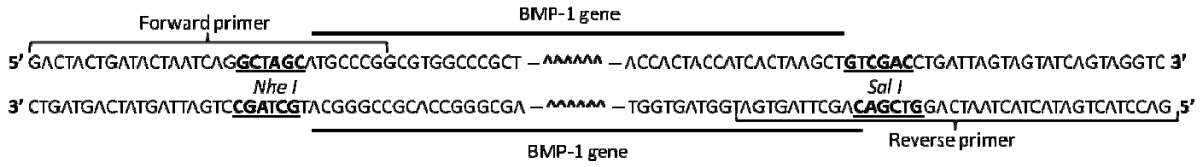



**Fig. 7.1.: BMP-1 gene and primers used for its amplification and insertion into the pIRES-AcGFP1 and pIRES-AcGFP1-Nuc vectors.**

The gel slice was weighed in a 2 ml tube and three volumes of Buffer QG to 1 volume of gel were added. Incubation for 10 min at 50 ºC was performed (until the gel slice was completely dissolved), to help dissolve the gel the content of the tube was mixed by vortexing every 2-3 min during the incubation. One gel volume of isopropanol was added to the sample and mixed. To bind DNA, the sample was applied to the QIAquick column, and the column was then centrifuged at maximum speed in a microcentrifuge for 1 min. After discarding the flow-through, the column was placed back in the same collection tube, 500 µl of Buffer QG were added, and the column was centrifuged for 1 min at full speed. The flow-through was discarded, and for the washing process, 750 µl of Buffer PE were added to the sample and left during three min. An additional centrifuge step at maximum speed of one min was carried out. To ensure the complete remove of residual ethanol, the flow-through from the previous step was discarded and the sample was centrifuged for 1 min once again. For the DNA elution, the column was placed in a clean 1.5 ml tube and 20 µl of Buffer EB were added to the center of the QIAquick membrane and centrifuged at maximum speed for 1 min. For an increased DNA concentration, this step was repeated.

### 7.1.4. Nhe I-BMP-1-Sal I Insert Amplification with pGEM-T Vector

In order to amplify the BMP-1 gene including the attached restriction sites, the gene was inserted into a pGEM-T vector with T4 DNA ligase. To proceed to the ligation a final volume of 10 µl, 2 µl of ligase reaction buffer, 1 µl of vector, 3 µl of the BMP-1 gene and 1 µl of ligase were required. The ligation took place at 14 ºC overnight and the ligase was

inactivated at 65 ºC for 10 min. Finally, competent cells were transformed with the resulting vector.

### 7.1.5. Nhe I + Sal I Double Digestion

Prior to the digestion, the ligated vector was purified with the GeneJET Plasmid Miniprepkit as described by the manufacturer. Since the restriction sites for the Nhe I and Sal I enzymes were attached to the BMP-1 insert, the excision of the insert from the pGEM-T vector was done by a double digestion with these two restriction enzymes. Due to the incompatible reaction conditions of both enzymes, the vector had to be digested in two different steps. The first digestion was carried out with Nhe I: 1 µl of the restriction enzyme, 2 µl of the specified reaction buffer (no. 4) and 3 µg of the vector adjusted to 20 µl volume with $H_2O$ and well mixed, were incubated at 37 ºC for 1 h. Nhe I was inactivated at 65 ºC for 20 min. To enable the second digestion, 2 µl of the specified reaction buffer (no. 10), and 1 µl of Sal I had to be added to the mixture. An additional 1 h incubation at 37 ºC took place. The enzyme was then inactivated as done previously.

### 7.1.6. Digestion Clean-up and Insert Purification

Once the digestion was completed, the resulting DNA was purified from all the enzymatic reagents with the MinElute Reaction Cleanup Kit: 300 µl of Buffer ERC were added to the enzymatic reaction and mixed. The mixture was applied to a MinElute column and centrifuged for 1 min to bind the DNA to the membrane. After discarding the flow-through, the bound DNA was washed by adding 750 µl Buffer PE and centrifuging for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min at maximum speed to remove completely the ethanol from the Buffer PE. The column was placed in a new 1.5 ml tube and the DNA was eluted with 20 µl Buffer EB, the elution was left for one min and the tube was then centrifuged for 1 min. To increase the DNA concentration a second elution step was performed. The product was analyzed by agarose gel electrophoresis to verify product sizes. The band corresponding to the Nhe I-BMP-1-Sal I construct was extracted from the gel as mentioned in section 7.1.3.

### 7.1.7. Alkaline Phosphatase Digestion

Dephosphorylation of the linearized cloning vectors (pIRES2-AcGFP1 and pIRES2-AcGFP1-Nuc) was carried out to prevent recircularization during ligation. A mixture containing 1 μg linear DNA, 2 μl of 10x FastAP buffer, 1 μl FastAP Thermosensitive Alkaline Phosphatase was adjusted with $H_2O$ to a volume of 20 μl and then incubated at 37 ºC for 10 min. The reaction was terminated by heating it at 75 ºC for 5 min, and the dephosphorylated plasmid was then cleaned-up as described in section 7.1.6..

### 7.1.8. Ligation of BMP-1 into pIRES-AcGFP1 and pIRES-AcGFP1-Nuc

The BMP-1 construct was ligated into the dephosphorylated vectors with T4 DNA ligase and under the supervision of Dr. A. Cid-Arregui (DKFZ, Heidelberg, Germany). For a final volume of 10 μl, 1 μl of ligase reaction buffer, 2 μl of vector, 4 μl of BMP-1 construct, and 1 μl of enzyme were required. The ligation, followed by a transformation of MAX Efficiency DH5α competent cells, took place as in previous transformation steps. The resulting colonies were selected and the ligated plasmids were purified. To verify successful ligation, the plasmids were digested with different restriction enzymes (Nhe I, Sal I, Dra I and Ssp I) and the sizes of the products were confirmed by agarose gel electrophoresis.

### 7.1.9. Transfection of Various Cell Types with the BMP-1 Encoding Plasmids by Electroporation

| Cell type | Cell number/ml | DNA (μg) | Buffer (μl) | Electroporation conditions(*) |
|---|---|---|---|---|
| Cal 72 | $5.6 \times 10^5$ | 10 | 1000 | 1220 V, 40 ms, 1 pulse |
| HAC | $6.4 \times 10^5$ | 10 | 800 | 800 V, 100 μs, 1 pulse |
| HeLa | $1 \times 10^6$ | 10 | 400 | 800 V, 100 μs, 1 pulse |

(*) Conditions that yielded best transfection efficiency and highest cell viability after thorough optimization.

Cells were cultured in monolayer to a confluence of 70-80%. After harvesting, counting and spinning (900 g for 5 min) the required number of cells, the pellet was resuspended in hypoosmotic or resuspension buffer and DNA was added to the suspension.

Once the electroporation was completed, the resulting solution was transferred to antibiotic-free DMEM in a 12-well plate. Selection of transfected cells was achieved by addition of G418 (400 μg/ml) to the medium (Van Raay et al., 2008).

### 7.1.10. Cell Culture

HACs in passages 2 – 3 were seeded and cultured for six days in pre-treated 24-transwell plates (5.1.1.1.) with Cal 72 cells. The cell seeding, cell ratio was adjusted to 1:5 as in our previous co-culture in transwell system (5.2.2. and 5.2.3.). There were six different culture conditions, with the chondrocytes always growing on the insert membrane:

a) chondrocytes (monoculture)
b) chondrocytes + Cal 72 cells
c) chondrocytes + transfected Cal 72 cells (pIRES2-AcGFP1)
d) chondrocytes + transfected Cal 72 cells (pIRES2-AcGFP1-Nuc)
e) chondrocytes + transfected Cal 72 cells (pIRES2-AcGFP1-BMP-1)
f) chondrocytes + transfected Cal 72 cells (pIRES2-AcGFP1-Nuc-BMP-1)

### 7.1.11. Analysis of Protein Expression

#### *7.1.11. 1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Protein extracts from the transfected HeLa cells and Cal72 osteosarcoma cells were separated according to molecular weight in denaturing polyacrylamide gel (Laemmli, 1970). The gels consisted of 7.5% and 12.5% resolving and 5% stacking gels. The resolving gel solution (7.5 % or 12.5 % PAA, 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.05% APS, 0.005% TEMED) was cast between two glass plates and n-butanol was put on top of the resolving gel solution layer. After gel polymerization n-butanol was removed, stacking gel solution (5 % PAA, 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% APS, 0.005% TEMED) was cast on top of the resolving gel and a comb was inserted between the glasses in order to obtain 10 wells after gel polymerisation. From 5 to 30 μg of protein were mixed with 1/4 volume of RotiLoad-1 loading buffer and the samples were denatured at 95 °C for 5 min and loaded into the wells of the stacking gel. Electrophoretic separation of proteins was carried out in SDS-Running Buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) at 25 mA per gel.

#### *7.1.11.2. Western blot*

After separation by SDS-PAGE proteins were transferred to a nitrocellulose membrane. The membrane and the gel were equilibrated in SDS-transfer buffer (25 mM Tris-HCl, pH 8.0, 100 mM Glycine, 25% MetOH), and then the gel was put on top of the membrane and covered with 3 sheets of wet filter paper from both sides. Finally, the gel was covered with wet sponges and put vertically into a holder of a PROTEAN Mini transfer chamber so that the membrane was oriented toward the anode. An ice container was inserted into the chamber and the chamber was filled with SDS-transfer buffer. The transfer of proteins from the gel to the membrane was carried out for 1 h at 350 mA. Afterwards protein transfer was verified by Ponseau S staining, which was then washed out with PBS. To block the unspecific binding sites the membrane was incubated 1 h at RT in blocking solution (5% Milk powder in PBS-0.2% (v/v) Tween 20). The membrane was then incubated with primary antibody (BMP-1 and GFP, diluted in blocking solution) for 2 h at RT or overnight at 4 °C and subsequently washed twice with PBS-0.2 % Tween 20 for 5 min each to remove unbound antibody. At the end the membrane was incubated with corresponding HRP-linked secondary antibodies and washed again (3 times for 5 min in PBS-0.2% Tween-20). The antibody bound to the target protein was detected with ECL detection reagents and high performance chemiluminescent film. To probe the same membrane with another antibody, membrane stripping was performed in stripping buffer (100 mM Glycine-HCl pH 2.8, twice 20 min) to remove previously bound antibodies.

## 7.2. Results

### 7.2.1. Ligation of the BMP-1 construct into the pIRES2-AcGFP1 and pIRES2-AcGFP1-Nuc vectors

Plasmids pIRES2-AcGFP1 and pIRES2-AcGFP1-Nuc containing the BMP-1 construct (Appendix I) were generated as mentioned in section 7.1. and tested via Nhe I-Sal I double digestion. An additional Dra I digestion was carried out as well, followed by agarose gel electrophoresis to verify the sizes of the plasmids and the insert (Fig. 7.2.).

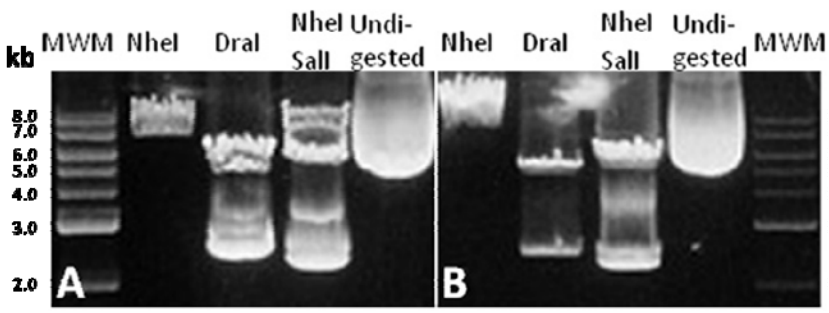



**Fig. 7.2.: Agarose gel electrophoresis after restriction digest of pIRES2-AcGFP1-BMP-1 (A) and pIRES2 AcGFP1-Nuc-BMP-1 (B).** Restriction enzymes and their digestion products: Nhe I (7.5 kb and 7.6 kb), Dra I (5.0+2.5 kb and 5.2+2.4 kb) and Nhe I+Sal I double digestion (BMP-1 gene 2.2 kb+5.3 and 5.4 kb respectively). Control: Undigested plasmid. MWM: 1kb DNA Ladder.

### 7.2.2. Transfection of HeLa Cells with pIRES2-AcGFP1-BMP-1 and pIRES2-AcGFP1-Nuc-BMP-1

Once the efficiency of the ligation was verified, an initial transfection of HeLa cells was performed by applying microporation under the supervision of Dr. A. Cid-Arregui (DKFZ, Heidelberg, Germany). After 72 h in culture, cells remained alive and a successful transfection was achieved, as the high GFP expression revealed (Fig. 7.3.).

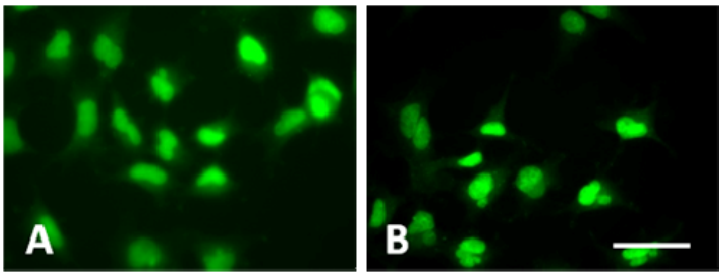



**Fig. 7.3.: Representative images of transfected HeLa cells after 72 h in monolayer culture.** HeLa cells were transfected with both pIRES2-AcGFP1-BMP-1 (A) and pIRES2 AcGFP1-Nuc-BMP-1 (B). Scale bar = 50 μm.

SDS-PAGE followed by Western blotting confirmed the successful transfection by electroporation and verified that the IRES sequence of the host vectors ensured the expression of BMP-1 precursor and GFP together (Fig. 7.4.) enabling further studies with our ligated plasmids.

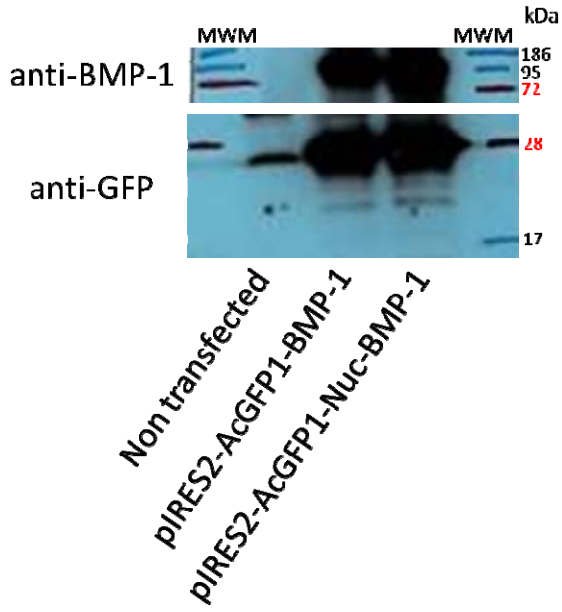



**Fig. 7.4. Western blot analysis of BMP-1 & GFP protein expression by HeLa cells transfected with pIRES2-AcGFP1-BMP-1 and pIRES2 AcGFP1-Nuc-BMP-1.** Transfected cells expressed BMP-1 precursor (110 kDa) and GFP (25 kDa) as revealed by specific antibody staining. Control: Non-transfected HeLa cells. MWM: PageRuler Prestained Protein Ladder.

### 7.2.3. Transfection of HACs with pIRES2-AcGFP1-BMP-1 and pIRES2-AcGFP1-Nuc-BMP-1

Once the plasmids had been tested and verified, to optimize the gene transfection protocol for HACs, these cells were transfected with both BMP-1 containing plasmids by various transfection methods, including a liposome-based protocol (Cid-Arregui et al., 2003) and commercial electroporation with various parameters (based on the manufacturers' instructions). Electroporation of HACs when conducted under the conditions mentioned in the "Methods" section (7.1.9), yielded the best results, although the transfection efficiency was extremely low after 72 h in culture (<10%) as shown in the representative fluorescence microscopic images presented in Fig. 7.5..

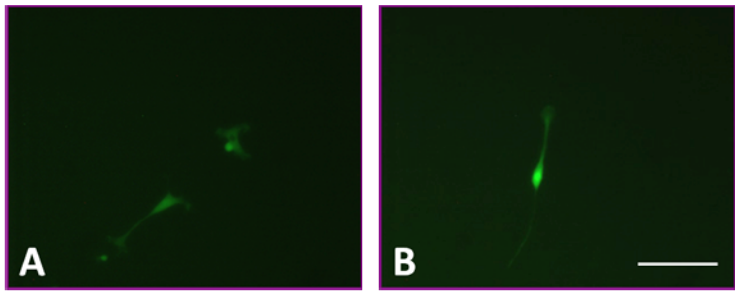



**Fig. 7.5.: Representative images of transfected HACs after 72 h in monolayer culture.** HACs were transfected with both pIRES2-AcGFP1-BMP-1 (A) and pIRES2 AcGFP1-Nuc-BMP-1 (B), showing very low transfection efficiency. Scale bar = 200 µm.

### 7.2.4. Transfection of Cal 72 Osteosarcoma Cells with pIRES2-AcGFP1-BMP-1 and pIRES2-AcGFP1-Nuc-BMP-1 vectors

Since the transfection of HACs did not lead to successful transient transfection (defined as rate of transfection $\geq$ 60%); in order to maintain the aim of the study, further attempts of transfections were made with Cal 72 cells. This cell type was susceptible to a much higher transfection efficiency (Fig. 7.6.) thus providing a high amount of transiently transfected Cal 72 cells for further co-culture studies with HACs.

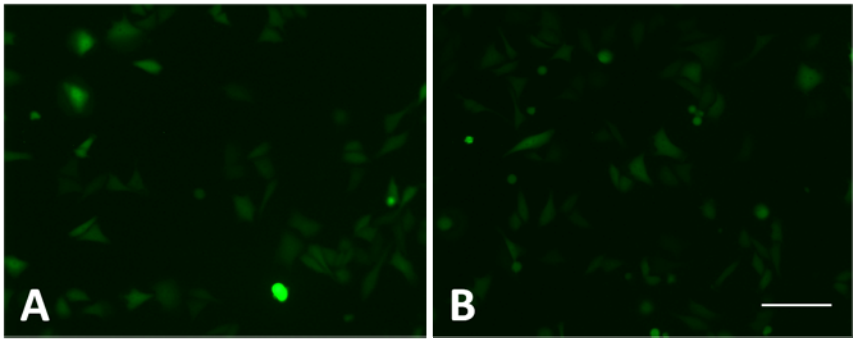



**Fig. 7.6.: Representative images of transfected Cal72 osteosarcoma cells after 72 h in monolayer culture.** HACs were transfected with both pIRES2-AcGFP1-BMP-1 (A) and pIRES2 AcGFP1-Nuc-BMP-1 (B). In contrast to HACs, this cell line displayed higher transfection efficiency, thus enabling further studies in co-culture with HACs. Scale bar = 150 μm.

Prior to initiating the co-culture studies, A. Sartorius (Universitaetsmedizin, Mainz, Germany) transfected Cal 72 cells with pIRES2-AcGFP1 and pIRES2-AcGFP1-Nuc plasmids as well as with our BMP-1 containing plasmids. SDS-PAGE followed by Western blot was carried out in order to verify the expression of BMP-1 and GFP within the transfected cells. The Western blots revealed GFP expression by all cell populations transfected with any of the four different plasmids, but BMP-1 expression could not be verified (Fig. 7.7.), although the plasmid IRES sequences should have also ensured the expression of BMP-1 in those cases in which its gene had been inserted into the multiple cloning site (MCS) of the expression vector. The inability to prove BMP-1 expression could be due to several factors, such as poor quality of the cell lysates, the large size of BMP-1 in comparison to GFP, etc. Regardless of the missing confirmation that BMP-1 was expressed by the transfected cells, we proceeded to the co-culture systems.

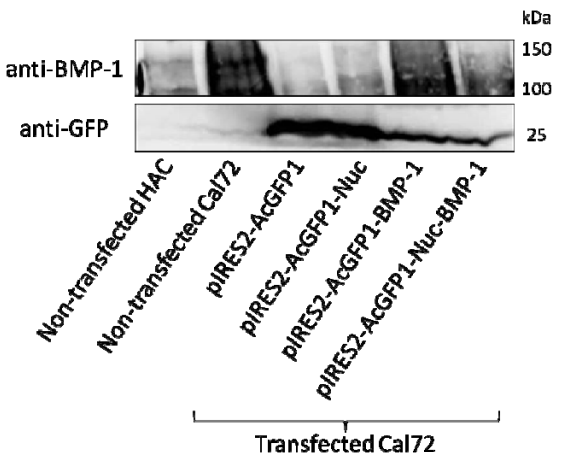



**Fig. 7.7.: Western blot analysis of HACs and Cal 72 cells transfected with pIRES2-AcGFP1, pIRES2 AcGFP1-Nuc, pIRES2-AcGFP1-BMP-1 and pIRES2 AcGFP1-Nuc-BMP-1.** Transfected cells expressed GFP (25 kDa) as indicated. There was no evidence of BMP-1 precursor (110 kDa) expression by any of the transfected cells. Control: non-transfected HACs and Cal 72 cells. Molecular weight marker: Precision Plus Protein Standards Dual Color.

### 7.2.5. Co-culture of HACs and Transiently Transgenic Cal 72 Osteosarcoma Cells using Transwell Systems

The present culture model was carried out resembling Nakaoka's et al.'s model with animal source cells (Nakaoka et al., 2006) as detailed in section 5.2.2..

Due to the short culture period of six days, HACs did not undergo much change over time under any of the culture conditions. However, H&E staining revealed that HACs in co-culture adopted a spherical morphology in contrast to the elongated morphology of those cells cultured in monoculture. Remarkable was the spherical morphology (in circles) adopted by those HACs co-cultured with Cal 72 cells tranfected with either pIRES2-AcGFP1-BMP-1 (Fig. 7.8. I and J) and the thin ECM in which they were embedded. Alcian blue staining showed an ECM poor in GAGs and other characteristic elements in the six different culture conditions (Fig. 7.9.).

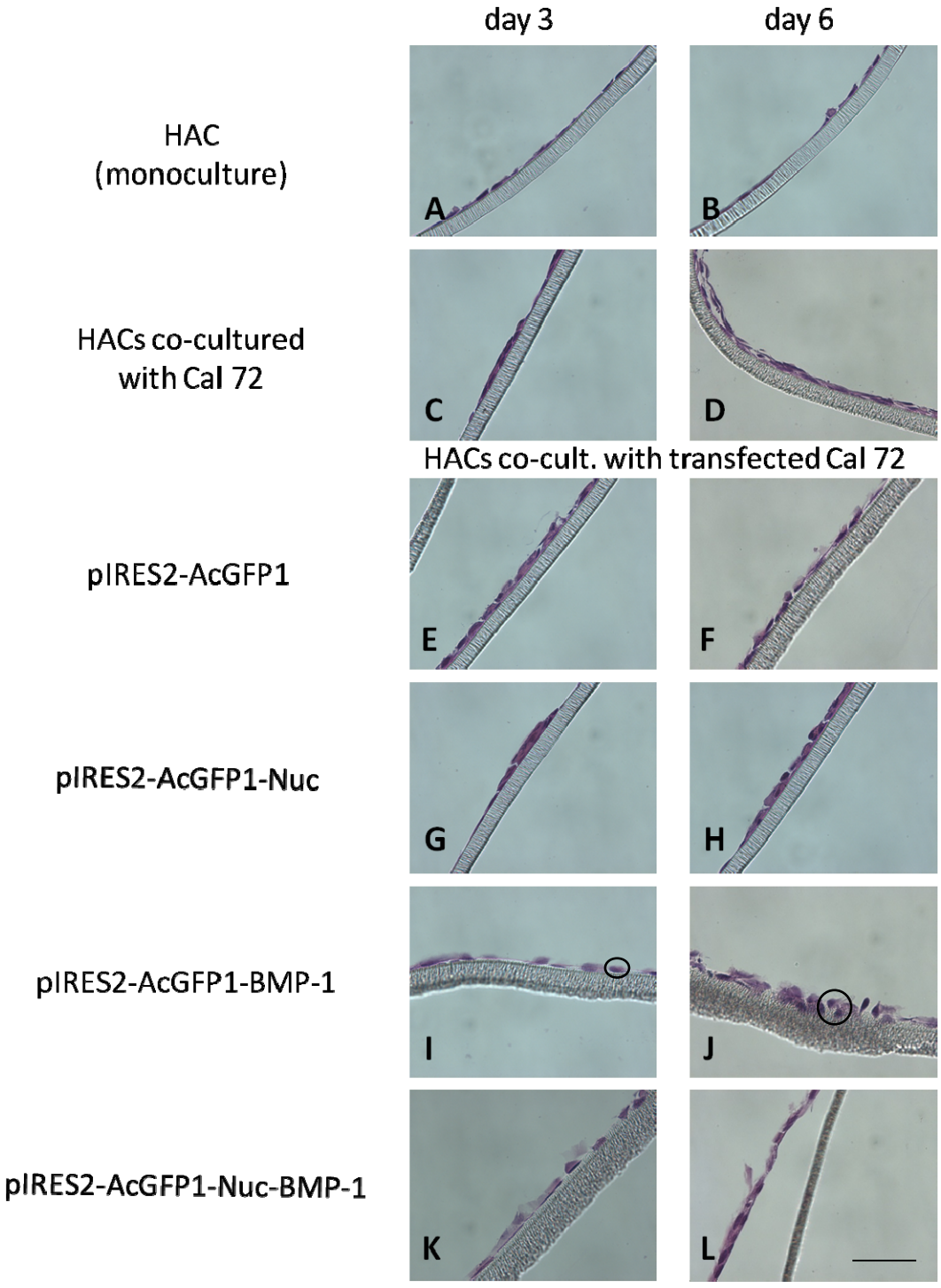



**Fig. 7.8.: Representative images of H&E stained HACs cultured on transwell membranes in monoculture (A and B) and co-culture (C, D, E, F, G, H, I, J, K and L).** Cells that were co-cultured with Cal 72 cells transfected with either pIRES2-AcGFP1-BMP-1 (I and J) or pIRES2 AcGFP1-Nuc-BMP-1 (K and L) displayed a spherical morphology and were embedded in a highly irregular ECM. Scale bar = 50 µm.

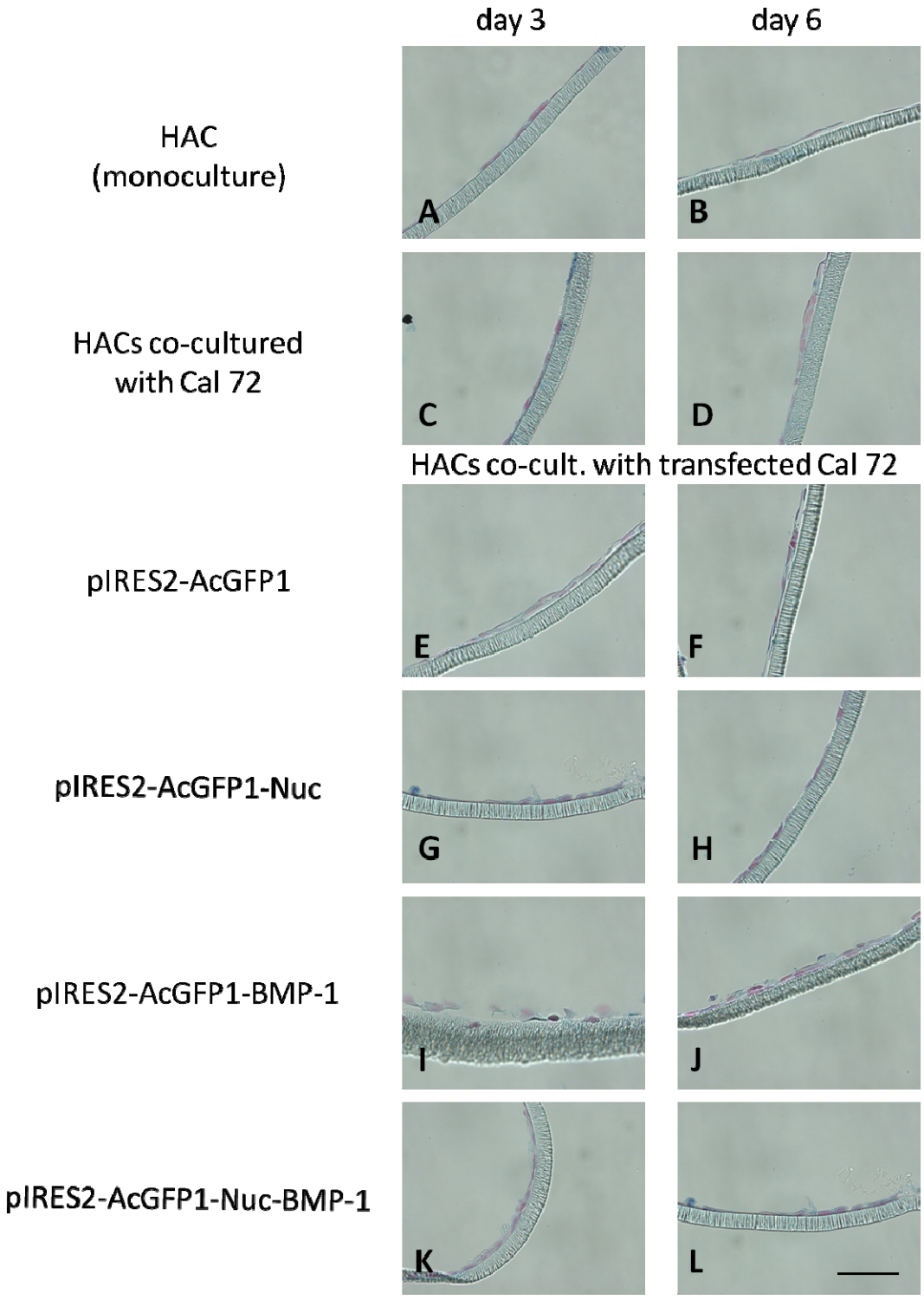
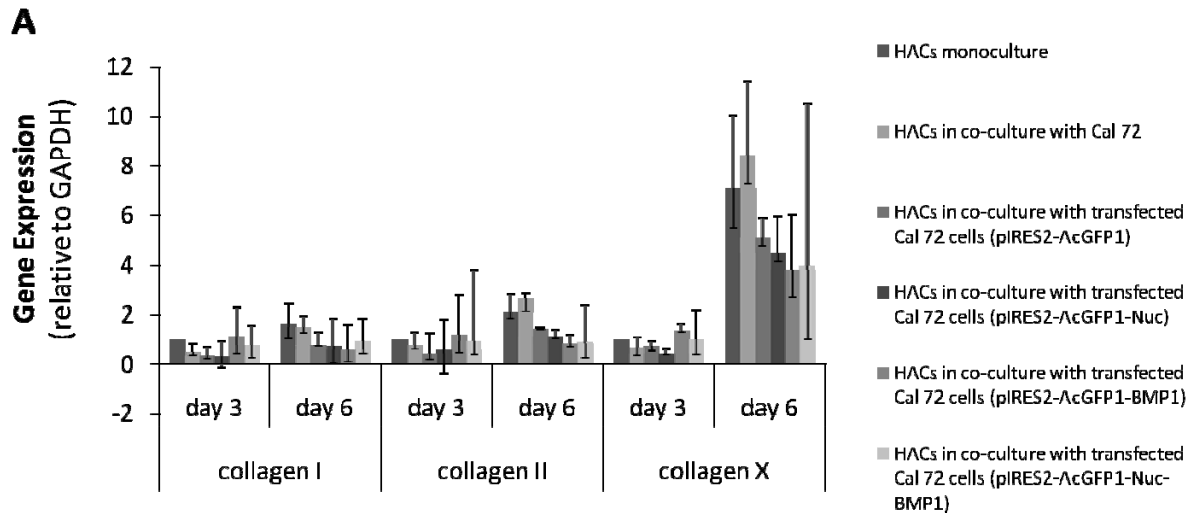



**Fig 7.9.: Representative images of Alcian blue stained HACs cultured on transwell membranes in monoculture (A and B) and co-culture (C, D, E, F, G, H, I, J, K and L).** The weakness of the light blue obtained from the staining was indicative of an ECM poor in GAGs and proteoglycans. Scale bar = 50 μm.

Quantitative analysis of mRNA expression of collagen types I, II and X, aggrecan, versican, and BMP-1 by HACs was performed by qrt-PCR. (Fig 7.10.). Up-regulation from 4 to 5-fold of collagen type X mRNA on day six denoted a trend of all HACs co-cultured with the different Cal 72 cells to undergo hypertrophy (Fig. 7.10. A). Versican expression, which points to HAC de-differentiation as well, increased 10 to 15-fold. This increase was not specific to those HACs co-cultured with Cal 72 cells transfected with BMP-1 plasmids, but it was observed in HACs co-cultured with all the transfected or untransfected Cal 72 cells. BMP-1 mRNA expression (a typical marker for osteoblasts) was also up-regulated 20 to 25-fold (Fig. 7.10. B); however, its expression was not higher in HACs in co-culture with Cal 72 cells transfected with pIRES2-AcGFP1-BMP-1 or pIRES2-AcGFP1-Nuc-BMP-1 than in those co-cultured with Cal 72 transfected with pIRES2-AcGFP1 or pIRES2-AcGFP1-Nuc as it might have been expected from the consequences of the effects of the transfection of the Cal 72 with BMP-1 plasmids.

It has to be mentioned that besides the fact that the culture period was too short to compare results with our previous co-culture results, these results revealing a HAC de-differentiation and hypertrophic behavior are not conclusive, due to the transient and ultimately unstable nature of the transfection and due to the fact that we have not proven BMP-1 expression at the protein level by the transfected Cal 72 cells.



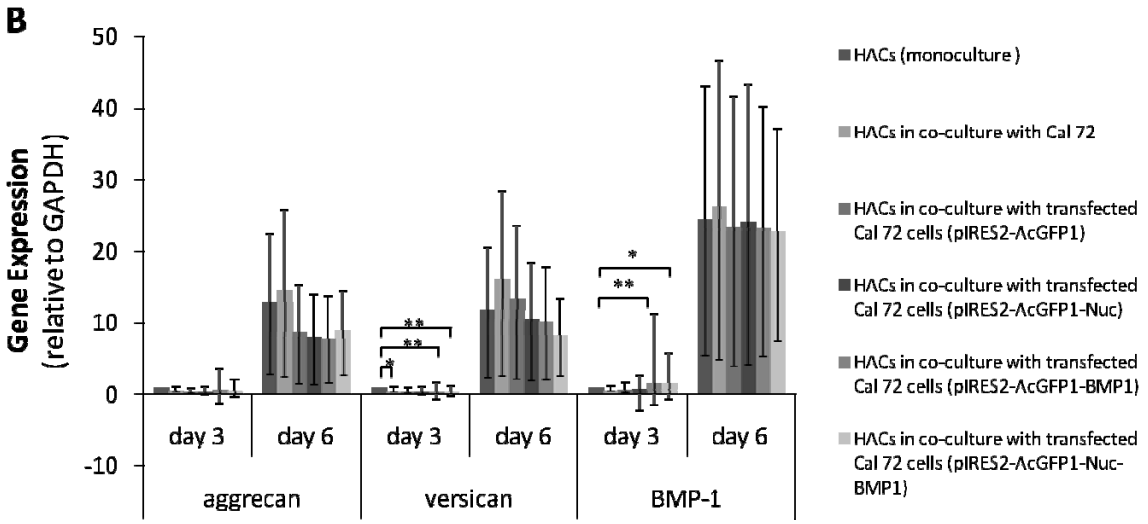



**Fig. 7.10.: Relative gene expression for relevant ECM markers of HACs and BMP-1 cultured in transwells.** Culture conditions: HACs monoculture, HACs in co-culture with Cal 72 cells, HACs in co-culture with transfected Cal 72 cells (pIRES2-AcGFP1 or pIRES2-AcGFP1-Nuc and pIRES2-AcGFP1-BMP-1 or pIRES2-AcGFP1-Nuc-BMP-1). The up-regulation of collagen type X, versican and BMP-1 indicates the trend of HACs undergoing de-differentiation and hypertrophy. Results are shown as the means of target gene expression relative to GAPDH $\pm$ 95% confidence interval, n=3. Values were normalized with respect to gene expression under HACs monoculture conditions on day 3 set at 1. For statistical analysis one-way ANOVA test for independent samples was performed on $\Delta\Delta$Ct values. * Statistically significant difference at $p < 0.05$ and ** at $p < 0.01$ from the corresponding monoculture on each time point.

# 8. Discussion

## 8.1. Phenotype Preservation of Human Articular Chondrocytes

Chondrocytes are a unique cell type in articular cartilage tissue and are essential for cartilage formation and functionality. Although native chondrocytes offer little assistance to injured or diseased cartilage, these cells are responsible for the synthesis and turnover of the cartilage ECM, which provides an environment of nutrition diffusion for chondrocytes and provides the joint surface with biomechanical competence. Numerous cytokines and transcription factors are required for chondrocyte maturation process, although the regulation and the specific mediators remain undefined (Archer and Francis-West, 2003; Aubin et al., 1995; de Crombrugghe et al., 2000; DeLise et al., 2000). Chondrocytes are responsible not only for the generation of ECM during growth and development, but also for the maintainance of tissue homeostasis during adult life. The chondrocyte in mature articular cartilage exhibits no mitotic activity under physiologischal conditions and a very low rate of matrix synthesis and degradation. Changes in the level of gene expression in injured and diseased cartilage are gene-specific and time-dependent. The quantity of specific proteins may be altered as a result of these changes in gene expression, which may eventually lead to degradation at the tissue level and cause a compromise in cartilage structure and function, thus compromising mechanical properties, and reducing chondrocyte viability (Hedbom and Hauselmann, 2002).

The major pathology associated with the chondrocyte is arthritis. Arthritis can be subdivided into two major classes: rheumatoid arthritis (RA), which is driven by inflammation of the synovial membrane, and predominantly non-inflammatory osteoarthritis (OA) or osteoarthrosis as it is also called, a term which describes the associated degeneration of the hyaline articular cartilage. OA is more common, and although it may affect juveniles, it is more associated with the older population. The cellular reaction pattern during ostearthritic disease is at first sight rather pleomorphic. However, it can be basically summarized in three categories. First, the chondrocytes can undergo cell death, whether programmed (apoptosis) or not (necrosis), or they can proliferate to compensate for cell loss or to increase their synthetic activity, as cells do in many other tissues of the body. Second, chondrocytes activate or deactivate their synthetic-anabolic activity by increasing or decreasing anabolic gene expression. Finally, chondrocytes undergo phenotypic modulation

implicating an overall severely altered gene expression profile of the cells in the diseased tissue (Aigner and McKenna, 2002). Hence, the isolation and growth of chondrocytes outside their matrix microenvironment has been researched for decades in the hope of better understanding this resilient cell and exploiting its function for therapeutic gain.

Cell culture is a basic experimental approach used in cellular and molecular biological studies of chondrocytes. Of these, the monolayer culture of articular chondrocytes is employed extensively but fails to maintain the chondrogenic phenotype during long-term culture (Benya and Shaffer, 1982; Bonaventure et al., 1994). Cells cultured under these conditions produce proteins normally seen in fibroblasts or in chondrocytes undergoing hypertrophy *i.e.* collagens type I and X and the large proteoglycan versican (Bonaventure et al., 1994) and adopt a fibroblastic morphology as well. Phenotypic instability of chondrocytes when removed from their matrix has been consistently observed in different species. Since the phenomenon is reversible, several culture systems have been found to promote the re-expression.

In the present study, HACs were cultured in monolayer conditions and studied over a period of 16 days. The loss of their characteristic rounded morphology and change to a flattened fibroblast-like morphology is evident from day seven on, thus indicating cell de-differentiation. An initial profile of the changes in gene expression during cell expansion was established. The level of mRNA expression of a number of ECM markers, including chondrocyte-specific ECM genes (collagen type II and aggrecan) and collagen types I and X (indicative of hypertrophy) was assessed using both RT-PCR and qrt-PCR. In addition Sox9, a transcription factor important in chondrogenesis and associated with further differentiation and hypertrophy of chondrocytes was also examined by RT-PCR. Expression of the typical marker for de-differentiated HACs, collagen type I, started from day seven on and was rapidly up-regulated, reaching a 40-fold increase on day 16, concordant with the cell elongation. However, the fact that the typical differentiation markers (collagen type II, Sox9 and aggrecan) remained present during the entire culture period and that cells did not undergo hypertrophy, as the low collagen type X expression revealed, gave qualitative reasons to assume a preservation of the phenotype in HACs, the source of which was diseased cartilage, suggesting that the potential for re-differentiation is not completely lost, even during prolonged monolayer culture, as verified in previous studies (Benya and Shaffer, 1982; Bonaventure et al., 1994; Bruckner et al., 1989; Horton et al., 1992; Zaucke et al., 2001).

One limitation of our study was that samples from different locations within the femoropatellar groove were pooled; thus, it was not possible to determine whether tissue from different depths and locations along the groove reacted differently to the disease. It would be necessary to extend the present study to identify any gender, age- or disease-degree-dependence of changes in gene expression caused by osteoarthritis and other diseases.

## 8.2. Biomaterials and Human Articular Chondrocyte Differentiation

Phenotypic instability of chondrocytes when they are removed from their cartilage matrix has been consistenly observed in different species (Benya et al., 1978; von der Mark et al., 1977).

Clinical bioengineering therapies such as cell and tissue engineering typically involve the use of material substrates that mimic the ECM and can serve as delivery systems to transplant cells into diseased, damaged or resected tissue. To stimulate native ECM, a tissue-engineered scaffold is fabricated as a 3-D structure that is capable of carrying out functions of the ECM. In this manner, the engineered scaffold component, fabricated in a desired shape and size, would provide physical protection and tissue growth guidance to the cells harbored within the scaffold. Once placed into the patient, hybrid material-biological constructs allow the transplanted cells, or induce cells at the periimplant site, to produce tissue ECM required for tissue regeneration or repair (Langer and Vacanti, 1993). Strategies employed to achieve the desired cellular response include use of exogenous growth factors and signaling molecules to enable differentiation potential to be realized (Tabata, 2003). Furthermore, the development of “smart materials” that are able to regulate the behavior of adherent or embedded cells by releasing bioactive molecules incorporated within the delivery substrate into the local environment to induce bioactive functionality (Chen and Mooney, 2003; Watzer et al., 2009) are also of major interest for future investigations in cell biology and tissue engineering.

Several basic properties, such as biochemistry, biocompatibility, biodegradation, structure, and mechanics are of vital interest in scaffold design. Nevertheless it is still difficult to provide definite specification for a material surface suitable for cartilage regeneration (Rotter et al., 2007). For example, chondrocytes embedded in 3-D gels such as collagen and

agarose can maintain cartilage-specific phenotype during *in vitro* culture, however their proliferation ability tends to be suppressed (Rahfoth et al., 1998; Schuman et al., 1995).

To date, many attempts have been made to use two types of scaffold for cartilage tissue engineering. The first is a solid type of scaffold including a honeycomb, porous body, mesh, sponge, and unwoven fabric (Lu et al., 2001). The use of solid-type scaffolds raises practical problems. The smaller the pore sizes are, the more difficult it will be for the cell suspension to infiltrate the scaffold. In contrast, when the pore sizes of solid scaffolds are increased, the chondrocytes attach to the walls of huge pores, but they are not placed in a 3-D condition. The second type of material for chondrocyte delivery is hydrogels. Various materials derived from animals or plants, *i.e.* collagen type I gel (Chaipinyo et al., 2004), fibrin glue (Ting et al., 1998), gelatin (Ibusuki et al., 2003), agarose (Benya and Shaffer, 1982) or alginate (Lee et al., 2007) are included in this type. The biological specificities of each hydrogel material have seldom been compared with each other. Although hydrogels often lack mechanical strength by themselves, this type of material can be mixed with cells and can completely surround the seeded cells. Hydrogel scaffolds are appealing for cell delivery and tissue development, because they are highly hydrated 3-D networks of polymers that provide a place for cells to adhere, proliferate, and differentiate (Drury and Mooney, 2003).

We have used 3-D culture systems of both solid type and hydrogel, to re-differentiate HACs which were first expanded in monolayer culture. HACs stimulated with different concentrations of $PGE_2$ de-differentiated when cultured on a 2-D surface (monolayer). In contrast, under the same stimulation conditions in 3-D gelatin-based solid scaffolds, HACs maintained their phenotype when stimulated with $PGE_2$ $10^{-6}$ M. Furthermore, HACs embedded in collagen type I hydrogels adopted spherical morphology and were isolated into their own *lacunae*, as in native cartilage. The up-regulation of proteoglycan synthesis in the latter system denoted that collagen type I gel favored proteoglycan synthesis. Based on these results, we can assume that, in principle, chondrocytic differentiation potential is maintained when these cells are placed in the proper 3-D environment. Thus, it is important to carry out an extensive study in which biomaterials of different nature are compared in order to achieve an optimal HAC culture environment.

## 8.3. Response of Human Articular Chondrocytes to $PGE_2$ exogenous stimulation

### 8.3.1. Effects of $PGE_2$ Exogenous Stimulation on HAC Proliferation

The role of prostaglandins in the metabolism of articular cartilage is still a matter of debate. Some reports have indicated that prostaglandins participate in the destruction of articular cartilage by degrading cartilage ECM (Fulkerson and Damiano, 1983; Lippiello et al., 1978), whereas others have showed that prostaglandins promote chondrogenesis and terminal differentiation (Biddulph et al., 2000; Kosher and Walker, 1983). The confusion may stem from the complexity of the family of prostaglandins and also from the complexity of the receptors. The effects of $PGE_2$ depend on the type of receptor, and sometimes completely opposite effects will be achieved by different EPs (Aoyama et al., 2005).

Several studies have addressed the effects of $PGE_2$ on HAC proliferation. Induction of DNA synthesis (cell proliferation) and reduction of apoptosis have been reported (Aoyama et al., 2005; Brochhausen et al., 2006; O'Keefe et al., 1992). Moreover, exogenous $PGE_2$ stimulation had a proliferation-inducing effect in a dose-dependent manner on cultured growth plate chondrocytes via the EP1 receptor (Brochhausen et al., 2006). These results opened up interesting new approaches to optimize the seeding of scaffolds via stimulation of cell proliferation by $PGE_2$ or EP1. However, these effects were reported in healthy and young animal models, whereas the current work (as well as other studies) in elderly and diseased HACs revealed the limitations of these models.

In our primary culture system, the limited number of samples (n=3) was insufficient to establish a trend for cell proliferation behavior. However, there was no evidence of a dose-dependent effect of $PGE_2$ on HAC proliferation, although there was indeed a manifest increase of the metabolic activity. Jakob et al. reported that $PGE_2$ reduced collagen type I mRNA expression, which paralleled a more rounded (less fibroblastic) cell morphology, without inducing proliferation (Jakob et al., 2004), thus suggesting that animal models provide incomplete information for clinical application.

### 8.3.2. Effects of Exogenous $PGE_2$ Stimulation on HAC Differentiation

$PGE_2$ can produce both catabolic and anabolic effects in articular chondrocytes (Aoyama et al., 2005).

Several studies have demonstrated the role of $PGE_2$ in stimulation of chondrocyte differentiation by increasing proteoglycan accumulation and up-regulating collagen type II mRNA expression via EP2 and EP4. $PGE_2$ also inhibits *colX* expression, preventing chondrocytes from undergoing hypertrophy and suppressing the maturation of growth plate chondrocytes (Li et al., 2004; Miyamoto et al., 2003). $PGE_2$ signalling through EP2 down-regulates mRNA expression of osteopontin, which is a major element in osteoarthritic cartilage, and one function is to destroy the tissue by activating the apoptotic pathway (Yumoto et al., 2002). $PGE_2$ is also associated with the suppressive effects of TGF-β2 in osteoarthritic cartilage, being capable of down-regulating collagen cleavage and hypertrophy in human osteoarthritic articular cartilage (Di Battista et al., 1997; Goldring and Berenbaum, 1999). Exogenous $PGE_2$ stimulation at concentrations much lower than those generated in inflammation are chondroprotective and yield a normalization of chondrocyte phenotypic expression (Tchetina et al., 2006; Tchetina et al., 2007).

In contrast, different studies establish the $PGE_2$ induction of cartilage degradation by decreasing collagen synthesis, increasing chondrocyte apoptosis (Martel-Pelletier et al., 2003), and modulating bone resorption through stimulation of osteoclast formation from precursor stem cells (Lader and Flanagan, 1998). Finally, $PGE_2$ signals through EP3 also promote IL-1β expression, thus amplifying the local inflammatory process (Lorenz et al., 1995).

These discrepancies can be explained by differences in methodology *i.e.* cell source, medium supplements, etc. Thus further investigation is required to determine the exact effects of $PGE_2$ on chondrocyte differentiation.

As part of the present study, HACs in monolayer conditions stimulated with $PGE_2$ $10^{-6}$ M underwent hypertrophy, thus confirming those results obtained by Martel-Pelletier *et al.* (Martel-Pelletier et al., 2003), in which $PGE_2$ was responsible for catabolic effects within chondrocytes from animal sources. However, the fact of culturing cells in gelatin-based scaffolds (Brochhausen et al., 2007; Zehbe et al., ; Zehbe et al., 2005) that emulated the physiological conditions of cartilage ECM gave major evidence in favor of a role for $PGE_2$ in

HAC phenotype preservation. HACs stimulated with $10^{-6}$ M $PGE_2$ displayed a rounded morphology in contrast to the fibroblast-like morphology displayed by cells stimulated with less or no $PGE_2$. These results, together with the decrease of collagen type I mRNA expression and collagen type II mRNA up-regulation over a culture period of two weeks, confirmed an enhancement of chondrocytic phenotype by $PGE_2$.

$PGE_2$ activity is very unstable. Its half-life in the circulatory system is approximately 30 s (Nomura et al., 2005; Watzer et al., 2009), so that repeated application of $PGE_2$ with each medium change was required to induce HAC re-differentiation. The reduced size of the $PGE_2$ molecule ensured that the gas chromatography-tandem mass spectrometry measured biologically active $PGE_2$, confirming that the re-differentiation of HACs was a consequence of the exogenous $PGE_2$ effects.

Our results indicated possible beneficial effects of administering physiological amounts of $PGE_2$ to HACs of diseased/defective cartilage, thereby stimulating cell differentiation, ECM deposition, and thus promoting appropriate cartilage repair.

These results together with those mentioned from other studies highlight the importance of $PGE_2$ and EP2 in cartilage maturation and in stimulating cell re-differentiation in ageing articular cartilage (Jakob et al., 2004; Li et al., 2004). This finding prompts further investigations of the possible combination of autologous chondrocyte implantation with a molecular therapy (*i.e.* the delivery in the joint of $PGE_2$ or EP2), or the potential use of $PGE_2$ as a medium supplement for the *in vitro* generation of engineered cartilage grafts, and the combination of suitable drug delivery systems with an EP2 agonist. Specific EP2 agonists are promising therapeutic compounds for the treatment of degenerative cartilage diseases to be used instead of $PGE_2$ itself, which can have undesirable additional effects through the EP3 receptor (Aoyama et al., 2005).

## 8.4. Responses of Human Articular Chondrocytes in Co-culture Systems

### 8.4.1. Co-culture of Human Articular Chondrocytes with Murine Preosteoblastic KS483 Cells

Murine preosteobalstic KS483 cells are stem-like cells and therefore are capable of self-renewal and multilineage differentiation (van der Horst et al., 2002). Since a major branch of regenerative medicine is the application of stem cells in cartilage tissue engineering and reconstructive surgery, the above mentioned properties make KS483 cells viable for attempting new cartilage repair strategies (Heng et al., 2004). The suitability and efficacy of co-culturing these cells with human primary chondrocytes to preserve the chondrogenic phenotype of the cultured chondrocytes were investigated in the present study.

In our co-culture system within a collagen type I hydrogel, where the cell-to-cell contact was the the key for an enhancement of chondrocytic differentiation, KS483 cells showed no major effects on the maintainance of a chondrocytic phenotype. These results were confirmed with co-cultures in transwell systems, where cells interacted in a paracrine manner.

Since the effects observed in this study may be species-specific and not necessarily transferable, future work to change the strategy, *i.e.* using MSCs from a human source, testing different cell ratios, and increasing culture periods to four or six weeks will provide more information regarding the re-differentiation of HACs via MSCs. This approach continues to be of major interest in developing proper differentiation protocols for tissue engineering therapies. A limitation of co-culturing cells from the same species is the difficulty of quantifying gene expression by individual subpopulations in a mixed culture via qrt-PCR, since cell-specific mRNA cannot be separated. Therefore, additional techniques, *e.g* using GFP-transfected cells (Yang et al., 2009), have to be optimized.

### 8.4.2. Co-culture of Human Articular Chondrocytes with Cal 72 Osteosarcoma Cells

A major challenge in tissue engineering is the integration of cartilage and subchondral bone in the same graft. The biological functions of osteoblasts and chondrocytes are much related, and this, together with the fact that they interact during cartilage development, suggest that they might regulate each other's growth and differentiation.

Several studies have addressed the interaction between osteoblasts and chondrocytes (Jiang et al., 2005; Mahmoudifar and Doran, 2005; Nakaoka et al., 2006), reporting beneficial effects of osteoblasts on the chondrocytic phenotype.

The osteosarcoma cell line Cal 72 is closely related to normal osteoblasts. Cal 72 cells grow in continuous culture and exhibit morphological, immunohistochemical and molecular characteristics of an osteoblastic lineage, osteocalcin and alkaline phosphatase being typical markers for these cells (Rochet et al., 1999). The aim of the present study was to determine the effects of Cal 72 cells on the expansion and re-differentiation of cultured primary chondrocytes.

Co-culture of osteoblasts and chondrocytes in transwell systems resulted in suppression of the regular de-differentiation process of passaged cultured chondrocytes in monolayer. The presence of Cal 72 enhanced the differentiation of chondrocytes, presumably via soluble factors secreted from Cal 72 cells, and reduced HAC expression of typical osteoblast- and de-differentiation markers, *i.e.* collagen type I, versican, osteocalcin and ALP. We also evaluated gene expression for collagen type X, which was not detected in any of the culture conditions. The effects of co-culture on chondrocytes are evident when taking into account ECM deposition. Thus, the difference in height between the mono- and the co-culture after two weeks was found to be up to two-fold.

Among others, TGF-β1 is a key factor in chondrogenic maturation (Denker et al., 1995; Han et al., 2005). Chondrocytes in monoculture do not express TGF-β1, whereas in co-culture the release of the growth factor into the culture medium reached 12-16 pg/ml, revealing that chondrocytes in the presence of osteoblasts underwent a maturation/re-differentiation process due to the diffusion of TGF-β1 (and other factors) secreted by osteoblasts into the chondrocytic culture medium.

The findings of our study suggest that during co-culture, rather than maintaining their individual phenotypes, osteoblasts and chondrocytes are instead responding to the presence of the other cell type, representing a novel, biological way to condition tissue engineered cartilage implants. Since the response of HACs to the presence of Cal 72 has been thoroughly analyzed, future research in order to complete the co-culture study could be to analyze the response of Cal 72 to the presence of HAC's.

## 8.5. Effects of BMP-1 on Human Articular Chondrocytes

In recent years, various candidate proteins, including TGF-β, BMPs and Sox9, have emerged as potential therapeutic agents to treat cartilage degeneration by stimulating matrix production (Masuda et al., 2004; Yoon et al., 2004). BMP-1 has been identified as having a positive effect on bone and cartilage development, mediating ECM formation by converting ECM precursors into mature functional proteins, and in morphogenetic patterning by cleaving the antagonist chordin to activate BMP-2/-4 (Pappano et al., 2003), which play a very important role during chondrocyte differentiation and which stimulate the synthesis of chondrocyte-specific macromolecules. It is therefore likely that the delivery of BMP-1 to monolayer cultures of HACs could stimulate expression of cartilage matrix genes, in which case this would be of major interest for cartilage regeneration (Getgood et al., 2009).

In the current study, the exogenous stimulation with recombinant BMP-1 initially tended towards a dose-dependent up-regulation of HAC metabolism, turning into a time-dependent effect on days two and three. A dose- and time-dependent trend was reflected in the gene expression as well, where after six days under the effects of BMP-1 stimulation, the mRNA expression of the de-differentiation markers (collagen type I and versican) was up-regulated. Since there was a proportional increase of mRNA expression of these markers up to 400 ng/ml of BMP-1 and then no further increase was observed, we suggested a hypothetical saturation point situated between 400 – 1000 ng/ml of BMP-1.

BMP-1-like proteinases are identified as regulators capable of orchestrating TGFβ signaling with ECM deposition, patterning, and negative feedback control of several vertebrate tissues (Ge and Greenspan, 2006; Lee et al., 1997). TGF-β1 expression was up-regulated by BMP-1, but HACs showed an assimilation limit: 200 ng/ml of exogenous BMP-1 was the optimal concentration for a 2.2-fold up-regulation of TGF-β1 with respect to the unstimulated control on the third day of cell culture.

In general, direct protein administration has less appeal due to the short half-life of the delivered protein, although longer half-life within chondrocytes is imaginable because of their avascular nature. However the same culture period (6 days) but with change of the time points (days 1, 2, 3, 4, 5, and 6), would have given a more evident trend of the effects of BMP-1.

As an alternative to protein delivery gene therapy enables prolonged, high-level, sustained protein expression (Chen et al., 2004). Since chondrocyte transfection suffers from several additional complications (Trippel et al., 2004), in an attempt to induce the paracrine effects of BMP-1 on HACs, co-culture of transiently transfected Cal 72 cells with HACs was included in the present study. Unfortunately, the transient transfection was not sufficiently optimized, and although the expression of GFP implied a successful transfection, there was no proof of BMP-1 expression in any of the transfected Cal 72 populations. Since the aim of the study was to elucidate the role of BMP-1, the mentioned co-cultures were not rigorous enough to obtain conclusive results. Nevertheless, the observed differences in gene expression between the mono- and co-cultured HAC populations point to the possibility that HACs can undergo de-differentiation due to BMP-1 signaling. This is consistent with the fact that BMP-1 is a major bone protein, and that the maturation process of bone and cartilage have many common factors (Steiglitz et al., 2006).

## 8.6. Possible Areas for Future Research

Cartilage biochemistry and cell biology is presented in this study in the context of OA and cartilage regeneration and repair. Success in current efforts towards cell-based orthopaedic treatment options in cases of cartilage trauma and early stages of osteoarthritic degeneration will strictly depend on strategies that rely on known mechanisms of chondrocytic regulation, hence the importance of a better understanding of HAC de-differentiation mechanism *in vitro*. An extensive research and characterization of *in vitro* cultured HACs, harvested from different sites, with different degrees of disease, from both genders, and taking a wide range of donor ages, would give a solid basis for further studies on cartilage regeneration and repair based on biomaterials, cells, and signaling molecules. It is important to emphasize the donor number, since a high number of samples avoids additional problems in the statistical analysis, as the results of the studies in the current work underline.

The development of the ACI technique and the progress in the tissue-engineering field in the early nineties, have suggested that the repair of human articular cartilage actually might be feasible, leading to the development of numerous strategies to repair or replace the damaged surfaces. In many approaches, cells are grown on biomaterial scaffolds and then implanted into the defect, where new functional tissue is formed, remodeled and integrated into the body. So-called “smart” biomaterials are produced by developing materials that can

deliver signaling molecules which are able to directly regulate cell differentiation and metabolism. Although numerous *in vitro* studies show the potential of signaling molecules to support tissue regeneration, several major problems must be solved before reliable human application can be considered: Despite the fact that cartilage regeneration is complex and affected by multiple factors that are released in a well-orchestrated manner, most studies deliver single molecules. Extremely little is known about which sequences and concentrations of factors are needed to optimize cartilage regeneration. Another problem is that most of the studies on biomaterials were performed using (mostly young adult or even fetal) animal cells, and not with cells from elderly osteoarthritis patients (as in the present study) (Stoop, 2008). Therefore more exhaustive research will be needed to determine if results can be extended to the human situation and used in a clinical setting for treating human cartilage defects. This may be the most difficult problem for these cell-regulating materials, to translate the results from *in vitro* and animal studies into clinical application and market introduction.

Besides the importance of the nature of the biomaterial and the selection of the appropriate signaling molecules, the cell source is a key element. Articular chondrocytes have already been deteremined towards the cartilage lineage, and MSCs have the ability of generating cells with features of stable chondrocyte multipotency. Furthermore, our results and several other studies (Jiang et al., 2005) have demonstrated a heterogenous effect on chondrocytic phenotype due to co-culture with osteoblasts. Future studies will be able to utilize this co-culturing system to investigate the importance of osteoblast-chondrocyte interactions in the regeneration of the osteochondral interface. However, additional work should be carried out to assess whether cells of non-articular origin can be successfully used for articular cartilage repair.

# 9. Appendices

## 9.1. Appendix I

### 9.1.1. Chemicals

| Name | Supplier |
|---|---|
| 2-Propanol | Fluka, Basel, Switzerland |
| 40% Acrylamide/Bisacrylamide solution, 19:1 | Bio-Rad, Hercules, US-CA |
| Activated charcoal | Sigma-Aldrich, St. Louis, US-MO |
| Agar | Invitrogen, Carlsbad, US-CA |
| Agarose | Biozym, Hessisch Oldendorf, Germany |
| Alcian blue 8GX | Sigma-Aldrich, St. Louis, US-MO |
| APS | Bio-Rad, Hercules, US-CA |
| Blocking reagent | Roche, Freiburg, Germany |
| Brij-35 detergent | Sigma-Aldrich, St. Louis, US-MO |
| BSA | Serva, Heidelberg, Germany |
| Butanol reagent | Sigma-Aldrich, St. Louis, US-MO |
| $CaCl_2 \times 2H_2O$ | Merck, Darmstadt, Germany |
| Calcein AM | Invitrogen, Carlsbad, US-CA |
| $CH_3COOH$ | Merck, Darmstadt, Germany |
| Chloroform | Sigma-Aldrich, St. Louis, US-MO |
| Citric acid | Sigma-Aldrich, St. Louis, US-MO |
| Collagen type I | BD Bioscience, San Jose, US-CA |
| Collagenase type I | Worthington, Lakewood, US-CO |
| Crystal violet | Merck, Darmstadt, Germany |
| Dextran | Sigma-Aldrich, St. Louis, US-MO |
| DMEM | Sigma-Aldrich, St. Louis, US-MO |
| DMSO | Sigma-Aldrich, St. Louis, US-MO |
| DNA Ladder (1 kb) | New England Biolabs, Ipswich, UK |
| DNA Ladder (100 bp) | New England Biolabs, Ipswich, UK |
| dNTP Mix | Qiagen, Hilden, Germany |
| ECL Western Blotting Detection Reagents | Amersham Pharmacia Biotech, Freiburg, Germany |
| EDTA | Sigma-Aldrich, St. Louis, US-MO |
| EDTA 0.5 M | Sigma-Aldrich, St. Louis, US-MO |
| Eosin | Sigma-Aldrich, St. Louis, US-MO |
| Ethidium bromide | Sigma-Aldrich, St. Louis, US-MO |
| EtOH | AppliChem, Darmstadt, Germany |
| FastAP Thermosensitive Alkaline phosphatase | Fermentas, Glen Burnie, US-MD |
| FCS | Sigma-Aldrich, St. Louis, US-MO |

| | |
|---|---|
| $FeCl_2$ | Sigma-Aldrich, St. Louis, US-MO |
| Fibrinogen | Fluka, Basel, Switzerland |
| Fibronectin | Roche, Freiburg, Germany |
| Fluoromount-G | SouthernBiotech, Birmingham, UK |
| Formalin | Sigma-Aldrich, St. Louis, US-MO |
| Fungizone | Gibco, Carlsbad, US-CA |
| G418 antibiotic solution | Biochrom AG, Berlin, Germany |
| Gelatin type A (porcine skin) | Sigma-Aldrich, St. Louis, US-MO |
| GenCarrier-1 Cell Transfection Reagent | Epoch Biolabs, Sugar Land, US-TX |
| GLC Mounting medium | Sakura, Torrance, US-CA |
| Glutamax I | Invitrogen, Carlsbad, US-CA |
| Glycin | Roth, Karlsruhe, Germany |
| $H_2O$ | Braun, Meslingen, Germany |
| $H_2O_2$ | Merck, Darmstadt, Germany |
| $H_2SO_4$ | Merck, Darmstadt, Germany |
| Haematoxylin solution | Sigma-Aldrich, St. Louis, US-MO |
| HCl | Merck, Darmstadt, Germany |
| HEPES 1M | Gibco, Carlsbad, US-CA |
| Hoechst 33342 | Sigma-Aldrich, St. Louis, US-MO |
| HotStar Taq Plus DNA Polymerase | Qiagen, Hilden, Germany |
| Hydrochloric acid | Sigma-Aldrich, St. Louis, US-MO |
| Hyposmolar buffer | Eppendorf, Hamburg, Germany |
| Iodiacetamide | Sigma-Aldrich, St. Louis, US-MO |
| Isopropanol | Fluka, Basel, Switzerland |
| Kanamycin sulfate | Gibco, Carlsbad, US-CA |
| Loading buffer 5x | Biorad, Hercules, US-CA |
| M199 | Sigma-Aldrich, St. Louis, US-MO |
| M199 10x | Sigma-Aldrich, St. Louis, US-MO |
| MetOH | VWR, Darmstadt, Germany |
| $MgCl_2$ | Merck, Darmstadt, Germany |
| $MgSO_4$ | Merck, Darmstadt, Germany |
| MTS | Promega, Madison, US-WI |
| $Na_2EDTA$ | Calbiochem, Darmstadt, Germany |
| $Na_2HPO_4$ | Roth, Karlsruhe, Germany |
| NaCl | Roth, Karlsruhe, Germany |
| $NaHCO_3$ 7.5% stock | Sigma-Aldrich, St. Louis, US-MO |
| NaOH | Roth, Karlsruhe, Germany |
| *n*-Butanol | Fluka, Basel, Switzerland |
| Neutral red | Sigma-Aldrich, St. Louis, US-MO |
| Nonfat dried milk powder | AppliChem, Darmstadt, Germany |
| PageRuler Prestained Protein Ladder | Fermentas, Glen Burnie, US-MD |
| Paraffin | Fisher Chemicals, Loughborough, UK |

| | |
|---|---|
| PBS | Gibco, Carlsbad, US-CA |
| PBS powder | AppliChem, Darmstad, Germanyt |
| Penicillin/streptomycin mix | Gibco, Carlsbad, US-CA |
| PFA | Merck, Darmstadt, Germany |
| $PGE_2$ | Cayman Chemical Company, Ann Arbor, US-MI |
| Ponceau S solution | Sigma-Aldrich, St. Louis,US-MO |
| Power SYBR Green | Applied Biosystems, Foster City, US-CA |
| Precision Plus Protein Standards Dual Colour | Bio-Rad, Hercules, US-CA |
| Precision Plus Protein Standards Western C | Bio-Rad, Hercules, US-CA |
| Precision StrepTactin-HRP Conjugate | Bio-Rad, Hercules, US-CA |
| Proteinase K | Sigma-Aldrich, St. Louis, US-MO |
| Random Primer, $d(N)_9$ | New England Biolabs, Frankfurt am Main, Germany |
| Recombinant human BMP-1/PCP | R&D Systems, Minneapolis, US-MN |
| RNAse-Free Dnase | Qiagen, Hilden, Germany |
| Rotiphorese TBE Buffer (10x) | Roth, Karlsruhe, Germany |
| Safranin O | Sigma-Aldrich, St. Louis, US-MO |
| SDS | Serva, Heidelberg, Germany |
| Sodium acetate | Sigma-Aldrich, St. Louis, US-MO |
| T4 DNA Ligase | Invitrogen, Carlsbad, US-CA |
| Taq DNase Polymerase | Qiagen, Hilden, Germany |
| TEMED | Bio-Rad, Hercules, US-CA |
| Thrombin | Sigma-Aldrich, St. Louis, US-MO |
| Tris | Roth, Karlsruhe, Germany |
| Tris-HCl 1M | Sigma-Aldrich, St. Louis, US-MO |
| Triton X-100 | Sigma-Aldrich, St. Louis, US-MO |
| TriZol | Invitrogen, Carlsbad, US-CA |
| Trypsin-EDTA | Gibco, Carlsbad, US-CA |
| Tryptone | Sigma-Aldrich, St. Louis, US-MO |
| Tween 20 | Serva, Heidelberg, Germany |
| Xylene reagent | Sigma-Aldrich, St. Louis, US-MO |
| Yeast extract | Sigma-Aldrich, St. Louis, US-MO |
| β-Mercaptoethanol | Sigma-Aldrich, St. Louis, US-MO |
| α-MEM | Gibco, Carlsbad, US-CA |

# 9.2. Appendix II

## 9.2.1. Plasmid Data Sheets

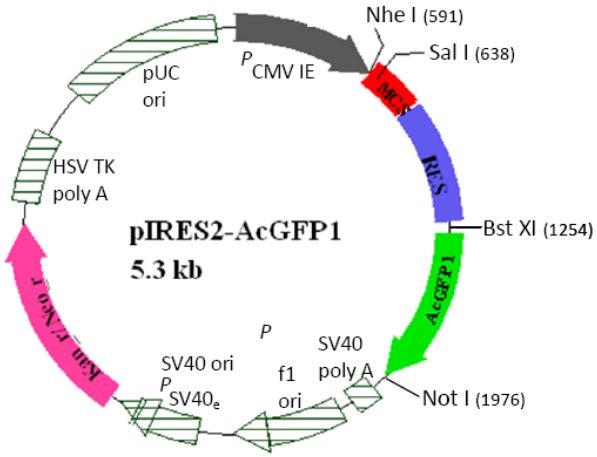
### *9.2.1.1. pIRES-AcGFP1-BMP1*

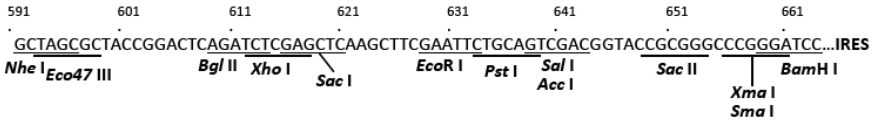
**Plasmid:** pIRES2-AcGFP1-BMP1

**Resistance:** Kanamycin/Neomycin

**Starting Plasmid:** pIRES2-AcGFP1 (Clontech PT3743-5). Catalog # 632435

**Host strain:** Competent lyophilized cells *E. coli*. InvivoGen (lyo-116-11)




**Restriction Map and Multiple Cloning Site (MCS) of pIRES-AcGFP1 Vector (591-665).** Unique restriction sites are in bold.

**Insert:** cDNA from human Bone Morphogenetic Protein-1 (2.2 kb) extracted from pIRESneo2-BMP1 (K. Aufenvenne / C. Wermter AK Stöcker) and amplified with primers containing Nhe I/Sal I restriction sites (NheI – BMP-1 – SalI 3': 5'-GACTACTGATACTAATCAG-**NheI**-ATGCCCG-3'; NheI – BMP-1 – SalI 5': 5'-GACTACTGATACTAATCAG-**SalI**-AGCTTAGTGAT).

**Cloning:** Nhe I/Sal I.

**Location of Features:**

- Human cytomegalovirus (CMV) immediate early promoter: 1-589
  Enhancer region: 59-465; TATA box: 554-560; transcription start point: 583
  C → G mutation to remove *Sac* I site:: 569.
- MCS: 591-665
- IRES sequence: 666-1250.
- *Aequorea coerulescens* green fluorescent protein (AcGFP1) gene:
  Start codon (ATG): 1254-1256; stop codon: 1971-1973
  Insertion of Val at position 2: 1257-1259
- SV40 early mRNA polyadenylation signal
  Polyadenilation signals: 2126-2131 & 2155-2160; mRNA 3' 3nds: 2164 & 2176.
- f1 single-strand DNA origin: 2223-2678 (Packages the noncoding strand of AcGFP1).
- Bacterial promoter for expression of $Kan^r$ gene:
  -35 region: 2740-2745; -10 region: 2763-2768
  Transcription start point: 2775
- SV40 origin of replication: 3019-3154
- SV40 early promoter/enhancer
  72-bp tandem repeats: 2852-2995; 21-bp repeats (3): 2999-3062
  Early promoter element: 3075-3081
- Kanamycin/neomycin resistance gene: 3203-3997
  G → A mutation to remove *Pst* I site: 3385; C → A (Arg to Ser) mutation to remove *Bss*H II site: 3731
- *Herpes simplex* virus (HSV) thymidine kinase (TK) polyadenylation signals: 4233-4251.
- pUC plasmid replication origin: 4582-5225

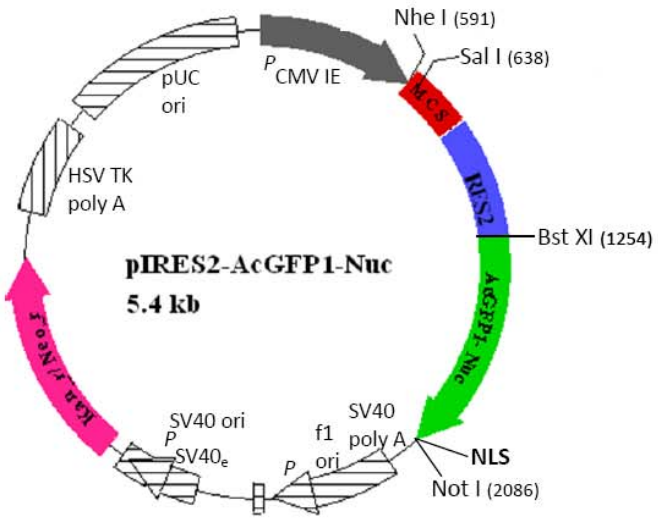
### *9.2.1.2. pIRES-AcGFP1-Nuc-BMP1*

**Plasmid:** pIRES2-AcGFP1-Nuc-BMP1

**Resistance:** Kanamycin/Neomycin

**Starting Plasmid:** pIRES2-AcGFP1-Nuc (Clontech PT3907-5). Catalog # 632515

**Host strain:** Competent lyophilized cells *E. coli*. InvivoGen (lyo-116-11).




TCCGCTAGCG CTACCGGACT CAGATCTCGA GCTCAAGCTT CGAATTCTGC AGTCGACGGT ACCGCGGGCC CGGGAT
**NheI** **SacI** **EcoRI** **PstI** **SalI** **AccI** **SacII** **SmaI** **XmaI**

**Restriction Map and Multiple Cloning Site (MCS) of pIRES-AcGFP1 Vector (591-661).** Unique restriction sites are in bold.

**Insert:** cDNA from human Bone Morphogenetic Protein-1 (2.2 kb) extracted from pIRESneo2-BMP1 (K. Aufenvenne / C. Wermter AK Stöcker) and amplified with primers containing Nhe I/Sal I restriction sites (NheI – BMP-1 – SalI 3': 5'-GACTACTGATACTAATCAG-**NheI**-ATGCCCG-3'; NheI – BMP-1 – SalI 5': 5'-GACTACTGATACTAATCAG-**SalI**-AGCTTAGTGAT).

**Cloning:** Nhe I/Sal I.

**Location of Features:**

- Human cytomegalovirus (CMV) immediate early promoter: 1-589
  Enhancer region: 59-465; TATA box: 554-560; transcription start point: 583
  C → G mutation to remove *Sac* I site:: 569.
- MCS: 591-661
- IRES sequence: 666-1250.
- *Aequorea coerulescens* green fluorescent protein (AcGFP1) gene:
  Start codon (ATG): 1254-1256; last codon of AcGFP1: 1968-1970
  Tandem repeat of the nuclear localization signal (NLS): 1971-2081
  Stop codon: 2082-2084
- SV40 early mRNA polyadenylation signal
  Polyadenilation signals: 2236-2286
- f1 single-strand DNA origin: 2223-2678 (Packages the noncoding strand of AcGFP1-Nuc).
- SV40 origin of replication: 3129-3264
- SV40 early promoter/enhancer: 2962-3230
- Kanamycin/neomycin resistance gene: 3313-4107
- *Herpes simplex* virus (HSV) thymidine kinase (TK) polyadenylation signals: 4343-4361.
- pUC plasmid replication origin: 4692-5335

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# 11. *Curriculum vitae*