CO-EXPRESSION OF INSULIN-LIKE GROWTH FACTOR-1 AND INTERLEUKIN-4 IN AN *IN VITRO* CANINE CHONDROCYTE AND MESENCHYMAL STEM CELL MODEL

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Kizzie Shawon Manning-Kroeger

MSc

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Reviewers:

1. Prof. Michael F.G. Schmidt, Institute of Immunology and Molecular Biology (IMB), Department of Veterinary Sciences, Freie Universität Berlin
2. Prof. Petra Knaus, Institute for Biochemistry, Department of Biology, Chemistry, and Pharmacy, Freie Universität Berlin

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Philippians 4:13

For Annabelle and Stephan

Summary

Osteoarthritis (OA) is a degenerative as well as inflammatory disease caused by an imbalance between both catabolic and anabolic factors, affecting both humans and canines alike. The main catabolic mediators, interleukin (IL) -1β and tumor necrosis factor α (TNFα), propagate the expression of other inflammatory mediators that are involved in the degradation of cartilage matrix proteins and ultimately the loss of cartilage. To combat inflammation and at the same time stimulate cartilage matrix synthesis, the presence of both anti-inflammatory and growth factor genes from the same species are needed. Currently there is no cure for OA. However, regenerative medicine coupled with gene therapy offers a good alternative in the treatment of arthritis, with more focus on co-expressing two genes to battle the disease processes. This study focuses on the effects of the co-expression of insulin-like growth factor-1 (IGF-1), and the anti-inflammatory cytokine, IL-4, on cartilage degrading and synthesizing factors in an *in vitro* canine chondrocyte and mesenchymal stem cell (MSC) model.

Regenerative and anti-inflammatory effects of IGF-1 and IGF-1/IL-4 in an *in-vitro* chondrocyte inflammatory model were analyzed. Co-expression of the transgenes was ascertained by immunoassay. Pro-inflammatory mediators like IL-1β, TNFα, matrix metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS), as well as IGF-binding proteins (IGFBPs), were analyzed by real-time qRT-PCR. The regeneration of extracellular matrix proteins was demonstrated on the mRNA and protein levels. Canine MSCs were isolated and characterized based on morphological as well as biochemical features utilizing real-time qRT-PCR, FACS, and immunocytochemistry. The stem cells were transfected with IGF-1/IL-4 to test the chondrogenic potential of IGF-1 compared to chondrogenic medium containing TGFβ3. Stable cells with the inflammatory sensitive pCOX2-IL-4 were super-transfected with pVitro2-IGF-1. Co-cultures with stably transfected cells and chondrocytes or MSCs were analyzed on their ability to produce extracellular matrix protein type II collagen and to reduce the expression of the collagen degradative mediator MMP-13.

Results from the chondrocyte pro-inflammatory model show that pro-inflammatory mediators as well as IGFBPs were down-regulated in samples transfected with IGF-1/IL-4 to levels comparable to the non-stimulated, non-transfected control. Also, those samples as well as samples transfected with IGF-1 alone showed signs of regeneration denoted by the expression of aggrecan, type II collagen and SOX9. Canine MSCs were shown to undergo

chondrogenesis utilizing chondrogenic medium with TGFβ3 as well as by transfecting them with IGF-1/IL-4. In both situations, chondrogenesis was proven by the expression of cartilage markers, namely aggrecan and type II collagen. Hypertrophy marker, type X collagen, was seen in the 2nd week of cultivation with TGFβ3. Co-cultures with stably transfected cells also demonstrated an up-regulation of type II collagen and a down-regulation of MMP-13 under pro-inflammatory conditions.

Overall, this study shows the ability of the combined expression of IGF-1 and IL-4 to stimulate proteoglycan and type II collagen synthesis and to down-regulate the degradative effects of IL-1β and TNFα, in chondrocyte and co-culture models. Co-expression of therapeutic genes in MSCs offers a dual role in stimulating differentiation of the stem cells and introducing therapeutic genes into the cells to balance catabolic effects in OA tissue. The use of multiple genes in chondrocytes or MSCs could better alleviate the signs and symptoms which are characteristic for the disease process. This study lays foundation for future studies where the use of more than one gene of interest would be necessary.

Zusammenfassung

Arthritis ist eine degenerative und entzündliche Krankheit, die durch ein Ungleichgewicht von katabolischen und anabolischen Faktoren verursacht wird. Sie tritt bei Menschen und Hunden gleichermaßen auf. Die wichtigsten katabolischen Mediatoren Interleukin (IL)-1β und Tumor Necrosis Faktor α (TNFα) stimulieren die Expression von weiteren entzündungsfördernden Mediatoren, die beim Abbau von Knorpelgewebeproteinen mitwirken und letztendlich zum Verlust von Knorpel führen. Die Natur der Krankheit Arthritis bedingt, dass für die gleichzeitige Bekämpfung der Entzündung und den Wiederaufbau des Knorpels sowohl entzündungshemmende als auch wachstumsfördernde Gene präsent sein müssen. Derzeit gibt es keine Behandlungsmethode für eine Heilung von Arthritis. Dennoch eröffnet die regenerative Medizin in Kombination mit der Gentherapie eine vielversprechende Behandlungsmöglichkeit in Gestalt einer gezielten Co-Expression zweier Gene zur Bekämpfung der Krankheit. Diese Forschungsarbeit konzentriert sich auf die Effekte der Co-Expression des Wachstumsfaktors Insulin-like Growth Faktor-1 (IGF-1) und dem entzündungshemmenden Zytokin IL-4 auf knorpelabbauende und knorpelregenerierende Faktoren in kaninen *in vitro* Knorpelzellen- und mesenchymalen Stammzellenmodellen. Analysiert wurden die regenerativen und entzündungshemmenden Effekte von IGF-1 und IGF-1/IL-4 in einem entzündlichen *in vitro* Knorpelzellenmodell. Die Co-Expression der Transgene wurde mit einem Immuntest überprüft. Die entzündungsfördernden Mediatoren IL-1β, TNFα, Matrix Metalleoproteinase (MMPs), Inducible Nitric Oxide Synthase (iNOS) sowie IGF-Binding Proteine (IGFBPs) wurden mit Hilfe von real-time qRT-PCR analysiert. Die Regeneration von extrazellulären Matrixproteinen wurde auf der mRNA- und Protein-Expressionsebene demonstriert. Unter Anwendung von real-time qRT-PCR, FACS und Immunzytochemie wurden mesenchymale Stammzellen (MSCs) von Hunden isoliert und basierend auf ihren morphologischen und biochemischen Besonderheiten charakterisiert. Die Stammzellen wurden mit IGF-1/IL-4 transfiziert, um das chondrogenetische Potential von IGF-1 im Vergleich zu einem chondrogenetischen Medium, das TGFβ3 enthält, zu testen. Stabile, mit pCOX2-IL-4 transfizierte Zellen wurden mit pVitro2-IGF-1 supertransfiziert. Co-Kulturen, die stabil transfizierte Zellen und Chondrozyten oder MSCs enthalten, wurden dahingehend untersucht, ob sie die Fähigkeit besitzen, das extrazelluläre Matrixprotein Type II Kollagen zu bilden und die Expression des Kollagen abbauenden Mediators zu reduzieren. Die Ergebnisse des pro-inflammatorischen

Chondrozytenmodells zeigen, dass in Proben, die mit IGF-1/IL-4 transfiziert worden waren, sowohl entzündungsfördernde Mediatoren als auch IGFBPs herunter-reguliert waren im Vergleich zu Kontollproben, die weder stimuliert noch transfiziert worden waren. Darüber hinaus wiesen die mit IGF-1/IL-4 transfizierten Proben und auch solche, die nur mit IGF-1 transfiziert wurden, Anzeichen für eine Regeneration auf, die sich in der Expression von Aggrecan, Type II-Kollagen und SOX9 ausdrückten. Im Anschluss an die Charakterisierung von cMSCs, konnte gezeigt werde, dass sich diese unter Verwendung eines chondrogenetischen Mediums mit TGFβ3 und des nicht-viralen Vektors, der IGF-1/IL-4 enthält, einer Chondrogenese unterziehen. In beiden Situationen wurde eine Chondrogenese durch die Expression von Knorpelmarkern, namentlich Aggrecan und Type II Kollagen, nachgewiesen. In der zweiten Woche der Kultivierung mit TGFβ3 wurde der Hypertrophie-Marker Type X-Kollagen nachgewiesen. Co-Kulturen mit stabil transfizierten Zellen zeigten zudem eine Hoch-Regulierung von Type II-Kollagen und eine Herunter-Regulierung von MMP-13 unter pro-inflammatorischen Bedingungen.

Insgesamt demonstriert diese Untersuchung die Eignung der kombinierten Expression von IGF-1 und IL-4 zur Stimulation der Synthese von Proteoglykan und Type II-Kollagen und der Herunter-Regulierung der degenerierenden Effekte von IL-1β und TNFα in Chondrozyten- und Co-Kulturmodellen. Die Co-Expression von therapeutischen Genen in MSCs ermöglicht einerseits die Stimulierung der Ausdifferenzierung von Stammzellen und andererseits den Ausgleich der katabolischen Effekte der Arthritis. Durch die Verwendung mehrerer Gene in Chondrozyten oder MSCs könnte eine bessere Linderung der für den Krankheitsverlauf bei Arthritis charakteristischen Symptome erreicht werden. Diese Arbeit legt den Grundstein für zukünftige Forschungsvorhaben, bei denen dann mehr als nur ein Gen von Interesse untersucht werden müsste.

Abbreviations

µg	Microgram
µl	Microliter
µmol	Micromole
2D	Two-dimensional
3D	Three-dimensional
ACLT	Anterior cruciate ligament transection
ACT	Autologous chondrocyte transplantation
ACCT	Autologous conditioned chondrocyte transplantation
APS	Ammonium persulfate
BLAST	Basic local alignment and search tool
BMP	Bone morphogenic protein
bp	Base pairs
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
cMSCs	Canine mesenchymal stem cells
Col I	Type I collagen
Col II	Type II collagen
CLS	Chemiluminescence
CMV	Cytomegalovirus
COMP	Cartilage oligomeric matrix protein
Cox-2	Cyclooxygenase-2
Ct	Cycle threshold
DAB	Diaminobenzidine
DMEM	Dulbecco’s modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2´-deoxynucleoside 5´-triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glycerinaldehyde 3-phosphate dehydrogenase
h	Hour
HRPO	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
IGFBPs	IGF binding proteins
IGFR1	IGF receptor-1
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
kDa	Kilo-Dalton
l	Liter
LINK	Biotinylated goat anti-mouse and anti-rabbit
LSAB	Labeled streptavidin biotin
min	Minute
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSCs	Mesenchymal stem cells
NCBI	National centre for Biotechnology information
NF-κB	Nucleofector-kappa B
ng	Nanogram
nm	Nano meter
NO	Nitric oxide
NOS	NO synthase
NSAIDs	Non-steroidal antiinflammatory drugs
OA	Osteoarthritis
OD	Optical density
OPDA	12-oxophytodienoic acid
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcriptase-PCR
rAAV	recombinate adeno-associated virus
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Second
Sox9	Sex-determining region Y (SRY)-box 9
STAT6	Signal transducer and activator of transcription 6
STET	Sucrose-Triton X-100-EDTA-Tris
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	TBS with Tween 20
TE	Tris-EDTA
TEMED	N,N,N,N Tetramethyl-ethylenediamine
TGFβ	Transforming growth factor-β
Th cell	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumor necrosis factor-α
UV	Ultraviolet
YT	Yeast extract tryptone

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1 Introduction

1.1 Osteoarthritis (OA)

Arthritis is a general term that refers to over 60 different conditions that involve pain and swelling of the synovial joints with the most prominent form being osteoarthritis (OA). OA is defined as a slowly progressive disease characterized by the degeneration of cartilage tissue (Fig.1). Even though OA was once thought to be a non-inflammatory arthropathy, signs of inflammation have been identified in human patients and animal models.

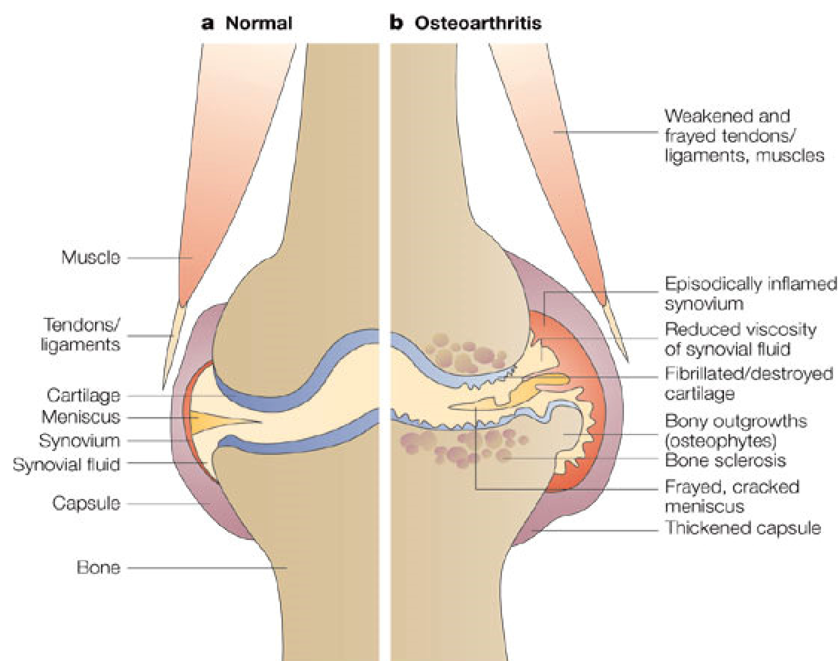


Figure 1: Normal and osteoarthritis joint. a) A normal joint has a smooth cartilage layer surrounded by the synovium, which is protected by the stable joint capsule. Cartilage tissue protects the bones from friction. The tendons help keep the joint in place as well as attach the muscles to the bones. **B)** The first signs of OA are signified by the degradation of cartilage and signs of inflammation. As the disease worsens patches of the bones are exposed (Wieland *et al.,* 2005).

The most common form of musculoskeletal disorders found throughout the world is OA. This type of arthritis has an incidence of about one in every seven individuals in the USA. According to statistics, approximately 12.1% of persons between the ages of 25 and 74 are

affected with OA whereas 20% of the canine population at large are affected [section 1.7] (Lawrence *et al.*, 1998).

Although the exact etiology is unknown, the multi-factorial disease is thought to be ultimately manifested by morphological, biochemical, molecular, and biomechanical changes of both chondrocytes and cartilage matrix terminally leading to softening and loss of articular cartilage. The initiators of the disease process include ageing, obesity, joint injury, mechanical stress, genetic factors as well as an imbalance between biological processes (Hinton *et al.*, 2002; Mansell and Bailey, 1998). Because OA lesions normally occur at sites of trauma or in weight-bearing joints, it is assumed that continual mechanical stress in these areas serves as the primary initiator of OA in combination with biochemical and genetic factors (Goldring, 2000). Regardless of the initiator of the disease, the pathological progression of OA involves an imbalance between synthesis and degradation of cartilage matrix proteins favoring catabolism (Hamerman, 1989).

1.2 Pathophysiology of OA

The pathophysiology of OA involves not only the break-down of cartilage but changes in the bone and synovium as well (Samuels *et al.*, 2008). Early observations of cartilage degeneration are mild raveling along the peripheral regions of the joint where little weight bearing occurs and progresses to gross erosion of cartilage and exposure to subchondral bone (Weiss, 1979; Gardner, 1983). Resulting intra-articular loose particles and catabolic mediators elicit a synovial inflammatory reaction made noticeable by pain and swelling of the joints (Bentley *et al.*, 1975). These symptoms result from the unregulated, increased expression of catabolic mediators, which contribute to the inflammation and degradation of cartilage.

A trademark for the disease process is the outweighing of T-helper 1 (Th1) cells to Th2 cells, where the shift is toward inflammatory cytokines (Liossis and Tsokos, 1998). Synthesized by Th1 cells, the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) are well accepted as the key players in the onset of OA (Fernandes *et al.*, 2002). They are produced in the surrounding synovial membrane and disperse into the cartilage tissue where they up-regulate additional catabolic mediators (Fig. 2). On the other hand, Th2 cells, which produce anti-inflammatory cytokines such as IL-4 and IL-13, are scarce in OA tissues, leaving the pro-inflammatory cytokines unregulated.

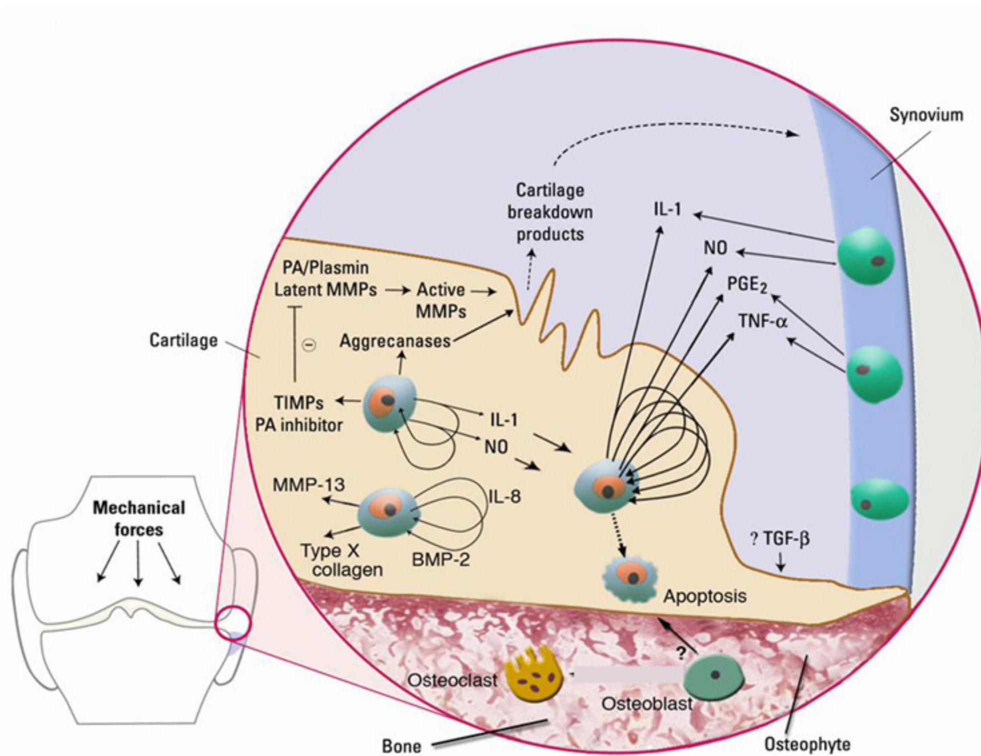


Figure 2: Key pathological events in OA. The disease processes involves three tissues, namely the synovium, bones, and articular cartilage. IL-1β and TNFα disperse into the cartilage tissue, where they up-regulate aggrecanases and matrix metalloproteinases (MMPs). This accelerates the degradation of cartilage (Abramson *et al.,* 2006).

In addition, increased expression of IL-1β and TNFα can lead to an up-regulation of transcription factors, such as nuclear factor (NF-) κB, which initiate the transcription of downstream mediators like matrix metalloproteinases (MMPs), nitric oxide (NO), prostaglandin E2 (PGE2), IL-6, IL-2 and IL-8 (Martel-Pelletier *et al.*, 1998) as well as the self-propagation of IL-1β and TNFα (Vincenti and Brinckerhoff, 2002). The effects of anabolic mediators like insulin-like growth factor-1 (IGF-1) and IL-4 are outweighed by the actions of the pro-inflammatory cytokines.

1.3 Role of catabolic mediators

1.3.1 Pro-inflammatory cytokines

IL-1β

IL-1β is an important cytokine belonging to the IL-1 superfamily, produced by macrophages and monocytes. It is released in its active form by way of proteolytic cleavage by the IL-1 converting enzyme (ICE-1 or caspase-1) (Black *et al.*, 1988; Kronheim *et al.*, 1992). This pro-inflammatory cytokine has been found in its active form in articular joint tissue, and in *ex vivo* experiments OA synovial membrane has been shown to secrete this cytokine. The biological activities of IL-1β are mediated through the association with the receptor type I IL-1R. It triggers an inflammation cascade mediated by MMPs and aggrecanases, leading to degradation of the cartilaginous matrix (Aigner *et al.*, 2007). By doing this, IL-1β indirectly suppresses the expression of genes associated with differentiated chondrocyte phenotype such as type II collagen. In normal cartilage tissue, this cytokine plays an important role in extracellular matrix turnover, and its catabolic actions can be counteracted by interleukin-1 receptor antagonist (IL-1Ra). IL-1Ra binds to the IL-1 receptor, blocking the binding and consequently the activity of IL-1β.

TNFα

TNFα is present in later stages of OA, where inflammation is prevalent. This cytokine is best known for its ability to promote the inflammatory processes, signifying its role as a principal mediator of matrix degradation. In addition, TNFα is synthesized as a precursor protein, which is activated by proteolytic cleavage through TNFα converting enzyme (TACE) (Black *et al.*, 1997). The pleiotropic effects of TNFα are similar to those of IL-1β and include recruiting other pro-inflammatory factors, up-regulating enzymes that cause cartilage erosion, and driving pathologic reactions such as over-proliferation or resistance to apoptosis that lead to synovial hyperplasia (Kim *et al.*, 2000; Kobayashi *et al.*, 1999).

IL-6

Not all catabolic processes in OA tissue are due solely to the effects of IL-1β and TNFα. Other pro-inflammatory cytokines like IL-6 are also involved in the disease process. IL-6 can induce the number of inflammatory cells in the synovial tissue (Guerne *et al.*, 1989) as well as amplify the IL-1β effects of inducing MMPs and inhibiting proteoglycan synthesis (Nietfeld *et al.*, 1990). Reversely, IL-6 has also been shown to up-regulate the expression of tissue inhibitors of metalloproteinases (TIMP) (Lotz and Guerne, 1991). This is believed to be a

feed-back mechanism that limits proteolytic damage in normal cartilage by the pro-inflammatory cytokine.

1.3.2 Matrix metalleoproteinases

Matrix metalloproteinases (MMPs), a group of approximately 20 family members, are zinc-dependent proteolytic enzymes that function as collagenases *in vivo*. They have been identified as the main enzymes that degrade extracellular matrix components (Nagase and Woessner, Jr., 1999; Vincenti, 2001). The majority of the MMPs are secreted into the extracellular matrix in a latent form needing proteolytic cleavage in order to become active while others are emitted into the extracellular matrix in an active form (Nagase and Woessner, Jr., 1999). Expression levels of these enzymes are regulated by TIMPs and are kept low in normal tissue, allowing for healthy tissue regeneration. Yet in pathological conditions such as OA, the levels of MMP expression exceed the expression of TIMPs causing an increase in cartilage destruction (Martel-Pelletier *et al.*, 1994).

The destructive processes of MMPs are well documented for OA conditions. Pro-inflammatory cytokines IL-1β, TNFα and IL-6 stimulate articular chondrocytes to produce MMPs such as MMP-1, MMP-3, and MMP-13 (Tetlow *et al.*, 2001; Nakamura *et al.*, 2006). MMP-3, also known as stromelysin, is the most commonly expressed MMP and may activate MMP-1 (collagenase-1) (Vincenti *et al.*, 1996) as well as other proenzymes to degrade proteoglycans (Okada *et al.*, 1992). MMP-13 (collagenase-3) is normally produced during development and by chondrocytes in OA (Borden *et al.*, 1996; Mengshol *et al.*, 2001; Vincenti *et al.*, 1998). Interstitial collagens, such as type II collagen, are the main targets for the destructive processes of both MMP-1 and MMP-13 (Billinghurst *et al.*, 1997).

1.3.3 Inducible nitric oxide synthase and nitric oxide

In addition to pro-inflammatory cytokines and degradative enzymes, another inflammatory mediator plays a major role in OA processes, namely nitric oxide (NO). NO is a free radical that is synthesized from L-arginine by enzymes of the NO synthase (NOS) family (Moncada and Higgs, 1993; Nathan, 1992). The synthesis of nitric oxide (NO) is increased by inducible NOS (iNOS), which is expressed in response to pro-inflammatory cytokines like IL-1β and TNFα, and has been detected in synovium and cartilage of patients with inflammatory joint diseases (Hashimoto *et al.*, 1998; Sakurai *et al.*, 1995; Farrell *et al.*, 1992). In a canine OA model, NO levels were found to be increased in the synovial fluid (Spreng *et al.*, 2001). This

increase in NO, which occurs thorough the iNOS pathway, leads to the production of prostaglandins via the cyclo-oxygenase-2 (COX-2) and ultimately pain (Amin *et al.*, 1997; Haupt *et al.*, 2005; Loeser *et al.*, 2003). Further, elevated levels of NO contribute to the disease processes of OA by partaking in processes such as apoptosis, by prolonging the expression of pro-inflammatory cytokines (Kobayashi *et al.*, 2001; Pelletier *et al.*, 2001; Sakurai *et al.*, 1995) and by intensifying cartilage destruction through the activation of MMPs (Murrell *et al.*, 1995; Pelletier *et al.*, 1998) as well as the inhibition of aggrecan and type II collagen synthesis in chondrocytes (Lee *et al.*, 2002; Studer *et al.*, 1999; Tomita *et al.*, 2001). In animal models, treatment with nitric oxide synthase inhibitors reduced the progression of cartilage destruction (Pelletier *et al.*, 1998). Overall, NO negatively affects the anabolic processes while promoting the catabolic processes in OA.

1.4 Role of anabolic mediators

1.4.1 Insulin-like growth factor-1

The anabolic activity in normal cartilage is maintained by growth factors and anti-inflammatory mediators like IGF-1 and IL-4 (Rachakonda *et al.*, 2008a; Tyler, 1989). In OA, the anabolic activity is suppressed despite an overall up-regulation of anabolic mediators in the affected cartilage (Aigner *et al.*, 1997; Hambach *et al.*, 1998; Lafeber *et al.*, 1992). Utilization of anabolic mediators offers an essential option to enhance cartilage regeneration and to reinstate the balance between synthesis and degradative mediators.

IGF-1, also known as somatomedin-c, is a 70 kDa protein which is primarily produced in the liver in response to growth hormones (GH). This growth factor is also produced locally in cells of mesenchymal origin in a variety of tissues. IGF-1 produced in those tissues has been found to be equally important as circulating IGF-1 originating from the liver (D'Ercole *et al.*, 1986; Han *et al.*, 1987). In chondrocytes, IGF-1 serves as a paracrine and autocrine regulator in the stimulation of matrix synthesis and in the inhibition of matrix degradation (Tyler, 1989).

Moreover, IGF-1 serves as one of the main anabolic mediators in articular cartilage, which naturally aids in the protection of cartilage from regular wear and tear. Its role in maintaining homeostasis in articular cartilage includes stimulating proteoglycan and type II collagen synthesis, counteracting their degradation during regular turnover, and preventing cell death (Luyten *et al.*, 1988; McQuillan *et al.*, 1986; Oh and Chun, 2003). In the presence

of IL-1β and TNFα, IGF-1 protein has been shown to stimulate the production of proteoglycans and decrease the cytokine-stimulated degradation of proteoglycans (Tyler, 1989).

The action of IGF-1 in cartilage is altered during ageing and pathologies such as OA. Although there is an increased degradation of cartilage seen in OA, up-regulation of IGF-1 expression levels has been detected in OA human cartilage (Middleton *et al.*, 1996; Middleton and Tyler, 1992; Olney *et al.*, 1996). Likewise in dogs, IGF-1 levels in a ruptured knee joint were shown to be fourfold higher than in the contralateral, unaffected joint (Fernihough *et al.*, 2003). It is assumed that the initial increased production of IGF-1 in OA is due to an attempt of cartilage to restore homeostasis. However, desensitization of the diseased tissue to IGF-1 could possibly be due to an increase in insulin-like growth factor binding proteins (IGFBPs) making IGF-1 unavailable to bind to its receptor. Alternatively, this hyporesponsiveness to IGF-1 could also in part be due to the reduced reparative ability of cartilage seen at later stages of the disease.

1.4.1.1 Insulin-like growth factor receptor 1

The biological effects of IGF-1 are displayed by the high affinity binding of IGF-1 to the specific IGF receptor type 1 (IGFR1). IGFR1 is a tyrosine kinase receptor, which can activate the mitogen-activated protein kinase/extracellular signal-regulated kinase-kinase 1/2 (MEK1/2), extracellular signal-regulated kinase 1/2 (Erk1/2), mitogen-activated protein kinase (MAPK), and the phosphatidylinositol-3-kinase-Akt (PI3K-Akt) pathways. IGF-1 binding to its receptor results in autophosphorylation of the IGFR1 on its tyrosine residues, which then perform as adaptor molecules to recruit and activate downstream signaling cascades (Backer *et al.*, 1992b;Backer *et al.*, 1992a;Baltensperger *et al.*, 1994). Expression of IGFR1 in OA cartilage has been shown to be normal or elevated (Dore *et al.*, 1994; Middleton *et al.*, 1996; Rachakonda *et al.*, 2008a; Tardif *et al.*, 1996), which is a contradiction to the theory that IGF-1 insensitivity in OA is caused by a decrease in receptor gene expression levels.

1.4.1.2 Insulin-like growth factor binding proteins

The accessibility of IGF-1 to its receptor is regulated by extracellular IGFBPs (Jones and Clemmons, 1995). IGFBPs are a family of 6 members, which modulate the activity and extend the half-life of IGF-1 (Baxter, 1991). IGFBP-7 has been identified in human biological fluids and breast cancer cells, but has shown less affinity for IGF-1 (Oh *et al.*, 1996; Wilson *et*

al., 1997). These binding proteins are some of the key players in the inability of cartilage to readjust to homeostasis during OA. Although the levels of IGF-1 are increased in OA (Dore *et al.*, 1995; Middleton and Tyler, 1992; Olney *et al.*, 1996), a hyporesponsiveness to IGF-1 in the diseased tissue has been attributed to an increase in IGFBPs (Dore *et al.*, 1994; Olney *et al.*, 1996; Tardif *et al.*, 1996; Olney *et al.*, 1996; Verschure *et al.*, 1996).

Almost all IGFs are found complexed with one of the six highly homologous IGFBPs (IGFBPs 1–6), which extend the half-life of the growth factor (Firth and Baxter, 2002; Jones and Clemmons, 1995; Rechler, 1993). IGF-1 has a greater affinity for the IGFBPs than to its receptor. Three of those IGFBPs (IGFBP-2, -3, and -4) are secreted by articular cartilage or chondrocytes with IGFBP-3 being the most predominant one (Bhaumick, 1993; Chevalier and Tyler, 1996; Morales, 2002; Tardif *et al.*, 1996). In case of need, IGF-1 is released from IGFBPs by proteolytic cleavage, which decreases the affinity of the binding proteins to IGF-1 (Clemmons, 2001) making the growth factor available for clearing or for receptor binding. Proteolytic cleavage of IGFBPs may occur by aspartic, serine, and metalloproteinases releasing IGF-1. The resulting IGFBP fragments are then reduced in their affinity to IGF-1 (Cohen *et al.*, 1992; Conover and De Leon, 1994; Fowlkes *et al.*, 1994; Olney *et al.*, 1996). An increased local production of certain IGFBPs like IGFBP-3 could affect the amount of free IGF-1 available to reach its receptors in cartilage. The functional role of the binding proteins has not been totally elucidated. However, one study has shown that the amount of IGFBP-3 in OA was three times higher than in healthy cartilage, and was positively correlated with the severity of the disease (Morales, 2002). Also, Sunic and co-workers (1995) have demonstrated further the involvement of IGFBPs in IGF-1 bioactivity *in vitro.* They have shown that a mutant form of IGF-1, which has a decreased affinity for IGFBPs, stimulated the production of proteoglycans by cultured chondrocytes more efficiently than wild-type IGF-1. By increasing the amount of wild-type IGF-1 as well as the mutant form of IGF-1, the production of proteoglycans was increased in cultured chondrocytes 2- and 3-fold, respectively. Reversely, the addition of IGFBP-3 protein to cell culture medium blocked the effects of IGF-1 (Martin *et al.*, 1997). Possible direct effects of IGFBPs, independent of their IGF binding capacity, have also been reported, but so far are not well understood (Oh, 1997).

1.4.2 Interleukin-4

Interleukin-4 (IL-4) is mainly produced by activated $CD4^+$ lymphocytes and promotes the maturation of Th2 cells, which participate in the first line of the humoral immune response

(Nelms *et al.*, 1999). The importance of IL-4 is noted by its anti-inflammatory effects that lead to a down-regulation of Th1 cytokines such as IL-1β and TNFα as well as IL-6, IL-8, IL-10, and IL-12 (Hart *et al.*, 1989; Standiford *et al.*, 1990). In addition, IL-4 antagonizes the action of IL-1β by inducing the production of IL-1 receptor antagonist (IL-1Ra) (Vannier *et al.*, 1992; Wong *et al.*, 1993) as well as a soluble form of IL-1 receptor IL-1 RII, which inhibits IL-1β activity by acting as a decoy target for IL-1 (Colotta *et al.*, 1993).

Due to its antagonistic effects on inflammatory and destructive mediators in OA, IL-4 is a good candidate gene to be used for the reduction of degeneration in cartilage (Rachakonda *et al.*, 2008b; Hegemann *et al.* 2005). Intra-articular IL-4 gene therapy has been shown to prevent cartilage degradation in experimental arthritis (Boyle *et al.*, 1999; Kim *et al.*, 2000; Lubberts *et al.*, 1999; Wong *et al.*, 1993). This could be a direct result of IL-4 down-regulating MMPs, including MMP-1, MMP-3 and MMP-13 (Rachakonda *et al.*, 2008b) as well as the expression of iNOS mRNA and consequently the production of NO (Nishisaka *et al.*, 2001; Schuerwegh *et al.*, 2003).

1.5 Characteristics of cartilage

Hyaline articular cartilage is an avascular and aneural tissue which has an increased compressive strength and allows for minimal friction in the movement of normal joints (Hunziker, 2002). Moreover, articular cartilage is composed of **1)** approximately 60-80% of water, which allows for load-dependent deformation as well as nutrition and medium for lubrication, **2)** 10-20% of collagen, with type II collagen as the principal component (90-95%), **3)** 10-20% of proteoglycans, and **4)** 1-5% of chondrocytes, the sole cell type in cartilage. Nutrients for these cells are supplied from the capillaries of the synovium which then diffuse into the synovial fluid before reaching cartilage tissue. This lengthy process limits the regenerative ability of cartilage in disease conditions.

OA is a disease of the cartilage tissue. The extracellular matrix proteins, type II collagen and proteoglycans, are degraded by erosive mediators, which dissolve into the tissue from the synovial fluid, resulting in softening and eventually loss of cartilage tissue. In early OA, there is an increased synthetic activity, which is regarded as an attempt to regenerate the extracellular matrix, accompanied by an increase in catabolic mediators. Phenotypic modulation is reflected in the presence of collagens not normally found in cartilage, namely type X collagen, the hypertrophic chondrocyte marker. Due to the avascular nature of

cartilage, the earliest phases of degeneration are left unnoticed. Only in later stages of OA, where inflammation and pain are present, is the degradation of cartilage tissues revealed.

1.5.1 Chondrocytes

Chondrocytes, the single sole cell type in cartilage, are specialized terminally differentiated mesenchymal stem cells that are responsible for the synthesis of cartilage matrix proteins, which provide tensile strength and resistance to mechanical loading in cartilage. These cells are responsible for maintaining tissue homeostasis by up-regulating synthetic activity or by increasing the production of inflammatory cytokines. They are the cellular target of action in cartilage and carry on their cell surface receptors for the pro-inflammatory cytokines IL-1β and TNFα. In mature cartilage, chondrocytes show little mitotic activity and a low rate of matrix synthesis and degradation. However, in early OA chondrocytes display similar differentiation events as growth plate chondrocytes including cell proliferation, hypertrophy, and cell clustering (Kouri *et al.*, 1996; Hulth *et al.*, 1972; Pfander *et al.*, 2001). These changes are initiated by cytokines and other catabolic mediators from the synovium and chondrocytes themselves (Fig. 3) (Aigner *et al.*, 2002; Pelletier *et al.*, 2001).

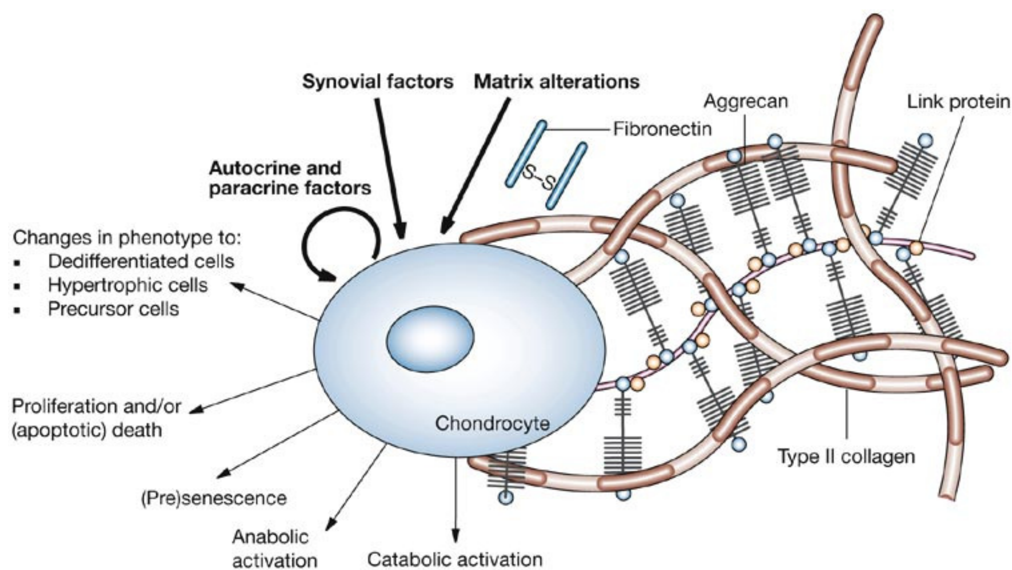


Figure 3: Chondrocyte response to OA. In OA chondrocytes are exposed to stimuli such as autocrine and paracrine factors, synovial factors and altered matrix components. Up-regulation of catabolic processes leads ultimately to apoptosis. Presenescence results in loss of chondrocyte function. Also affected are cartilage matrix proteins like aggrecan and type II collagen. Other collagens and proteoglycans are also affected by the disease processes (not shown)(Aigner *et al.,* 2007).

In a previous work, canine chondrocytes were characterized (Rai, 2008b). It was shown that canine chondrocytes produced type II collagen up to passage 4. Thereafter, the cells became dedifferentiated. However, this process was reversible following seeding these cells in 3D alginate culture system (section 1.5.4). More importantly, it was demonstrated that inducing inflammation by canine specific cytokines caused an up-regulation of pro-inflammatory cytokines like IL-1β and TNFα. Inflammation was subdued in cells transfected with canine IL-4.

1.5.2 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multipotent cells capable of differentiating into a variety of lineages, namely cartilage, bone, tendon, ligaments and adipose tissue (Barry and Murphy, 2004; Friedenstein *et al.*, 1966; Pittenger *et al.*, 1999). These non-hematopoietic cells can be isolated from many adult tissue types including bone marrow, skin, muscle, trabecular bone, and synovium (Baksh *et al.*, 2004; Friedenstein *et al.*, 1974) and from many different animals, including canine, porcine, equine and rabbit species (Fortier *et al.,* 1998; Kadiyala *et al.,* 1997; Koga *et al.,* 2008; Ringe *et al.,* 2002). In rabbits, a higher chondrogenic potential was seen in MSCs isolated from bone marrow and synovium (Koga *et al.* 2008). Of those two, bone marrow is more widely accepted as a source for MSCs in animal studies due to the ease at which the cells can be harvested. Up to now mostly bone marrow protocols have been used in dogs for pre-clinical studies. Canines provide a large animal system which is necessary when studying therapeutic potential of stem cells.

The morphology of non-differentiated MSCs is spindle-shaped sharing resemblance to fibroblasts (Friedenstein *et al.*, 1966; Pittenger *et al.*, 1999). There are no specific cell surface markers which can be used to identify MSCs. However, a consensus has been found amongst available data which standardizes the characterization of MSCs. Research shows that *in vitro* expanded MSCs adhere to cell culture flasks and do not express hematopoietic or endothelial surface markers such as CD11b, CD14, CD31, CD34 or CD45, but that they are positive for markers like CD29, CD44, CD73, CD90 and CD105 (Barry and Murphy, 2004; Jorgensen *et al.*, 2003; Pittenger *et al.*, 1999).

Studies show that MSCs are capable of differentiating into a variety of cell lineages when different cell culturing conditions are applied like administering certain cytokines and growth factors as well as increasing cell density. MSCs are able to differentiate into a variety of ectodermal, mesodermal and endodermal tissue cells (Fig. 4). They are however naturally

prone to differentiate into cells of mesodermal origin (Uccelli *et al.*, 2008). From bone marrow isolated cells, only a very finite number are stem cells, which are able to differentiate. Only 1 out of every 10,000–100,000 mononuclear cells isolated from bone marrow is capable of undergoing chondrogenesis, osteogenesis or adipogenesis (Pittenger *et al.*, 1999).

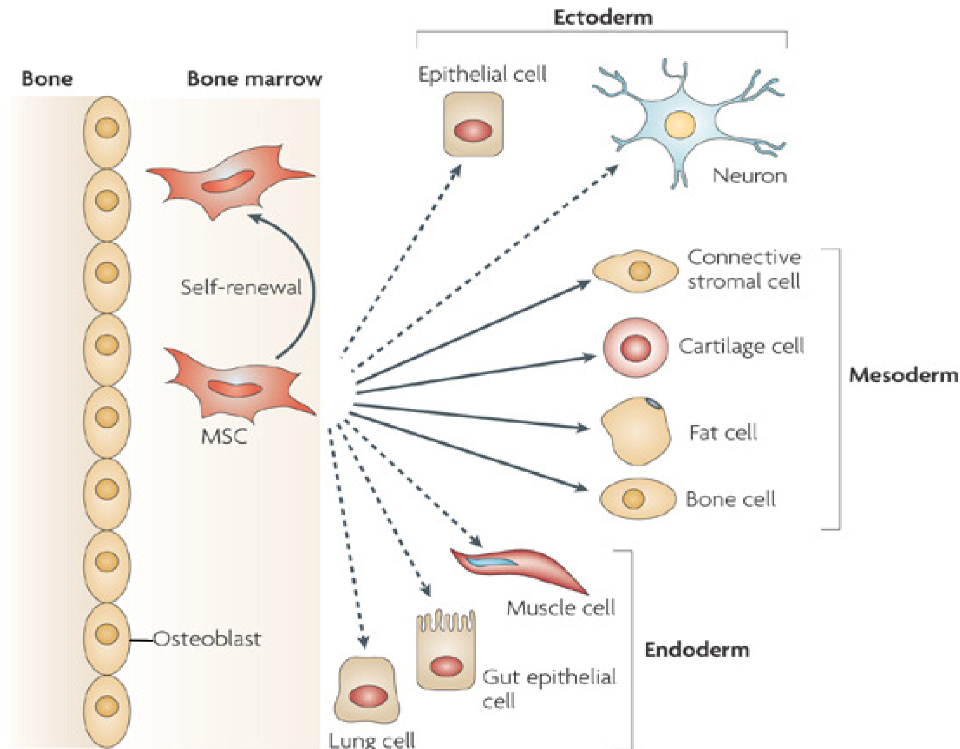


Figure 4: **Different cell lineages of mesenchymal stem cells (MSCs)**. MSCs are capable of self-renewal and are known for their ability to differentiate into a number of different cell lineages of the mesoderm (solid arrows). It is now known that MSCs can also differentiate into lineages of the ectoderm and endoderm in a process known as transdifferentiation (dashed arrows), which is controversial *in vivo* (Uccelli *et al.*, 2008).

The chondrogenic potential of MSCs has been demonstrated in *in vitro* pellet cultures and in alginate cultures treated with transforming growth factor β (TGFβ) (Johnstone *et al.*, 1998; Bosnakovski *et al.*, 2006). TGFβ possesses the ability to induce signal transduction pathways specific for chondrogenesis (Sekiya *et al.*, 2002). Moreover, MSCs respond to all isoforms of TGFβ, however the different isoforms have been shown to have different effects on the chondrogenic differentiation of MSCs. TGFβ2 and TGFβ3 have been shown to promote chondrogenesis more effectively than TGFβ1 (Barry, 2003). The appearance of a cartilage

phenotype is demonstrated by the greater accumulation of proteoglycans and the expression of type II collagen. *In vitro* experiments show that markers for chondrogenesis, such as aggrecan, type II collagen, and cartilage oligomeric matrix protein (COMP), are expressed in MSC cultures (Johnstone *et al.*, 1998; Martin *et al.*, 1997; Bradham *et al.*, 1995). Little research has been done to date, concerning the chondrogenic potential of canine MSCs (cMSCs). However, one study showed that canine MSCs were able to undergo chondrogenesis following treatment with chondrogenic medium consisting of TGFβ (Csaki *et al.*, 2007a). Chondrogenesis was verified by alcian blue staining of the proteoglycans and detection of type II collagen and SOX-9 expression in the differentiated cells. Despite the lack of available information, a better understanding of cMSCs will offer opportunities for better cell based therapies and tissue engineering approaches.

Not only can TGF-β enhance MSC chondrogenesis, IGF-1 and bone morphogenic proteins (BMPs) can also increase extracellular matrix synthesis of MSCs (Dore *et al.*, 1994; Moskowitz *et al.*, 1991; Worster *et al.*, 2001). The role of IGF-1 in the stimulation of chondrogenesis of MSCs is controversial. Some studies have shown that IGF-1 treatment has no effect on MSCs or only in combination with TGFβ (Baddoo *et al.*, 2003; Milne *et al.*, 2001; Indrawattana *et al.*, 2004). Others have shown that IGF-1 can induce chondrogenesis of limb bud MSCs (Oh and Chun, 2003) as well as periosteal MSCs (Fukumoto *et al.*, 2002). Another study has shown that IGF-1 induces the chondrogenic potential of bone marrow derived MSCs similar to TGFβ1 (Longobardi *et al.*, 2006). Further analyses of the affects of IGF-1 on differentiation of MSCs will have to be performed to elucidate the magnitude to which this gene can stimulate chondrogenesis.

1.5.3 Chondrocyte/MSC markers

1.5.3.1 Collagen

Type II collagen is the primary collagen in healthy cartilage tissue, making up 90–98% of the total tissue (Martel-Pelletier *et al.*, 2008). There are two forms of type II pro-collagen that can be generated, namely type IIA procollagen, which is mostly associated with newly formed cartilage, and type IIB pro-collagen, which is present in all cartilage. Type II collagen serves as a good marker for chondrocyte differentiation as well as the chondrogenic differentiation of MSCs. Pre-cartilaginous mesenchyme synthesizes type I and type III collagen. Non-differentiated MSCs lack the expression of type II collagen and express

typically type I collagen. Commitment to the chondrogenic lineage is characterized by an up-regulation of mRNA synthesis of type II collagen followed by the development of extracellular matrix proteins. Dedifferentiated chondrocytes show a down-regulation of type II collagen and an increased expression of type I collagen. However, seeding those cells in a 3D-matrix can stimulate their redifferentiation (Rai *et al.*, 2008). Type X collagen is a marker of the hypertrophic chondrocyte. Its expression is normally absent in normal adult articular cartilage. However, in OA the expression of type X collagen is increased. In MSCs, the expression of type X collagen has been noted as early as two weeks following induction of chondrogenesis (Johnstone *et al.*, 1998), signifying the need to further optimize *in vitro* conditions of MSCs before utilizing them for therapeutic treatment.

1.5.3.2 Aggrecan

The proteoglycan aggrecan is the second most common macromolecule in cartilage and is associated with hyaluronan and link protein in the form of proteoglycan aggregates, which allows cartilage to withstand compressive loads. Its composition and concentration are not the same throughout cartilage tissue and change with increasing age, injury and disease (Setton *et al.*, 1999). In OA, proteases are able to attack proteoglycan monomers, which lead to the diffusion of fragments into the synovial fluid leaving behind normal proteoglycans that can still form aggregates. However, the reduction of hyaluronan in OA cartilage causes a reduction in the size of the aggregates, making the proteoglycans more vulnerable to terminal degradation (Martel-Pelletier *et al.*, 2008). The increased loss of aggrecan fragments is due to the action of not only aggrecanases but also MMPs (Buttner *et al.*, 1998; Lark *et al.*, 1997). In early stages of OA, new aggrecan synthesis does occur as an attempt by chondrocytes to initiate repair. However in later stages, these fragments also are diffused into the synovial fluid (Lohmander *et al.*, 1999). In MSCs, aggrecan serves as a marker gene validating the chondrogenic differentiation of MSCs.

1.5.3.3 SOX9

Transcription factors are important regulators of cartilage metabolism noted through their ability to stimulate chondrogenesis in physiologic and pathologic conditions. One essential transcription factor for articular chondrocytes is sex-determining region Y-type high mobility group box-9 (SOX9). SOX9 plays a critical role in the regulation of skeletal and cartilage formation (Bi *et al.*, 1999) as well as chondrocyte differentiation (Ikeda *et al.*, 2004).

Moreover, this transcription factor has been shown to be co-expressed with type II collagen during fetal development in mice (Ng *et al.*, 1997) and to prevent hypertrophic differentiation of chondrogenic cells (Akiyama *et al.*, 2002). In OA, SOX9 gene expression levels were found to be decreased (Aigner *et al.*, 2003; Salminen *et al.*, 2001). This explains what is seen for the expression levels of type II collagen in OA conditions. Research has demonstrated that SOX9 binds directly to the type II collagen enhancer element resulting in the transcription of type II collagen in chondrocytes (Bell *et al.*, 1997; Lefebvre *et al.*, 1997; Ng *et al.*, 1997; Zhou *et al.*, 1998). Mutations in this binding site lead to a loss of transcriptional activation of the type II collagen gene at least *in vitro* (Matsuno *et al.*, 1997). Dedifferentiation of chondrocytes *in vitro* has been linked to the down-regulation of SOX9 and type II collagen mRNA levels (Lefebvre and de Crumbrugghe , 1998). SOX9 is not only thought to be involved in the regulation of type II collagen expression (Bridgewater *et al.*, 1998) but also in the regulation of other cartilage specific genes like aggrecan (Bell *et al.*, 1997; Lefebvre *et al.*, 1997; Ng *et al.*, 1997; Xie *et al.*, 1999).

1.5.4 Three-dimensional (3D) cultures

One problem associated with using chondrocytes for the treatment of joint defects is the lack of a sufficient number of cells. In an effort to increase the number of cells available for cartilage repair, chondrocytes are isolated and expanded *in vitro*. Once released from their native matrix and propagated in monolayer, chondrocytes become dedifferentiated and lose their native chondrogenic phenotype. Moreover, they tend to have a fibroblastic morphology and no longer synthesize type II collagen and aggrecan (Bonaventure *et al.*, 1994; Stewart *et al.*, 2000). Nevertheless, redifferentiation of passaged chondrocytes has been achieved by culturing the cells in pellet cultures (Borge *et al.*, 1996; Lubke *et al.*, 2005), agarose (Benya and Shaffer, 1982), collagen gels (Muller-Rath *et al.*, 2007), and in alginate beads (Bonaventure *et al.*, 1994).

The alginate 3D-culture system is the most readily used culture *in vitro* for investigating chondrocytes and MSCs (Bosnakovski *et al.*, 2006; Ma *et al.*, 2003). Alginate is a polysaccharide that is composed of D-mannuronic acid residues and L-gluronic acid residues, which contribute to the elasticity and mechanical strength as well as to the stability and porosity of the gel, respectively. The highly porous nature of alginate provides a way for nutrient and protein diffusion (Williams *et al.*, 2003). Within the alginate gel cells can attach, proliferate and differentiate. Another advantage is that cells are easily recovered from the

alginate using calcium chelating agents, allowing for analyses of matrix proteins (Hauselmann *et al.*, 1994; Madry *et al.*, 2003).

Production of an extracellular matrix with morphological and functional characteristics similar to that of articular cartilage has been seen in chondrocytes cultured in 3D matrices (Cook *et al.*, 1997; Buschmann *et al.*, 1992; Thompson *et al.*, 1985). Chondrocytes grown in alginate beads have been shown to regain their spherical shape and to begin to produce chondrocyte specific proteins, such as type II collagen (Hauselmann *et al.*, 1994; Liu *et al.*, 1998; Lemare *et al.*, 1998; Guo *et al.*, 1989). Research has shown that chondrocytes cultured in alginate beads retained their native morphology and continually expressed type II collagen and proteoglycans for up to 8 months (Hauselmann *et al.*, 1994).

In addition, the use of 3D-cultures is also important for analyzing MSCs *in vitro*. Two representative culture systems, which have been developed to promote chondrogenic differentiation of MSCs are the pellet culture system and alginate beads. The pellet culture system was originally described for its ability to prevent dedifferentiation of chondrocytes (Johnstone *et al.*, 1998). This system involves the formation of cell aggregates by centrifugation and allows for cell-cell interactions, which are comparable to those seen during pre-cartilage condensation without the use of additional biomaterials. Among the different types of carrier culture systems (agarose, alginate, collagen gels), alginate was found to have the necessary physical characteristics and handling properties necessary to support the cells and to serve as a carrier to fill osteochondral defects *in vivo* (Diduch *et al*. 2000). Bovine MSCs seeded in alginate beads maintained their spherical form and increased the expression of chondrocyte specific genes (Bosnakovski *et al.*, 2006). Not only in alginate, but cells cultured in both systems are able to express type II collagen, one important marker for differentiated chondrocytes. Cultivation of MSCs in pellets or alginate matrices is not enough to stimulate chondrogenesis of MSCs (Barry *et al.*, 2001; Johnstone *et al.*, 1998). Chondrogenesis of MSCs require a defined medium containing certain bioactive factors like insulin, pyruvate, dexamethasone, and TGFβ.

1.6 Treatment of OA

1.6.1 Medication

Currently, there is no cure or effective treatment for OA. The use of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are the most common treatments used to

dampen the symptoms associated with the disease (Bannwarth, 2006). Unfortunately, long-term, extensive usage of NSAIDs can lead to other health ailments, including drug intolerance, gastro-intestinal tract disturbances and ultimately ulcers. In chronic OA, NSAIDs are more often substituted with COX-2 inhibitors. Long-term intake of COX-2 inhibitors has been associated with systematic side effects, resulting from the down-regulation of the entire synthesis of prostaglandins (Kawano *et al.*, 2006). This blockage disrupts regulatory processes associated with the cardiovascular system and the brain.

1.6.2 Surgical options

If left untreated, large focal defects may lead to end stage arthritis. Common treatment for patients with severe joint erosion is partial or total joint replacement. One of the main problems associated with total joint replacement is the limitation of movement in that particular joint. For this reason, this treatment is reserved for elderly patients with a sedentary life style. Gene therapy in combination with autologous cell transplantation offers an alternative treatement for focal defects and joint replacement.

1.6.3 Gene therapy

Gene therapy is defined as the insertion of genetic material into cells in order to improve cellular function or structure at the molecular level (Anderson, 1998). Due to their crucial role in regenerative processes, such as soft tissue repair and cartilage healing, efforts have been made to use immunomodulatory agents as a means of gene therapy (Trippel, 1997). An alternative to genetically modifying cells would be to apply recombinant proteins directly into the defect. However, utilizing growth factor or anti-inflammatory recombinant proteins presents problems such as short half-life, enzymatic inactivation, and high costs (Tepper and Mehrara, 2002). Also, the use of recombinant proteins is associated with repeated systematic intake or injection, which could lead to adverse side-effects. For this reason, gene transfer techniques using viral or non-viral vectors present an attractive alternative to have localized, long-term production of the therapeutic protein.

1.6.3.1 Viral vectors

There are a number of viruses which may be used for gene delivery, including adenoviruses, adeno-associated viruses, retroviruses and herpes simplex virus. Overall viral vectors are known for their higher gene transfer efficiencies compared to the non-viral counterparts.

Nevertheless, viral gene transfer systems have been found to be immunogenic and more toxic than non-viral vectors, which possess the risk of recombination resulting in diseases caused by replication-competent virus (Bleiziffer *et al.*, 2007). Due to the inflammatory response normally seen by direct injection of viral vectors, *ex vivo* transduction has risen as an alternative. Despite this, there are still concerns about cytotoxicity.

1.6.3.2 Non-viral vectors

Usage of non-viral vectors offers some specific advantages over viral vectors, including better safety profiles (lower toxicity and non-infectious properties), the capacity to transfer large genes and low production costs. Even though there are concerns associated with relatively low gene delivery and transgene expression efficiencies of non-viral vectors, efforts are continually being made to overcome these obstacles. Transfection of chondrocytes with FuGENE6 resulted in a transfection efficiency of 35% and therapeutic levels of IGF-1 for 32 days (Madry *et al.*, 2004). Yet, MSCs are only mildly receptive to transfection with non-viral vectors and highly receptive to transduction with various viral vectors. Obtaining high transfection efficiencies in stem cells is challenging. Transfection of MSCs with non-viral vectors via nucleofection, however, has yielded transfection efficiencies between 40 and 94%, where the use of FuGENE6 yielded a mere 4% (Aluigi *et al.*, 2006). Despite the challenges faced transfecting MSCs, research has shown that MSCs transfected with a non-viral vector expressing the transgene TGFβ2 undergo chondrogenesis (Wang *et al.*, 2003). In addition, those cells express cartilage specific genes and proteins, underlining the ability of progenitor cells to undergo chondrogenesis from transient transfection. In addition, periosteal MSCs transfected with a non-viral vector containing the transgene BMP-7 have shown a greater amount of hyaline cartilage than non-transfected cells (Grande *et al.*, 2003).

1.6.3.3 Combined expression of therapeutic genes

Due to the multi-facet pathological nature of OA, regeneration of cartilage tissue and restoring the balance between catabolic and anabolic mediators may require more than one therapeutic gene (Loeser *et al.*, 2003). In synoviocytes, an adenoviral vector containing both IGF-1 and IL-1 receptor antagonist was necessary to lessen the disease processes resulting in not only a reduction in inflammatory mediators but also an increase in proteoglycan expression (Haupt *et al.*, 2005). These effects were not seen in synoviocytes transduced with

IGF-1 alone. In another study, the transgenes FGF2 and SOX9 were delivered simultaneously by separate recombinant adeno-associated viral (rAAV) vectors showing that not only could fibroblast growth factor-2 (FGF2) maintain its mitogenic characteristics but also co-expression with SOX9 resulted in the stimulation of type II collagen and proteoglycan synthesis (Cucchiarini *et al.*, 2008). In each case, the combined effects of the genes were greater than what was observed using single genes.

1.6.3.4 Stable transfection and co-cultivation of cells

Low transfection efficiencies and short-term gene expression are characteristics of non-viral gene transfer. This may be advantageous in clinical settings where gene expression is only needed for short periods, like in wound healing or bone regeneration (Bleiziffer *et al.*, 2007). However, in cases where extended gene expression is desired, selection of a cell line stably expressing a gene or a combination of genes over a long period of time offers an alternative to viral vectors. Stably transfected cells co-cultivated with chondrocytes or MSCs could provide a possibility to increase the number of cells available for joint repair as well as enhance and maintain tissue formation of a chondrogenic lineage needed in OA therapy.
Co-cultivation of cells provides a means of cell-cell interaction, which plays an important role in tissue development and influences cell proliferation, differentiation and physiology (Bhatia *et al.*, 1997; Gerstenfeld *et al.*, 2003). Because cell-cell interactions normally occur at the outer surface of cartilage, co-culture research has based on studying the development of OA by focusing on the interactions between articular chondrocytes and synovial cells (Goldring *et al.*, 1984; Lubke *et al.*, 2005; Wu *et al.*, 2005) or chondrocytes and osteogenic cells (Jiang *et al.*, 2005; Sanchez *et al.*, 2005). Co-cultivation of chondrocytes or MSCs with stably transfected chondrocytes has not yet been studied. However, co-cultivation of MSCs with non-transfected bovine articular chondrocytes has been shown to up-regulate the cartilaginous phenotype of the latter (Tsuchiya *et al.*, 2009).

1.6.4 Cell therapy

1.6.4.1 Autologous cell transplantation

Autologous Cell Transplantation (ACT) is a cell-based articular cartilage repair therapeutic procedure, which utilizes autologous chondrocytes or differentiated mesenchymal stem cells as a cell source (Fig.5). When chondrocytes are used, two surgical sessions are necessary. In

the first session, healthy cartilage from non-weight bearing areas of the joint is removed from the patient and the chondrocytes are isolated and expanded *ex vivo* followed by placement in a chosen matrix (see section 1.5.4). In the following surgical session, the autologous cells seeded in matrix material are placed into the cartilage defect. The use of autologous chondrocytes is important to avoid the risk of onsetting an immune reaction (Elves and Ford, 1974; Heyner, 1969; Kawabe and Yoshinao, 1991). However, locating a suitable donor site to obtain enough cartilage for chondrocyte isolation is problematic.

In this situation, utilization of MSCs as a cell source is more advantageous as autologous chondrocytes because damage to healthy cartilage donor sites can be avoided and expansion in monolayer is possible without the loss of the chondrogenic potential of the cells at early passages. In addition, the possibility of using allogenic MSCs has also been suggested for use due to their ability to escape immune responses since they lack major histocompatibility class II complex (MHC class II) (Ryan *et al.*, 2005). In using MSCs for the repair of joint defects, they are stimulated to undergo chondrogenic differentiation and stabilized as chondrocytes. In a rabbit model, the chondrogenic potential of MSCs as well as the regenerative potential of MSCs were shown *in vivo* (Wakitani *et al.*, 1994). Nevertheless, the challenge when using MSCs as a cell source in cartilage tissue engineering is to maintain the MSC-derived chondrocytes in a pre-hypertrophic state and to prevent them from undergoing terminal differentiation (Steinert *et al.*, 2007).

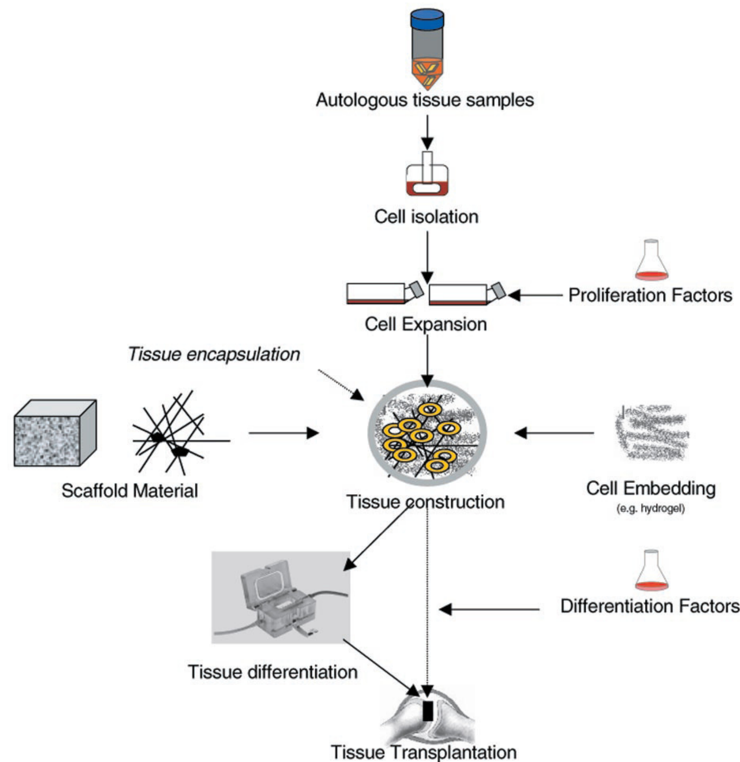


Figure 5: Schematic overview of autologous cell transplantation (ACT). Cells (chondrocytes or MSCs) are isolated and expanded *ex vivo*. Then, those cells are encapsulated in a biomaterial matrix and used to fill defects in the joints (Schultz *et al.*, 2000).

1.6.4.2 Autologous conditioned cell transplantion

Cartilage defects that arise from disease processes such as OA are distinct from focal cartilage lesions that result from acute injury. The latter often occur in possibly young patients with otherwise healthy joints that do not bear signs of inflammation. On the other hand, OA patients tend to be older, and the entire cartilage surface may require treatment. Repairing such defects with unconditioned transplants (transplants without therapeutic genes) may result in temporary pain relief and delay of OA processes, however this does not offer a long-lasting control of the disease mediators. In order to reach the ultimate goal of cartilage repair, it becomes necessary to implement a gene therapy approach. In doing so, it would ideally be possible to down-regulate pro-inflammatory cytokines and to up-regulate regenerative mediators while eliminating pain and restoring the function of the joint. ACT combined with gene therapy, i.e. conditioned with a therapeutic transgene, presents a possibility to aid in restoring the balance in OA tissue. This technique, termed autologous

conditioned cell transplantation (ACCT), involves *ex vivo* modification of cells (chondrocytes or MSCs) and transplantation of these cells into the diseased joint. *Ex vivo* gene-delivery allows for control of modified cells and quality control before reimplantation (Evans *et al.*, 2004).

1.7 Canine OA model

Obtaining cartilage tissue from human patients is difficult, making it challenging to study the disease process of OA. For this reason, the use of animal models provides a practical way to examine the underlying mechanisms which cause the imbalance between matrix synthesis and degradation as well as the inflammatory processes in OA. The greater the similarity of OA in the animal model compared to that of humans, the more desirable the model. Attributable to the evidence that the canine form of OA mimics the disease form seen in humans, canines make a good model for the study of OA. Models based on surgically induced laxity of the knee joint have been most frequently utilized in dogs. The most studied surgical models are the anterior cruciate ligament model (ACL) (Marshall and Chan, 1996; Pond and Nuki, 1973) and the bilateral small Groove model (Intema *et al.*, 2008). The natural processes of OA in these models make it attractive for use in studying the pathology of the disease (Brandt, 1994).

Moreover, in Germany, there are approximately 5 million dogs of which 15−20% are affected with arthritis (Section 1.2). A majority of these dogs are middle-aged or older and belong to large breeds. Larger breeds of dogs are more susceptible to rupture of cranial cruciate ligament, hip dysplasia or osteochondrosis, which can subsequently lead to OA. Also, in certain breeds, OA can develop due to genetical pre-dispostion. Labrador retrievers with hip dysplasia developed OA as early as at 2 years of age (Burton-Wurster *et al.*, 1999). A loss of proteoglycan content and collagen fibrils has been shown under these conditions (Burton-Wurster *et al.*, 1982; Lust *et al.*, 1972). This is very similar to what has been shown for human OA.

1.8 Research Aim

As of today, there is no complete cure for OA. Available treatments are based upon minimizing the pain and symptoms associated with the disease, but are unable to heal defected cartilage. The main mediators in the disease process are IL-1β and TNFα, where IL-1β is believed to be responsible for cartilage degradation and TNFα for driving the inflammatory processes. Both up-regulate the expression of catabolic mediators, such as other pro-inflammatory cytokines, matrix metalloproteinases, and inducible nitric oxide synthase. This in return leads to a degeneration of cartilage tissue. A treatment that is able to restore the balance between both catabolic and anabolic mediators is needed to combat the disease processes. Implementation of gene therapy utilizing the combined expression of two anabolic mediators offers the possibility of not only pain reduction but also regeneration of cartilage defects.

The overall research aim of this study is to down-regulate catabolic mediators while stimulating the synthesis of extracellular matrix components in canine chondrocytes and cMSCs by co-expressing the anabolic mediators IGF-1 and IL-4. This aim can be further divided into three sections as specified below.

1) **To analyze the dual expression of anabolic mediators in canine chondrocytes:**

In chondrocytes, both IGF-1 and IL-4 are known for their ability to induce the synthesis of type II collagen and the proteoglycan aggrecan as well as down-regulate destructive catabolic mediators, respectively. It is hypothesized that the combined expression of these genes from a single vector would down-regulate the effects of the pro-inflammatory mediators in an *in vitro* OA model more effectively than the use of IGF-1 alone, resulting in regeneration of extracellular matrix proteins and suppression of pro-inflammatory mediators.

2) **To characterize canine mesenchymal stem cells and their ability to undergo chondrogenesis utilizing TGFβ3 and transfecting them with a non-viral vector expressing IGF-1 and IL-4:**

Mesenchymal stem cells offer a good alternative cell source for application in tissue engineering. Following characterization of the canine MSCs, questioned was whether these cells could undergo chondrogenesis utilizing TGFβ3 and if transfection with a non-viral

vector expressing both IGF-1 and IL-4 from a single vector would be able to stimulate chondrogenesis.

3) To analyze the co-cultivation of stably transfected canine chondrocytes with chondrocytes or MSC.

To further analyze the effect of the dual expression of IGF-1 and IL-4 in canine chondrocytes and MSCs, a stable chondrocyte cell line expressing both genes would be prepared. Those cells would then be co-cultivated with chondrocytes or MSCs and analyzed for their ability to down-regulate catabolic affects of IL-1β and TNFα and to up-regulate type II collagen expression.

2 Material and Methods

2.1 Material

2.1.1 Animal samples

Articular cartilage used in this study was taken from the knee joints of euthanized dogs (n = 5) supplied by the Clinic for Small Animals of the Freie Universität Berlin. The animals used in these experiments were euthanized for reasons not pertaining to osteoarthritis. Moreover, bone marrow was aspirated from the posterior iliac spine of dogs (n = 5) undergoing joint replacement.

Table 1: Animal Samples

Patient/Breed	Age	Sex	Sample Type
Russian Terrier	7 years	Male	Cartilage
Great Dane	6 months	Male	Cartilage
Shar Pei	2 years	Female	Cartilage
Labrador	4 months	Male	Cartilage
Weimaraner Welpen	3 months	Male	Cartilage
Doberman	5 years	Male	Bone Marrow
Bordeauxdogge	5 months	Male	Bone Marrow
German Shepherd	9 years	Male	Bone Marrow
German Shepherd	1 year	Female	Bone Marrow
Bernese Mountain	1 year	Female	Bone Marrow

2.1.2 Reagents

Chemicals not listed were purchased from Sigma-Aldrich (Taufkirchen) or Carl Roth (Karlsrühe).

Agarose	Invitrogen, Karlsruhe
Acrylamide	Carl Roth, Karlsruhe
Alcian Blue 8GX	Carl Roth, Karlsruhe
Alginate Disulfide Salt	Sigma-Aldrich, Taufkirchen
APS	National Diagnostics, Munich
Bisacrylamide	Carl Roth, Karlsruhe
Coomassie Brillant Blue R 250	SERVA Feinbiochemica, Heidelberg
Collagenase type CLS	Biochrom AG, Seromed, Berlin
Collagenase P	Roche Diagnostics, Mannheim
Dexamethasone	Sigma Aldrich, Taufkrichen
DMEM	Pan-Biotech, Aidenbach
FCS	BioWhittaker, Walkersville, USA
FuGENE 6	Roche Diagnostic, Mannheim
HEPES	Biochrom AG, Berlin
Hyaluronidase	Roche Diagnostics, Mannheim
ITS	Sigma Aldrich, Taufkrichen
L-Ascorbic Acid	Wako Chemicals, Neuss
L-Glutamin	Biochrom AG, Berlin
Low Glucose-DMEM	Biochrom AG, Berlin
Na-Pyruvate	Sigma Aldrich, Taufkrichen
Non-fat Dry Milk	AppliChem, Darmstadt
Oligonucleotide Primers	TIB Molbiol, Berlin
OPDA	Carl Roth, Karlsruhe

Percoll	Biochrom AG, Berlin
PBS	Biochrom, Berlin
PMSF	AppliChem, Darmstadt
RPMI	PAN Biotech, Aidenbach
SDS	AppliChem, Darmstadt
TEMED	National Diagnostics, Munich
Tween 80	SERVA Feinbiochemica, Heidelberg

2.1.3 Enzymes

Deoxyribonuclease I (DNase I)	Fermentas, St. Leon-Rot
Lysozym	Carl Roth, Karlsruhe
Mango Taq DNA Polymerase	Bioline, Luckenwalde
Restriction Endonucleases	New England BioLabs, Frankfurt/M
RevertAid M-MuL V Rev Transcriptase	Fermentas, St. Leon-Rot
Ribolock Ribonuclease Inhibitor	Fermentas, St. Leon-Rot
T4 DNA-Ligase	Fermentas, St. Leon-Rot

2.1.4 Vectors

pcDNA 3.1	Invitrogen, Karlsruhe
pGEM-T	Promega, Mannheim
pVitro2	InvivoGen, Toulouse, France

2.1.5 Reagent systems (Kits)

cDNA Synthesis Kit	Fermentas, St. Leon-Rot
GeneMATRIX Universal RNA Purification Kit	Roboklon, Berlin
Griess Reagent System	Promega, Mannheim
Human IGF-1 Quantikine Immunoassay	R+D Systems, Wiesbaden-Nordenstadt
Invisorb Spin Plasmid Mini Kit	Invitek, Berlin

Jetsorb Gel Extraction Kit 600	Genomed, Bad Öyenhausen
DAKOCytomation LSAB2 System-HRP	Dako Deutschland, Hamburg
SensiMix*Plus* SYBR & Fluorescein	Quantace, Berlin
SuperSignal West PICO Chemiluminescent Substrate	Pierce Biotechnology, Jena
Z-Competent E. coli Transformation Kit	Zymo Research, California, USA

2.1.6 Competent cells

BL21 (DE3)p(Lys)	Novagene, Darmstadt
DH5α	Invitrogen, Karlsruhe
XL1-Blue	Stratagene, California, USA

2.1.7 Media and antibiotics

2 YT-Broth Medium	Invitrogen, Karlsruhe
Agar	Invitrogen, Karlsruhe
Ampicillin	Carl Roth, Karlsruhe
Fast-Media TB Media & Agar	InvivoGen, Toulouse, France
G418 Disulphate	AppliChem, Darmstadt
Kanamycin	Carl Roth, Karlsruhe

2.1.8 DNA/protein markers

DNA λ Eco91 (Bst E II)	Fermentas, St. Leon-Rot
HyperLadder IV DNA	Bioline, Luckenwalde
O'RangeRuler 100 bp	Fermentas, St. Leon-Rot
Pre-stained Marker III	AppliChem, Darmstadt

2.1.9 Antibodies

Goat anti-IL-4	R+D Systems, Wiesbaden-Nordenstadt
Goat anti-collagen type I	Chemicon International, Hofheim
Rabbit anti-bovine collagen type II	Chemicon International, Hofheim

CD34-FITC	Acris Antibodies, Hiddenhausen
CD45RO-FITC	Acris Antibodies, Hiddenhausen
CD105-FITC	Acris Antibodies, Hiddenhausen
CD44	Acris Antibodies, Hiddenhausen
CD90	Acris Antibodies, Hiddenhausen
CD29-FITC	Acris Antibodies, Hiddenhausen
2° Antibody Rat IgG	Acris Antibodies, Hiddenhausen

2.1.10 Recombinent proteins

TGF-β3	R+D Systems, Wiesbaden-Nordenstadt
rh bFGF	Tebu-bio, Offenbach

2.1.11 Buffers/solutions/media components

All buffers and solutions were prepared in autoclaved, deminerilised water (milipore).

2.1.11.1 Cell culture

DMEM growth medium	DMEM, 10% FCS, 1% pen/strep
$DMEM_{MSC}$ Complete Medium	Low-glucose DMEM, 10% FCS 1 M Hepes, 4 mM L-glutamine, 1% pen/strep, 2 ng/ml rh bFGF
$DMEM_{MSC}$ Chondrogenic Medium	DMEM, 1x ITS, 1 mM Sodium Pyruvate, 0.1 mM L-ascorbic acid, 1x 10^{-7} M dexamethasone, 1% pen/strep, 10 ng/ml TGF-β3

2.1.11.2 ELISA

Blocking Solution	PBS, 3% BSA
Coating Buffer	0.14g, $NaCO_3$ (pH 9.6)
1x PBS (pH 7:42)	8 g NaCl, 0.2 g KCl, 1.26 g Na_2HPO_4, 0.2 g H_2PO_4
Substrate Buffer, OPDA (pH 5.0)	30 mg OPDA in 30 ml citrate buffer
Wash Buffer	PBS, 0.05% Tween

2.1.11.3 Western blot

Coomassie Solution	45% Methanol, 10% glacial acetic acid, 0.2% (w/v) Coomassie Brillant Blue, 10% Ethanol, 10% acetic Acid
Destaining Solution	10% Ethanol, 10% Acetic Acid
3X Reducing SDS loading Buffer	62.5 mM Tris (pH 6.8), 2% SDS, 10% Glycerin, 0.01% Bromphenol blue, 5% β-Mercaptoethanal
10X Transfer Buffer	25 mM Tris; 192 mM Glycin
1X Transfer Buffer	1X Transfer Buffer; 20% (v/v) Methanol
Wash Buffer	1X PBS; 0.1% (v/v) Tween, 5% (v/v) Dry Milk
Acrylamide Gel Running Buffer (pH 8.3)	25 mM Tris, 192 mM glycine, 0.1% SDS
Resolving gel 4X Buffer (pH 8.8)	1.5 M Tris HCl, 0.4% SDS
Stacking gel 4X Buffer (pH 6.8)	0.5 M Tris HCl, 0.4% SDS, pH 6.8

2.1.12 Plastic-ware/miscellaneous

Plastic-ware (eg. Epis, cell culture flasks) was purchased from PEQLAB Biotechnologie (Erlangen) or Greiner Bio One (Frickenhausen) unless stated otherwise.

Falcon Cell Strainer (100 µm)	Becton Dickinson Labware, Claix, France
Falcon Chamber slides (8 Chambers)	Becton Dickinson Labware, Claix, France
CL-X Posure Film	Pierce Biotechnology, Jena

ELISA Plates 96-well flat bottom	NUNC, Wiesbaden
OCT Carbowax	Science Services, Munich
PVDF-Transfer Membrane	Amersham, UK
Real-Time PCR 96-well plates	Bio-Rad Laboratories, Munich
Sterile Filter (0.22 µm)	Millipore S.A. Molsheim, France
SuperFrostPlus Slides	Science Services, Munich
Syringes/Needles	Versandapotheke, Frankenthal
TissueTek Cryomold	Science Services, Munich
Whatman Paper	Biometra, Göttingen

2.1.13 Instruments

BIO-TEK Synergy Microplate Reader/Washer	Bio-Tek Instruments, Bad Friedrichshall
Biofuge Stratus Centrifuge	Heraeus Instruments, Düsseldorf
Flow Cytometer	Beckman Coulter, Canada
iQ5 real-time PCR Detection System	Bio-Rad Laboratories, Munich
MicrosonTM Ultrasonic Cell Disrupter XL	Microsonic, Dortmund
NanoDrop Spectrophotometer ND1000	PEQLAB Biotechnologie, Erlangen
PCR-*Mastercycler Gradient*	Eppendorf, Hamburg
Tecan Microplate Reader	Tecan Group Ltd., Crailsheim
Thermomixer 5436	Eppendorf, Hamburg

2.2 Methods

2.2.1 Chondrocyte isolation

Articular cartilage tissue was aseptically removed from euthanized dogs (n = 5), which had no previous history of joint disease. Chondrocytes were isolated under a laminar flow hood according to a previously described protocol (Kaps, et al, 2004) with slight modifications. Briefly, superfluous tissue covering the joint was first excised. Then, the cartilage surface area of the joint was disinfected with 70% ethanol and cartilage was carefully dissected from the joint. Care was taken to prevent excision into the subchondral bone. Thereafter, the cartilage pieces were washed three times with HANK's salt solution and 1% penicillin/streptomycin, then for 15 s with 70% ethanol, and placed in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. Following dicing of the cartilage pieces (1−3 mm^3), chondrocytes were isolated enzymatically with an enzyme cocktail consisting of 1 U/ml collagenase P, 300 U/ml collagenase CLS II and 30 U/ml hyaluronidase for approximately 18 h in a spinner flask at 37°C and 5% CO_2. Following digestion, the sample was filtered through a nylon mesh (pore size 100 µm) into a sterile tube and centrifuged at 400xg for 10 min. The pellet was washed with HANK's salt solution, centrifuged at 400xg for 10 min, and then resuspended in growth medium. Isolated primary chondrocytes were counted and their vitality assessed using trypan blue staining.

2.2.2 Chondrocyte monolayer cultures

Isolated canine chondrocytes were seeded in cell culture flasks at a density of 2×10^4 cells/cm^2 and maintained at 37°C in a 5% CO_2 incubator until they were confluent. Growth medium was changed 2−3 times per week. Once 80% confluency was reached, cells were trypsinized using 0.05% trypsin/0.02% EDTA in PBS without Ca^{2+} and Mg^{2+}. Following trypsinization, the cells were either passaged for additional experiments or cryopreserved. For cryopreservation, 1×10^6 cells/ml were suspended in DMEM, 10% FCS, and 10% dimethylsulfoxide (DMSO). The cells were first stored at −80°C and within two days transferred to liquid nitrogen until further use.

2.2.3 Primer design

The mRNA sequences specific for the canine genes of interest were obtained from the National Center for Biotechnology Information (NCBI) gene database (www.ncbi.nlm.nih.gov). Oligonucleotide primers for quantitative real-time PCR (qRT-PCR)

were designed using Primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). For all primer sequences a Basic Local Alignment Search Tool (BLAST) search was performed to confirm gene specificity. Primers were subsequently synthesized by TIB Molbiol (Table 2).

Table 2: Oligonucleotide Primer Sequences

Gene	Accession No.	F/R	Sequence 5´-3´	Position ORF	Amplicon Size (bp)
Aggrecan	U65989	F	CTATGAGGACGGCTTTCACC	573-592	194
		R	AGACCTCACCCTCCATCTCC	747-766	
COMP	XM_860228	F	GAACCCAGACCAACGCAAT	1011-1029	221
		R	CATCTCCTACACCGTCACCA	1212-1231	
GAPDH	NM_01003142	F	TAT TGT CGC CAT CAA TGA CC	81-100	195
		R	TAC TCA GCA CCA GCA TCA CC	261-275	
*IGF-1	XM_848024.1	F	CTTGTAAGATCTGTCATGGGAAAAATCAGCAGTCT	259-278	492
		R	TTAGAGCTCGAG-CTTCTACATTCTGTAGTTCTTGTTTC	698-720	
IGF-1	XM_848024.1	F	CAGCAGTCTTCCAACCCAAT	12-31	105
		R	CAAGCACAGTGCCAGGTAGA	98-117	
IGFBP-1	XM_537927	F	CATCCTCTGGAACGCTGTC	432-450	157
		R	GCAGTTGGGCAGGTAGAAAC	569-588	
IGFBP-2	XM_545637	F	ACAATGGCGACGAGCACT	281-298	144
		R	TGACCTTCTCCCTGAACACG	405-424	
IGFBP-3	XM_548740	F	CCGGCTCAAGTTCTTGGATA	1482-1501	137
		R	ACTTGTCCACACACCAGCAG	1599-1618	
IGFBP-4	XM_845091	F	CCCAACAACAGCTTCAGTCC	949-968	291
		R	ATCTAAGGCCGGGTGACACT	1220-1239	
IGFBP-5	XM_847792	F	AGCAAGCCAAGATCGAGAGA	657-676	164
		R	CTGGGTCAGCTTCTTTCTGC	801-820	
IGFBP-6	XM_844250	F	AGGATGTGAACCGCAGAGAC	411-430	180
		R	TAGGCACGTAGAGGGTGTGA	571-590	
IGFR1	XM_545828	F	AGTGCTGTATGCTTCCGTGA	2938-2957	181
		R	CACCCTTGTTTCAGGCTCAT	3099-3118	
IL-1β	DQ251036	F	AGTTGCAAGTCTCCCACCAG	149-169	177
		R	TATCCGCATCTGTTTTGCAG	325-345	
*IL-4	NM_001003159	F	AAGCTTGGATCCGATCTATTAATGGGTCTCAC	55-74	417
		R	GTCGAGCCTAGGCCTATTCAGCTTCAATGCCT	454-471	
IL-4	NM_001003159	F	CTCACCTCCCAACTGATTCC	70-89	156
		R	CTTGACAGTCAGCTCCATGC	206-225	
IL-6	NM_001003301	F	GGCTACTGCTTTCCCTACCC	108-128	198
		R	TTTTCTGCCAGTGCCTCTTT	305-325	
iNOS	AF068682	F	GGAGGAGCAGCTACTGTTGG	1227-1246	178
		R	GTCATGAGCAAAGGCACAGA	1385-1404	
MMP-1	XM_546546	F	TTCGGGGAGAAGTGATGTTC	866–885	194
		R	CTGACCCTGAACAACCCAGT	1059–1078	
MMP-13	AF201729	F	CAGACTTCATGACGGCACT	438-456	104
		R	AAGCATGAGCCAGAAGACC	523-541	
MMP-3	NM_001002967	F	CATTTATGGAGATGCCCACT	594-613	124
		R	GTCAGCCGAGTGAAAGAGAC	698-717	
SOX-9	NM_001002978	F	CACCGAACAGACGCACAT	576-593	185
		R	GCTTCAGGTCAGCCTTGC	743-760	
TNFα	NM_001003244	F	TCATCTTCTCGAACCCCAAG	235-255	157
		R	ACCCATCTGACGGCACTATC	391-411	
Type I Collagen	NM_001003090	F	GAACCTGGCAAACAAGGTC	3017-3035	150
		R	AGGAGAACCATCTCGTCCA	3148-3166	
Type II Collagen	NM_001006951	F	GAAACTCTGCCACCCTGAAT	3878-3897	160
		R	GCTGCTCCACCAGTTCTTCT	4018-4037	

* Primers used for cloning in pVitro2 vector; F: forward; R: reverse; ORF: open reading frame; bp: base

2.2.4 Conventional RT-PCR

Oligonucleotide primers, which were used for real-time quantitative (q) RT-PCR, were first analyzed by conventional RT-PCR. This was done to check their specificity and their hybridization ability at 55°C. Each 25 µl reaction was comprised of 1x NH_4 buffer, 1.5 mM $MgCl_2$, 500 µM dNTP mix, 0.5 µM, 0.05 U/µl Taq polymerase. PCR conditions were as follows: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed by a final extension at 72°C for 10 min.

RT-PCR was also performed to obtain the full length sequences of cIL-4 and cIGF-1 for cloning in the double expression vector, pVitro2. The reaction mixture remained the same as mentioned above with slight modifications in the PCR conditions, namely: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min.

2.2.5 Agarose gel electrophoresis

The specificity of RT-PCR products was analyzed by agarose gel electrophoresis. Amplicons were separated on a 2% Tris-acetate-EDTA (TAE) agarose gel supplemented with 0.5 µg/ml ethidium bromide. The gel was run electrophoretically at 110 V for approximately 1 h, depending on the desired separation. RT-PCR product bands were visualized with the use of UV-radiation.

2.2.6 Amplification and sequencing of cIGF-1 and cIL-4

Following primer design based on the published NCBI dog genome sequence, RT-PCR was performed to amplify the full length cIGF-1 from MDCK cells. Full length canine IL-4 was amplified from existing pcDNA3.1-IL (Wondimu *et al.*, 2001). The resulting amplicons were first visualised via agarose gel electrophoresis, purified using JetSorb Extraction Kit according to manufacturer’s instructions, and then 8 µl of the eluate were submitted for sequence analysis (Services in Molecular Biology, Berlin). Sequence data files were analyzed using Chromas software (www.technelysium.com.au/chromas.html). Resulting sequences were confirmed using Basic Local Alignment Service Tool (BLAST).

2.2.7 Restriction analysis

In order to analyze the ubiquitous and constitutive co-expression of two genes, a dual promoter plasmid, pVitro2, was selected. This vector consists of two constitutive promoters that drive high levels of expression of each gene inserted into the two different multiple cloning sites. This allows for the expression of both genes in relatively equal amounts. Minimization of transcriptional interference is achieved by using promoters that are coordinately activated, in this case ferritin promoters. To eliminate the regulation by iron, the 5'untranslated regions (5'UTR) of FerH and FerL had been replaced by the 5'UTR of the mouse and chimpanzee EF1α genes. By using SV40 and CMV enhancers, which yield activities similar to the CMV promoter, the promoter activities are augmented (InvivoGen, Toulouse).

The pVitro2 vector was used for the single expression of cIGF-1 as well as for the combined expression of cIGF-1 and cIL-4. In 20 µl reaction volume, 1 µg of pVitro2 plasmid DNA was incubated with 1x NEBuffer, and 1.5−5 units of the restriction enzymes *Bgl*II and *Xho*I for cIGF-1 insertion and *BamH*I and *Avr*II for cIL-4 insertion. The reaction was incubated at 37°C for 60 min.

2.2.8 Ligation

The ligation of both cIGF-1 and cIL-4 in one pVitro2 vector occurred in succession. First, cIGF-1 was ligated into the vector. The 10 µl reaction consisted of 1x T4 ligase buffer, 90 fmol purified PCR product, 30 fmol plasmid DNA, and 0.1 units of T4 DNA ligase. Following incubation at 16°C for 16 h, the enzyme was inactivated by heating at 65°C for 15 min. The ligation product was stored at −20°C until transformation in bacteria. Once the purified plasmid DNA was obtained for cIGF-1-pVitro2, this plasmid DNA was again submitted to restriction enzyme assay with the enzymes for cIL-4. Then, the cIL-4 purified PCR product was ligated into the freshly cut vector as mentioned above.

2.2.9 Transformation and selection with antibiotics

Highly competent *E. coli* XL-1 blue cells were transformed with the ligation products using heat shock. First, a 200 µl aliquot of competent cells were thawed on ice. Approximately 1 µg of DNA or 5 µl of the ligation reaction were added to the competent cells and mixed gently. The mixture was incubated on ice for 30 min followed by heating at 42°C for 45 sec. The reaction was allowed to cool on ice for 2 min. Then, 800 µl of yeast extract triptone (2YT-broth) medium pre-heated to 37°C was added and incubated for 45 min at 37°C with

constant shaking at 150 rpm. Afterwards, 100 µl of the reaction mixture was plated onto Teriffic Broth (TB)/Kanamycin agar plates, which were incubated overnight at 37°C. Colonies were picked and propagated in 5ml TB/Kanamycin overnight at 37°C following colony screening.

2.2.10 Colony screening

Insertion of cIGF-1 or cIGF-1/cIL-4 into pVitro2 was checked by colony screening. Colonies were picked from the agar plates and dipped into a reaction tube containing PCR components, followed by PCR. Positive colonies were propagated in 5 ml TB/Kanamycin overnight at 37°C. Mini-plasmid preparation was performed to isolate plasmid DNA.

2.2.11 Isolation of plasmid DNA—Mini-Prep

Plasmid DNA was isolated from the overnight cultures utilizing the Invisorb Spin Plasmid Mini Kit according to manufacturer's instructions. The concentration of the plasmid DNA was determined using a NanoDrop spectrophotometer. Proper insertion of the genes into pVitro2 was analyzed by restriction enzyme assay and sequence analysis.

2.2.12 Isolation of plasmid DNA—Maxi-Prep

A volume of 250 ml TB/Kanamycin was inoculated with 1 ml of the mini-overnight culture and incubated overnight at 37°C under constant shaking. Pure plasmid DNA was isolated using the Qiagen Maxi-Preparation Kit according to instructions. Purity and concentration of the plasmid DNA were determined using a NanoDrop spectrophotometer. The presence of the proper inserts was determined by restriction enzyme assays, RT-PCR and sequencing of the products.

2.2.13 Transfection

Transfection was performed using FuGENE6 as previously described elsewhere (Madry and Trippel, 2000). Briefly, canine chondrocytes and cMSCs in passage 3−4 were seeded at a density of 2.5×10^5 cells/well in 6-well plates using DMEM growth medium. Following 24 h cultivation, cells were washed twice with 1x PBS and cultured with DMEM containing 1% FCS and 1% penicillin/streptomycin. Also, chondrocytes were pre-treated with hyaluronidase (4 U/ml) for 6 h before and during transfection. Approximately 2–6 µg of endotoxin-free plasmid DNA (1 µg/µl; from maxi-prep) were used for transfection studies. Chondrocytes were transfected with cIGF-1, cIGF-1/cIL-4 or pVitro2. cMSCs were transfected with

cIGF-1/cIL-4 or pVitro2. Non-transfected cells and cells transfected with the empty vector (pVitro2) served as controls. For each reaction, FuGENE6 and plasmid DNA were used at a ratio of 3:2. Non-transfected cells were treated with FuGENE6 without plasmid DNA. Reaction components were prepared in serum-free medium and added dropwise onto the cells.

2.2.14 Stimulation with pro-inflammatory cytokines

Chondroycytes transfected with cIGF-1, cIGF-1/cIL-4 or pVitro2 empty vector were stimulated with the pro-inflammatory cytokines IL-1β (100 ng/ml) and TNFα (50 ng/ml) 24 h post-transfection. Cells were then incubated for an additional 48−96 h in the presence of pro-inflammatory cytokines. Thereafter, conditioned medium was collected, and cells were submitted to RNA isolation or protein extraction.

2.2.15 RNA isolation and cDNA synthesis

Total RNA was extracted from articular chondrocytes using the GeneMatrix RNA Purification Kit according to the manufacturer's instructions. Extracted RNA eluted in diethylpyrocarbonate (DEPC)-treated H_2O was quantified, RNAse-free-DNAse I treated, and stored at –80°C until further use. One microgram of RNA was subjected to first-strand cDNA synthesis using RevertAid M-MuL V reverse transcriptase at 42°C for 60 min according to the supplied protocol.

2.2.16 Real-time quantitative RT-PCR

Quantification of the various gene products was performed using real-time qRT-PCR on an iCycler iQ-5. Typically, reactions were carried out using a 20 µl reaction volume (in 96 well plates sealed with film) containing 10 µl of 1x SensiMix containing SYBR green, fluorescein and 3 mM $MgCl_2$. Primers were used at a concentration of 500 nM, and cDNA was added at a concentration of 0.5 µg/µl in triplicates. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as endogenous reference for normalization of fluorescence threshold (C_t) values of target genes. PCR conditions after initial denaturation at 95°C for 3 min were: denaturation at 95°C for 15 sec; annealing at 55°C for 30 sec; extension at 72°C for 30 sec for 40 cycles. The melting curve analysis was carried out with the following parameters: denaturation at 95°C for 30 sec (1 cycle), annealing at 65°C for 30 sec (1 cycle) and finally cooling at 55°C for 10 sec (41 cycles). Thereafter, melting curves were inspected for amplification specificity. Threshold cycle (C_t) values were determined automatically by the optical system software v2.0.

2.2.17 Protein sample preparation

Samples used for protein assays were collected 48 h or 72 h post-transfection. First, cell conditioned medium was removed and stored at −80°C for further analyses. Then, the cell layers were washed with 1x PBS, trypsinized and centrifuged as mentioned previously. The cell pellets were then lysed using radioimmunoprecipitation assay (RIPA) buffer containing 1% triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 20 mM Tris, 10 mM EDTA, 10 mM iodoacetamide, 1 mM phenylmethane sulfonyl fluoride (PMSF) and protease inhibitor cocktail. Lysates were incubated for 30 min on ice and then centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentration was determined by BioRad Lowry microassay.

2.2.18 IGF-1 immunoassay

To determine IGF-1 protein levels, samples were analyzed by Quantikine IGF-1 Immunoassay Kit according to instructions. The assay has a detection limit of 0.007 ng/ml.

2.2.19 IL-4 enzyme linked immunosorbent assay (ELISA)

Sandwich ELISA was performed using house raised polyclonal and monoclonal antibodies (Wondimu *et al.*, 2001) against cIL-4 in a 96-well plate. Initially, anti-rabbit cIL-4 polyclonal antibody (2.4 µg/ml) in 20 mM sodium carbonate buffer (pH 9.6) was used to coat the 96-well plate for 1 h at RT. After washing in 1x PBS, the plate was blocked with 200 µl of 1% BSA in 1x PBS for 2 h at RT followed by additional washing with 1x PBS. The standard cytokine, *E. coli* expressed recombinant IL-4 protein, was serially diluted in blocking buffer (1 ng protein as starting material) and dispensed in triplicates. Similarly, lysates from test samples and controls were also diluted in blocking buffer and dispensed in separate wells in triplicates. Following incubation for 1 h at RT, the plate washed with 1x PBS/0.1% tween and the secondary antibody (anti-mouse cIL-4 monoclonal) was added in a 1:10 dilution in 20 mM sodium carbonate buffer and incubated for 1−2 h at RT. Subsequently, wells were rinsed and 50 µl of biotin-labeled anti-mouse antibody (1:2500 dilution) was applied and allowed to incubate for 30 min at RT. Streptavidin conjugated horse-radish peroxidase (1:4000 dilution) was added to the samples and incubated likewise for 30 min at RT. Finally, 100 µl of chromogenic substrate (12-oxophytodienoic acid [OPDA] in citrate buffer with hydrogen peroxide) was given to each well of the plate and incubated in dark for 20−30 min. Color development was measured at 492 nm after stopping the reaction with 1 M sulfuric acid. In this experiment, the washing was carried out in an ELISA washer.

2.2.20 Immunocytochemistry

Type I and type II collagen expression in canine chondrocytes and cMSCs were analyzed on the protein level by immunocytochemistry. The Dakocytomation system was employed with slight modifications (Kaps *et al.*, 2004). Cells were cultivated in chamber slides with DMEM growth medium until they were approximately 70% confluent. Medium was removed and cells were washed twice with 1x PBS. Antigens on cMSC were demasked with pepsin for 12 min at RT. Both chondrocytes and cMSC were then fixed with methanol/acetone (1:1) for 5 min and then dried for 20 min at 37°C. Hydrogen peroxide was added for 5 min at RT followed by washing twice with 1x PBS for 5 min. Primary antibodies were then added, goat anti-collagen type I (1:800) or rabbit anti-bovine type II collagen (1:300), and incubated for 1 h at RT. Slides were afterwards washed three times with 1x PBS for 5 min each and then, incubated for 15 min at RT with LSAB/LINK. Slides were then washed again three times with 1x PBS for 5 min each followed by treatment with LSAB/HRP streptavidin. Following washing with 1x PBS three times for 5 min, the slides were treated with diaminobenzidine (DAB) and counterstained with hematoxylin.

2.2.21 Alcian blue staining

Sulfated glycosaminoglycans of the cells were stained using Alcian Blue as described elsewhere (Asahina *et al.*, 1996). Briefly, cells were washed with PBS and fixed with 95% methanol for 2 min at 20°C. Following incubation, the cells were stained with 0.1% Alcian Blue 8GX in 0.1M HCl overnight. After washing 3 times with aqua dest, the stain was extracted with 500 µl of 6 M guanidine-HCl for 2 h at room temperature. Optical density was measured at 620 nm using a microplate reader.

2.2.22 NO assay

Nitric oxide (NO) production was estimated by measuring nitrite (NO^{2-}), a stable end product of NO, using the Griess reagent system according to protocol. Briefly, conditioned media from test samples and controls were incubated with equal volumes of Griess reagent, and absorbance was measured at 562 nm. Total nitrite concentration was determined utilizing a standard curve and expressed as µM nitrite. All assays were performed in triplicate.

2.2.23 cMSC isolation

Canine mesenchymal stem cells (cMSCs) were harvested from bone marrow samples provided from the Small Animal Clinic of the Freie Universität Berlin. Bone marrow aspirates

were stored at room temperature (RT) for no longer than 2 h. Isolation of cMSCs proceeded according to a modified protocol established for the retrieval of human MSCs from bone marrow (Haynesworth et al, 1992). Briefly, bone marrow aspirates stored with heparin were transferred into sterile 50 ml Falcon tubes containing 50 ml 1x PBS without Ca^{2+} and Mg^{2+} and centrifuged twice for 10 min at 310 xg at RT. Following centrifugation, the supernatant was discarded and the pellet was resuspended in 20 ml $DMEM_{msc}$ complete medium (low-glucose DMEM, 10% FCS, 1% penicillin/streptomycin, 1 M HEPES, 4 mM L-glutamine, 2 ng/ml rh bFGF). The fundamental isolation step is based upon separation of cMSCs, which have a lower density level, from the other blood cells. This is achieved by using a percoll density gradient of a defined density (1.073 g/ml). Four sterile Falcon tubes containing 20 ml of percoll were layered each with 5 ml of cell suspension and centrifuged at 900 xg for 32 min without braking. The upper red layer including the cloudy phase-boundary was transferred into a centrifugation-tube along with 2.5 volumes of 1x PBS without Ca^{2+} and Mg^{2+} and centrifuged for 10 min at 310 xg at RT. Cell pellets were resuspended in 10 ml $DMEM_{msc}$ complete medium. Cell vitality was assessed using trypan blue and the cell number was determined.

2.2.24 cMSC monolayer culture

Freshly isolated cMSCs were cultured at a density of $1.5-3.5 \times 10^6$ cells/cm^2 in $DMEM_{msc}$ complete medium and incubated 37°C and 5% CO_2 until confluent. Medium was changed 72 h after isolation and all non-adherent cells were removed. All subsequent medium changes occurred every 2−3 days thereafter. Once cells had reached 80% confluence, they were passaged and seeded at a density of 5×10^3 cells/cm^2 to avoid spontaneous cell differentiation due to a high cell density. Cryopreservation of cMSCs was possible for cells in passage 1 or higher. This was done as described in section 2.2.2. with a minor modification, namely the storing medium for cMSC consisted of DMEM, 80% FCS, and 10% DMSO.

2.2.25 Alginate cultures of cMSCs

Induction of chondrogenesis in cMSCs occurred in three-dimensional (3D) alginate cultures. Monolayer cultured cMSCs in passage 2−4 were trypsinized and the cell number and cell vitality was determined. Preparation of 3D-alginate cultures was performed as previously described (Madry *et al.*, 2003). Briefly, 1 ml of sterile 1.2% alginate was added to the cell pellet per 4×10^6 cells. The mixture was aspirated into a 1 ml syringe (needle 21 gauge) without the needle. After replacing the needle, the viscous solution was given dropwise,

under constant shaking, to a T75 flask containing 15 ml of sterile 102 mM $CaCl_2$ and was incubated for 10 min at RT. The beads were then washed shortly with 15 ml of sterile 1x PBS without Ca^{2+} and Mg^{2+} followed by three washes with 20 ml DMEM and incubations for 5 min at RT. Alginate beads were cultivated in either 20 ml $DMEM_{cmsc}$ complete medium (2.2.23) or in $DMEM_{chond}$ complete medium (low-glucose DMEM, 1x ITS, 1 mM Na-pyruvate, 0.1 mM L-ascorbic acid, 10^{-7} Dexamethasone, 1% penicillin/streptomycin and 10 ng/ml TGFβ3). The medium was changed three times per week. Cells were released from the alginate by solubilizing each bead separately in a reaction tube containing 100 µl 55 mM sodium citrate, 90 mM NaCl (pH 6.8) for 20 min at RT. Cell number per bead and cell vitality was checked after alginate beads were formed.

2.2.26 Histology

Alginate beads were removed from the cell culture medium and placed in the middle of a TissueTek Cryomold. Then, the bead was embedded in carbowax (OCT) without making bubbles and assuring that the bead remained in the middle of the mold. The cryomold was dipped slowly into liquid nitrogen until frozen. Embedded beads were then stored at −80°C until further usage. The beads were cut utilizing a cryostat and sections were placed on SuperFrostPlus slides. Resulting cryosections (5 µm thick) were dried at RT for 12 h and immunocytochemistry was performed.

2.2.27 Fluorescence activated cell sorting (FACS)

Due to the fact that there are no specific MSC markers, a number of parameters has to be analyzed to exclude certain parameters not associated with MSCs. Some of these parameters include cell surface markers, which can be detected by fluorescence activated cell sorting (FACS). For phenotyping of the cells, monoclonal antibodies directed against mouse CD90+, mouse CD29+/FITC, canine CD44+, CD45RO-/FITC, and CD34-/FITC were used. Briefly, cells cultivated in monolayer were trypsinized as previously described. Cells were then washed twice with 1x PBS/1% BSA and centrifuged at 1500 rpm for 5 min. Approximately 1×10^5 cMSCs were used per analysis. Each antibody was added at a concentration of 2.5−3 µg/ml and incubated for 15 min in the dark. Then, 1 ml 1x PBS/1% BSA was added to each sample and centrifuged for 6 min at 2000 rpm. For antibodies conjugated with FITC, the supernatant was removed and the pellet was resuspended in 400 µl 1x PBS/1% BSA and placed on ice in the dark. As for the other

antibodies (CD90 and CD44), the supernatant was removed and the pellet was resuspended in 100 µl 1x PBS/1% BSA. Approximately 5 µl (0.2 µg/ml) of rat polyclonal secondary antibody conjugated to IgG-fluorescein was added and the samples were incubated for 15 min in the dark. Following incubation, 1 ml 1x PBS/1% BSA was added followed by centrifugation for 6 min at 2000 rpm. The supernatant was removed and the pellet was likewise resuspended in 400 µl 1x PBS/1% BSA. Samples were placed on ice and FACS analysis was performed.

2.2.28 Western blot

Expression of type I collagen and type II collagen in non-differentiated and differentiated cMSC was measured by Western blot. For the analysis, 20 µg protein from each sample was loaded on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to electrophoresis. Components used for the preparation of the resolving and stacking gel are shown in Table 3. An equal volume of 2x sample buffer was added to the samples (1:1), followed by incubation for 5 min at 95°C. Samples were mixed, centrifuged briefly, and loaded onto the gel. Electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for 90 min at 110 mA (1 mA/cm^2). The membrane was then blocked in blocking buffer for 1 h at RT before incubating overnight at 4°C with goat anti-collagen type I polyclonal or rabbit anti-bovine type II collagen polyclonal antibody in 1x PBS/0.1% tween. The membrane was washed three times with blocking buffer followed by addition of secondary antibodies (anti-goat horseradish peroxidase−1:8000 or anti-rabbit horseradish peroxidase−1:10000) in blocking buffer for 1 h at RT. The membrane was then washed twice with blocking buffer and once with 1x PBS/0.1% tween. Antigen-antibody reactions were visualized using a Chemiluminescence (CLS) system according to instructions by means of X-ray films.

Table 3: **SDS Mini-Gel Preparation Scheme**

Running gel

Components	Volume (ml) (7.5% gel)
Acrylamide solution	
Resolving gel 4X buffer	3.75 ml
Water	3.75 ml
TEMED	15 µl
APS (10%)	80 µl

Stacking gel

Components	Volume (ml) (5% gel)
Acrylamide solution	0.65
Stacking gel 4X buffer	1.25 ml
Water	3.05 ml
TEMED	7.5 µl
APS (10%)	40 µl

2.2.29 Stable cell line

Canine chondrocyte stable cell line was generated previously in this lab which stably expresses cIL-4 under the disease sensitive COX-2 promoter. This cell line was super transfected with cIGF-1-pVitro2 using FuGENE6. Antibiotics, G418 and kanamycin were given to the cells for selection 48 h post-transfection. Cells with both cIL-4 and cIGF-1 survived following 3 weeks of treatment with the antibiotics. Thereafter, colonies were picked and grown in 6 cm plates with DMEM growth medium and antibiotics.

2.2.30 Pellet Cultures

In an effort to control the ratio of each cell type in the co-cultures, the experiment for the co-culture studies occurred in pellet cultures. Monolayer cultured cMSCs (passage 2−4) or canine chondrocytes (passage 4−6) were trypsinized and the cell number and cell vitality was determined. The cells were then centrifuged at 1000 rpm for 10 min, resuspended in culture

medium, and counted. Thereafter, the cells were mixed in the respective ratios and placed in 15 ml conical tubes. Then, 2 x 10^5 cell aliquots were centrifuged at 1800 rpm for 5 min. For the co-culture experiments the following ratios were implemented (cMSCs or chondrocytes/stably transfected chondrocytes): 0/100, 100/0, 80/20, 70/30, 50/50.

2.2.31 Statistical analysis

Results from the present study are based on experiments performed in triplicates. Statistical analysis was carried out with GraphPad Prism V.5 software (GraphPad, San Diego, California, USA) utilizing the Student's t-test. When the p-value was less than or equal to 0.05 ($p \leq 0.05$), the results were rendered significant. All values are presented as the mean ± standard deviation (± SD).

3 Results

3.1 Dual expression of anabolic mediators in canine chondrocytes

In OA, the effects of catabolic mediators outweigh those of anabolic mediators. In an effort to restore the balance between the two, the anabolic mediators IGF-1 and IL-4 were inserted into a single expression vector. The effects of the co-expression of the therapeutic genes were analyzed in an *in vitro* canine chondrocyte pro-inflammatory model and the following results were obtained. Of importance was the down-regulation of pro-inflammatory mediators and stimulation of extracellular matrix proteins synthesis.

3.2.4 Cloning of IGF-1 and IGF-1/IL-4 in pVitro2

To analyze the effect of the co-expression of IGF-1/IL-4 in canine chondrocytes both genes of interest were cloned into the vector pVitro2 (Fig. 6). RT-PCR was performed to amplify the mRNA sequence coding for IGF-1 and IL-4. Since the expression of IGF-1 was not detectable in canine chondrocytes using RT-PCR, the sequence was amplified from the Madin-Darby canine kidney II (MDCK II) cell line. Using the primers listed under material and methods, an amplicon with the size of 492 bp was obtained. IL-4 was amplified from the pre-existing pcDNA-IL-4 vector with the resulting amplicon having the size of 417 bp. IGF-1 was cloned alone in the pVitro2 vector to serve as a comparison for the combined studies. Following cloning of both genes into the vector the products were sequenced and the sequences were confirmed by Basic Local Alignment Search Tool (BLAST). Respective genes were found to be present in the correct multiple cloning sites in the correct orientation.

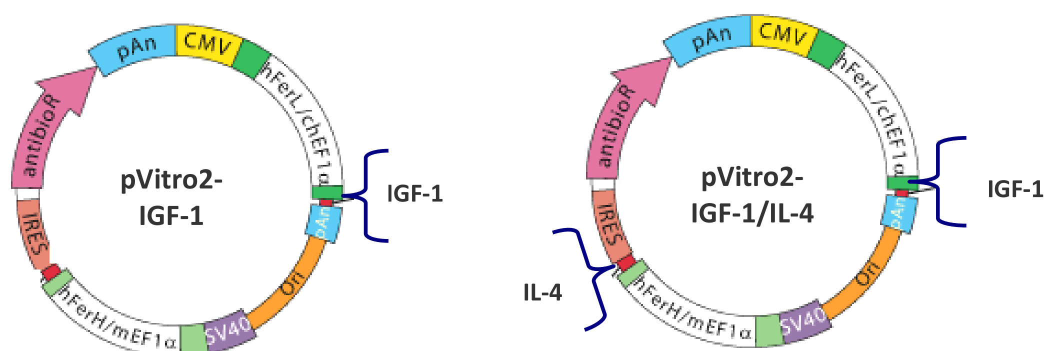


Figure 6: Plasmid constructs. The plasmid contains two human ferritin promoters FerH (heavy chain) and FerL (light chain), which are connected to enhancers SV40 and CMV, respectively. Both mouse

and chimpanzee elongation factor α (mEF1α, chEF1α) eliminate iron regulation. (Modified from www.invivogen.com)

3.2.5 Expression of IGF-1 and IL-4 proteins

Following successful cloning of the respective genes into the pVitro2 vector, immunoassays were performed in duplicate to analyze whether the transfected chondrocytes produced the respective therapeutic proteins. Due to the lack of canine IGF-1 antibodies, the immunoassay was performed using human IGF-1 antibodies. High homology (90%) of IGF-1 protein sequence between human and canine forms compensated for the non-availability of canine specific antibodies.

Immunoassay results in Fig.7 show that the human IGF-1 antibody could detect canine IGF-1. Incidentally, expression of IGF-1 was found to be more than 5-fold higher in cells co-expressing IGF-1 and IL-4 under stimulation with IL-1β and TNFα compared to expression of IGF-1 alone under identical conditions. The concentration of IGF-1 detected in chondrocytes transfected with IGF-1 and IGF-1/IL-4 was 1.3 ng/ml and 7.5 ng/ml, respectively. On the other hand, minimal amounts of IGF-1 were seen in the stimulated, non-transfected control and none in the non-stimulated, non-transfected control cells.

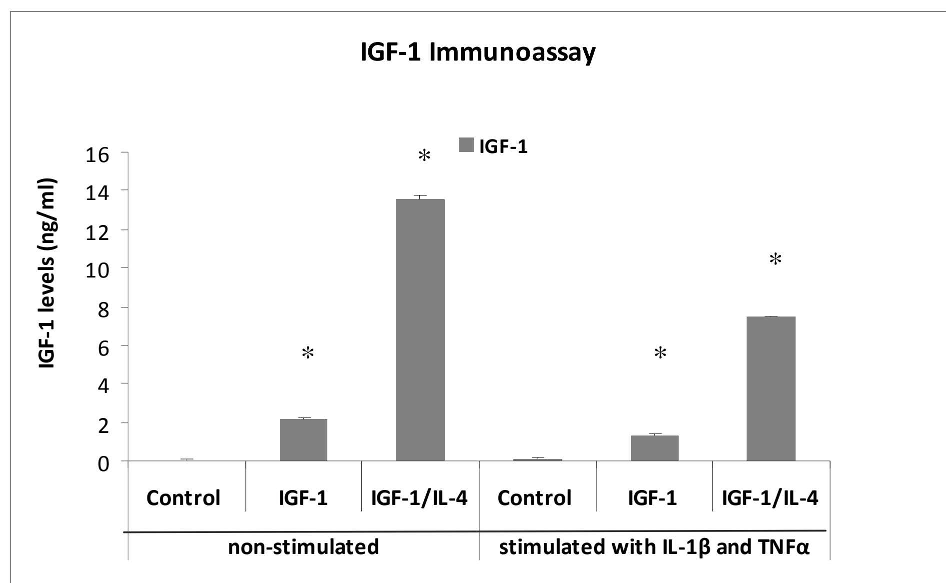


Figure 7: IGF-1 immunoassay. Canine chondrocytes in passage 3–4 were transfected with IGF-1 or IGF-1/IL-4 plasmid DNA, and non-transfected cells served as the control. The cells were stimulated 24 h post-transfection with 100 ng/ml IL-1β and 50 ng/ml TNFα proteins for 48 h. Cell lysates were collected and analyzed using a radioimmunosassy (R&D Systems). IGF-1

protein was detected in cells transfected with IGF-1 and IGF-1/IL-4. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the respective controls ($p<0.05$).

A similar trend was observed for the expression of IL-4 in the same samples (Fig.8). Sandwich ELISA using house-raised canine specific IL-4 polyclonal and monoclonal antibodies detected higher IL-4 expression in canine chondrocytes co-expressing IGF-1 and IL-4 under stimulation with pro-inflammatory cytokines than under non-stimulatory conditions. Surprisingly, IL-4 expression was also observed in chondrocytes transfected with IGF-1 alone, however, only when stimulated with the pro-inflammatory cytokines. In contrast, minimal expression of IL-4 was observed in the control cells. The results obtained from the IGF-1 and IL-4 immunoassays correlate to the results from IGF-1 and IL-4 real-time qRT-PCR (results not shown).

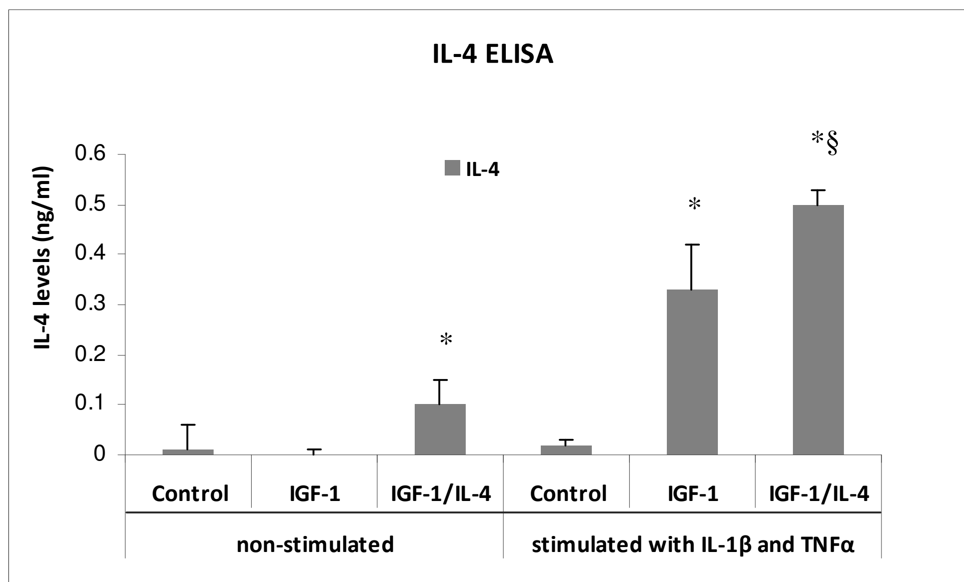


Figure 8: IL-4 ELISA. Canine chondrocytes in passage 3–4 were transfected with IGF-1 or IGF-1/IL-4 plasmid DNA, and non-transfected cells served as the control. Following transfection for 24 h, stimulated cells were incubated with 100 ng/ml IL-1β and 50 ng/ml TNFα proteins for 48 h. Cell lysates were collected, and sandwich ELISA was performed. IL-4 was detected in non-stimulated cells transfected with IGF-1/IL-4 and in stimulated cells transfected with IGF-1 and IGF-1/IL-4. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the respective controls ($p<0.05$); **§** indicates statistical significance compared to stimulated, IGF-1 transfected cells.

3.2.6 Regulation of IGFBP and IGFR1 expression

Differential regulation of IGF-binding proteins (IGFBPs) is of prime importance in arthritis conditions (Martel-Pelletier *et al.*, 1998) due to their effects on IGF-1 expression. *In vivo* IGF-1 is found bound to at least one of the six binding proteins until it is released by enzymatic cleavage and made available for receptor binding. Because it has been shown that IGFBP and IGFR1 levels are increased in OA, it was of importance to determine what effect IGF-1 and IL-4 would have on their expression. Accordingly, IGFBP levels in canine chondrocytes transfected with IGF-1 alone and with IGF-1/IL-4 were compared to the canine chondrocyte control. Fig. 9 shows that IGFBP3, -4, -5, and -6 were profoundly up-regulated in stimulated cells transfected with IGF-1 alone, denoting the effect of the up-regulation of IGF-1 on the expression of those IGFBPs. Reversely, cells transfected with IGF-1/IL-4 showed a down-regulation in the expression of those IGFBPs, excluding IGFBP4 which was only marginally affected. Moreover, the anabolic activity of IGF-1 is only possible following binding to its receptor. Under pro-inflammatory conditions, control cells showed an up-regulation of IGF receptor 1 (IGFR1). The expression level of this gene remained the same in cells transfected with IGF-1. However, IGFR1 was down-regulated in stimulated samples transfected with IGF-1/IL-4. It appears that the expression of IGF-1 in the cells increases the expression of certain IGFBPs. On the other hand, the expression of anti-inflammatory cytokine IL-4 regulates them to levels seen in the non-stimulated control cells.

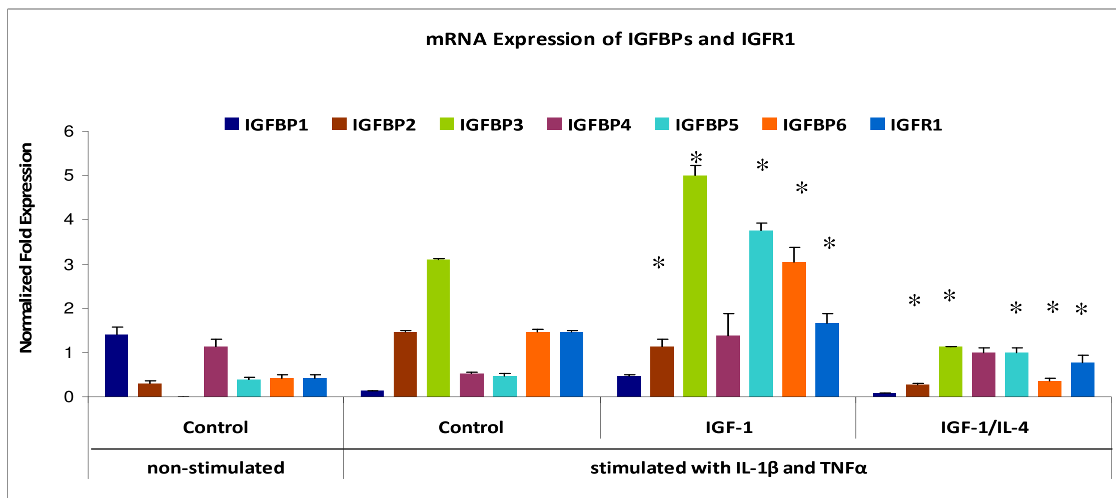


Figure 9: Real-time qRT-PCR of IGFBPs and IGFR1. Canine chondrocytes (passage 3–4) were transfected with IGF-1 or IGF-1/IL-4 and were stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα 24 h post-transfection. Stimulated and non-stimulated control cells were not transfected with the vector. Cells were stimulated for 48 h followed by RNA isolation, cDNA synthesis and real-time qRT-PCR. Values were normalized using GAPDH as endogenous control and are shown as fold changes relative to GAPDH. Cells transfected with IGF-1 showed an up-regulation of IGFBP3, -4, -5, and -6. The expression of IGF-1/IL-4 in the canine chondrocytes resulted in down-regulation of these binding proteins. IGFR1 expression was down-regulated likewise in cells transfected with IGF-1/IL-4. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated non-transfected control ($p<0.05$).

3.2.7 Down-regulation of pro-inflammatory cytokines and MMPs

In order to better understand the necessity of utilizing IGF-1 in combination with the anti-inflammatory cytokine IL-4, some of the main pro-inflammatory cytokines and enzyme mediators in OA were analzyed by real-time qRT-PCR. The effect of expressing IGF-1 alone and in combination with IL-4 was studied. Under stimulatory conditions, transfection of canine chondrocytes with the therapeutic genes IGF-1 and IL-4 repressed the expression of pro-inflammatory cytokines IL-1β, TNFα and IL-6, three of the main catabolic mediators in the inflammatory pathway of the disease process (Fig.10). The decrease in the expression levels of these cytokines was comparable to the levels seen in the non-stimulated control. This was not the case for the stimulated control and IGF-1 transfected cells, where the expression levels of the pro-inflammatory cytokines were still higher than in the non-

stimulated control. Cells transfected with IGF-1 alone demonstrated an up-regulation of TNFα and a minimal decrease in IL-1β when compared to the control cells. On the other hand, the expression of IL-6 was notably reduced in samples transfected with IGF-1 alone when compared to the stimulated control (Fig. 10).

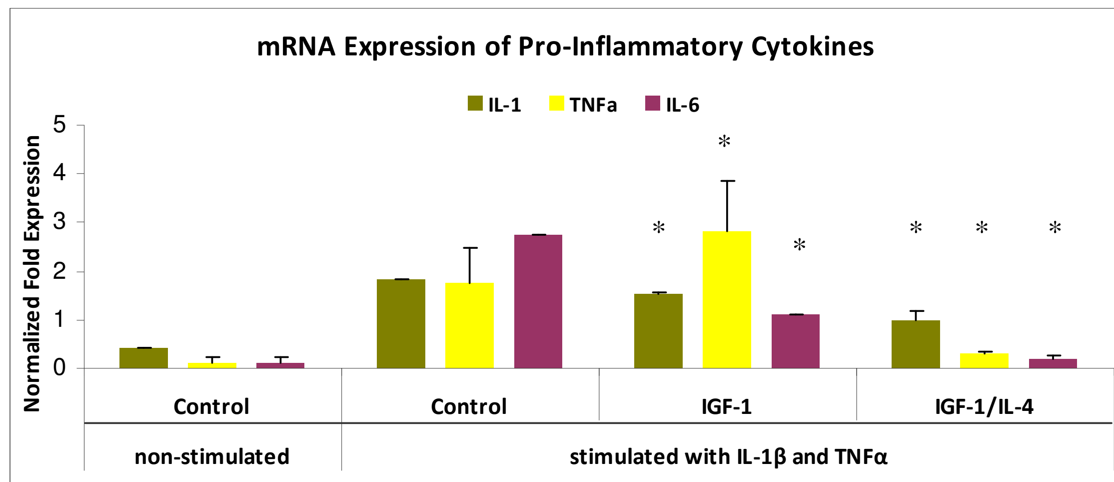


Figure 10: Real-time qRT-PCR of IL-1β, TNFα and IL-6. Following transfection for 24 h, chondrocytes (passage 3–4) were transfected with IGF-1 or IGF-1/IL-4 and were stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα for 48 h. Non-transfected cells served as the control. Following RNA isolation and cDNA synthesis, real-time qRT-PCR was performed. Values were normalized using GAPDH as endogenous control and are shown as fold changes relative to GAPDH. The expression of pro-inflammatory cytokines was reduced in cells transfected with IGF-1/IL-4 to levels comparable to the non-stimulated control. IGF-1 up-regulated the expression of TNFα but reduced the mRNA expression level of IL-6 compared to the stimulated non-transfected control. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

In addition, three matrix metalloproteases, namely MMP-1, -3 and -13, were analyzed by real-time qRT-PCR. These proteolytic enzymes are known for their role in cartilage degradation. The effect of expressing IGF-1 alone and together with IL-4 was analyzed. All three MMPs studied were suppressed in cells transfected with IGF-1 and IGF-1/IL-4 compared to the stimulated non-transfected control cells (Fig. 11).

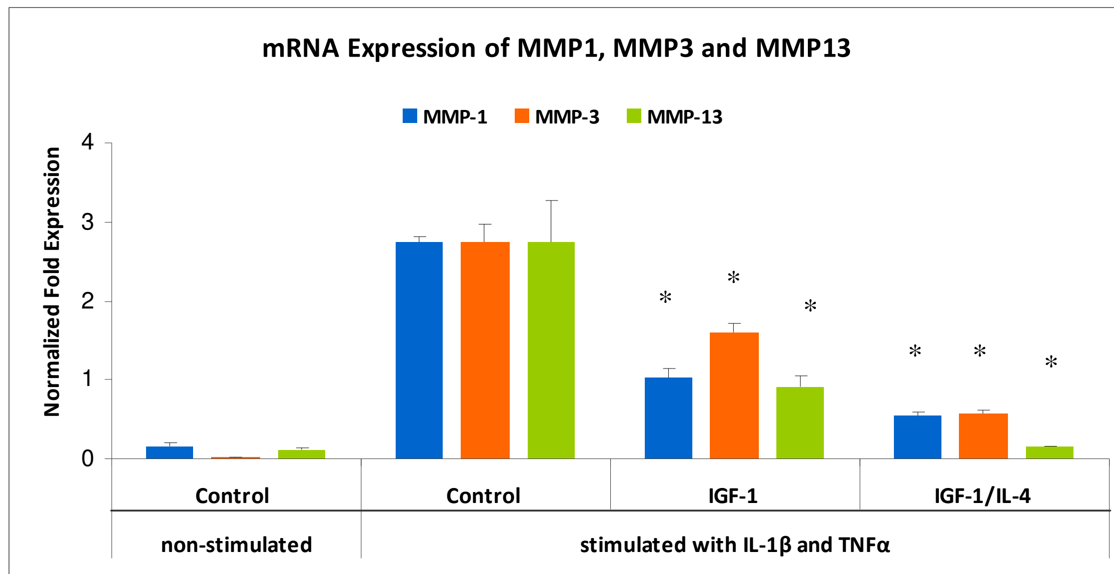


Figure 11: Real-time qRT-PCR of MMP-1, MMP-3 and MMP-13. Canine chondrocytes (passage 3–4) transfected with IGF-1 or IGF-1/IL-4 were stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα 24 h post-transfection. NFollowing stimulation for 48 h, RNA was isolated from cell pellets and cDNA was synthesized. Real-time qRT-PCR allowed for quantification of the mRNA expression utilizing GAPDH as housekeeping gene. Non-transfected cells served as control. The expression of MMP-1, -3, and -13 was decreased in cells transfected with IGF-1 and IGF-1/IL-4, however greater in cells transfected with IGF-1 and IL-4. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

3.2.8 Down-regulation on iNOS and NO

Estimation of endproducts of a signal cascade gives a good measure of the effect of therapeutic genes on inflammatory cytokines. Inducible nitric oxide synthase (iNOS) is an important catabolic mediator in OA. As known, iNOS levels correlate to the production of NO. While iNOS mRNA levels were analyzed by real-time RT-PCR, nitrite, a stable catabolite of NO, was assayed using the Griess reagent system.

Cell culture supernatants from stimulated and non-stimulated transfected chondrocytes and the non-transfected control cells were analyzed. As shown in Fig. 12, the non-transfected stimulated control showed an increased level of nitrite compared to the non-stimulated control. Samples from stimulated cells transfected with IGF-1 alone had only slightly reduced levels of nitrite. However, samples transfected with IGF-1 and IL-4 showed a more significant decrease in the level of nitrite. This result is in line with the qRT-PCR data depicted in Fig. 13, which demonstrate that iNOS mRNA expression levels are up-regulated in control cells

stimulated with pro-inflammatory cytokines. However, this trend is reversed in stimulated chondrocytes transfected with the therapeutic genes IGF-1 and IL-4. A minimal reduction in the mRNA levels of iNOS is seen in stimulated samples transfected with IGF-1 alone.

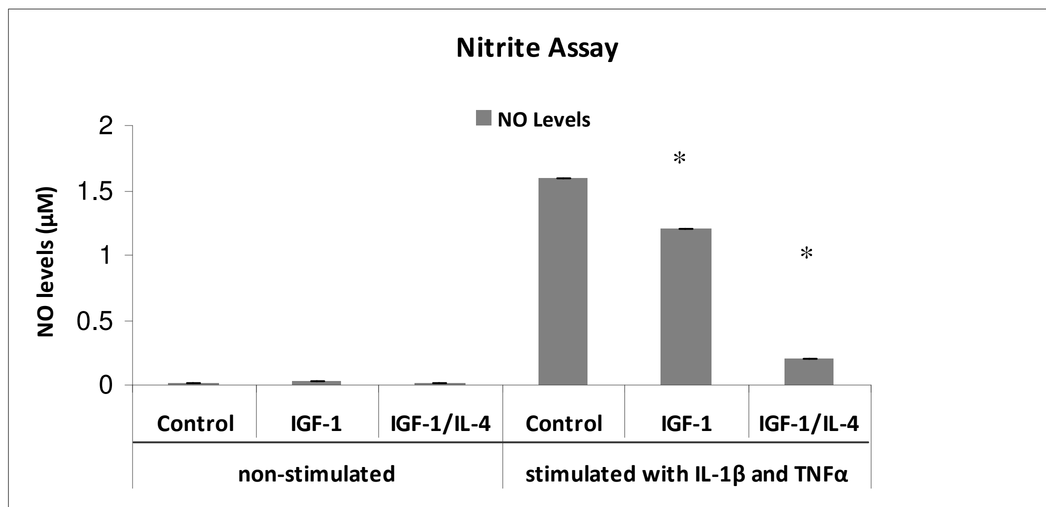


Figure 12: Effect of IGF-1/IL-4 on nitrite levels. Chondrocytes were transfected with IGF-1 or IGF-1/IL-4. Cells transfected with the empty vector served as control. The samples were either stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα for 48 h or left untreated post-transfection for 24 h. Conditioned medium from each sample was collected and NO assay was performed using Greiss reagent system (Promega). The level of detected NO was reduced in stimulated samples transfected with IGF-1 alone and even more in samples transfected with IGF-1 and IL-4. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

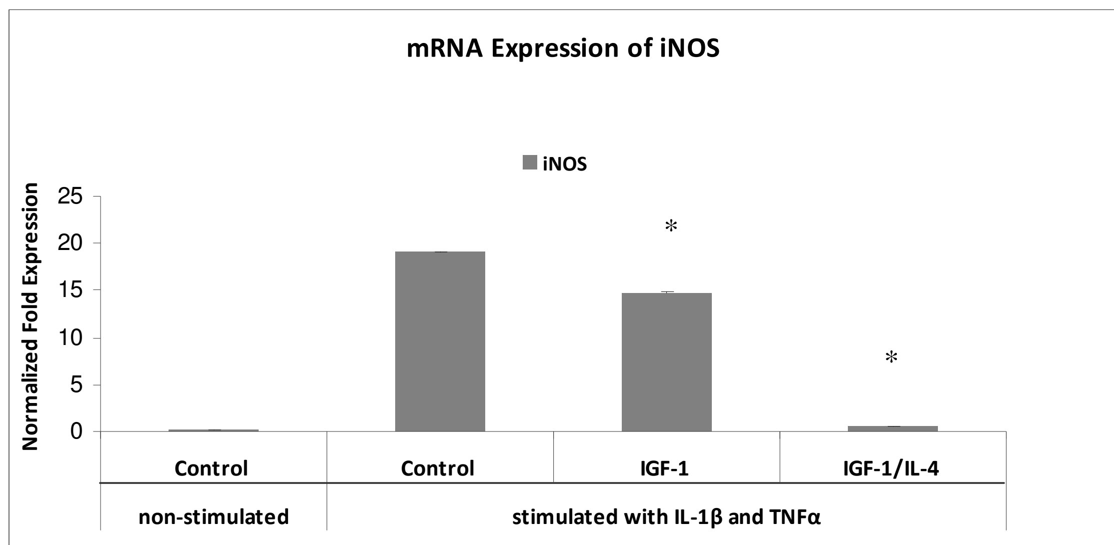


Figure 13: Real-time qRT-PCR of iNOS. Chondrocytes (passage 3–4) were transfected with IGF-1 or IGF-1/IL-4. Non-transfected cells served as control. The samples were stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα 24 h post-transfection. RNA was isolated from the cell pellets followed by cDNA synthesis. Real-time qRT-PCR allowed for quantification of the mRNA expression utilizing GAPDH as housekeeping gene. The expression of iNOS was reduced in samples transfected with IGF-1/IL-4 to levels comparable to the non-stimulated control, and only a slight reduction was seen in samples transfected with IGF-1 alone. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

3.2.9 Regenerative potential of IGF-1/IL-4

IGF-1 is known for its ability to stimulate the regeneration of extracellular matrix proteins. While it is expected that the presence of IL-4 could hamper the expression of inflammatory mediators, it remains to be known whether IL-4 in combination with IGF-1 has any positive influence on cartilage regeneration. In this study, the expression of type II collagen, aggrecan and SOX9 (transcriptional factor sex-determining region Y-box 9) were investigated. These genes were selected due to their acceptance as good marker genes for differentiated chondrocytes. Samples transfected with IGF-1 and IGF-1/IL-4 showed an increase in the mRNA expression level of aggrecan and type II collagen. Both transfected samples showed an increase in the SOX9 expression when compared to the control cells, with SOX9 expression being greater in samples transfected with IGF-1 (Fig. 14).

To further investigate the influence of IGF-1 on the regeneration of proteoglycans alcian blue staining was performed where the amount of proteoglycans is reported as optical density at 620 nm. This method is an easy way to measure the amount of proteoglycans in the cells. Compared to the stimulated control, the amount of proteoglycans was greater in samples transfected with IGF-1 alone. Those cells transfected with IGF-1/IL-4 showed an increased expression of proteoglycans compared to the non-transfected control. However, it was not as high as in cells transfected with IGF-1 alone (Fig. 15). In addition, transfected cells did not express the level of proteoglycans as seen in the non-transfected, non-stimulated control.

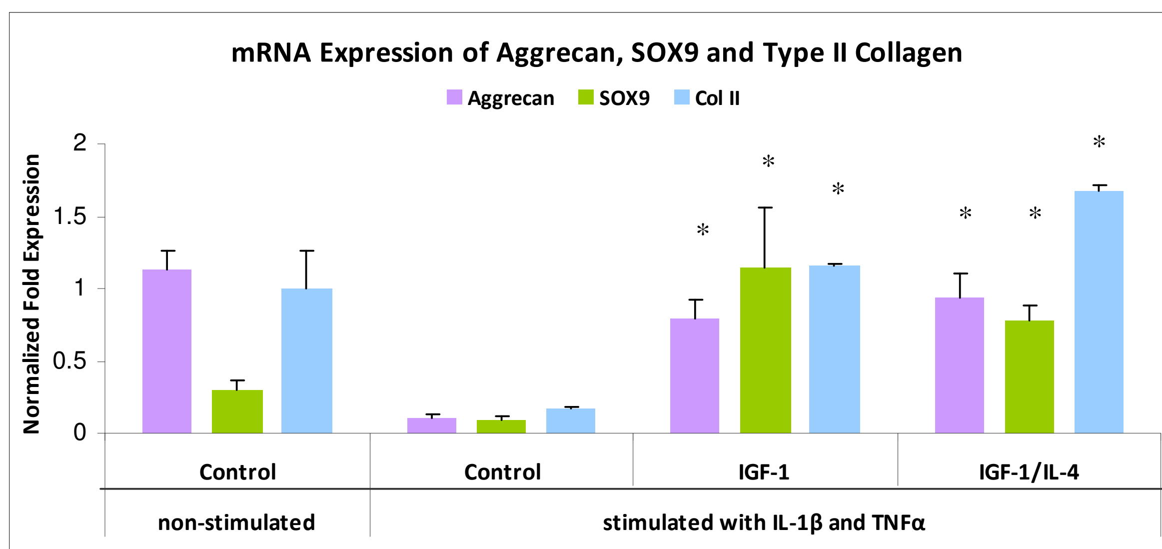


Figure 14: Real-time qRT-PCR of aggrecan, SOX9, and type II collagen. Chondrocytes (passage 3–4) were transfected with the respective genes and stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα 24 h post-transfection. Incubation with the pro-inflammatory cytokines proceeded for 48 h and then, RNA was isolated and cDNA was synthesized from the samples. Real-time qRT-PCR allowed for quantification of the mRNA expression utilizing GAPDH as housekeeping gene. Samples transfected with IGF-1 and IGF-1/IL-4 showed an up-regulation of the cartilage matrix proteins type II collagen and aggrecan. Transfection with therapeutic genes resulted in an increase in the expression of SOX9 compared to the stimulated control. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

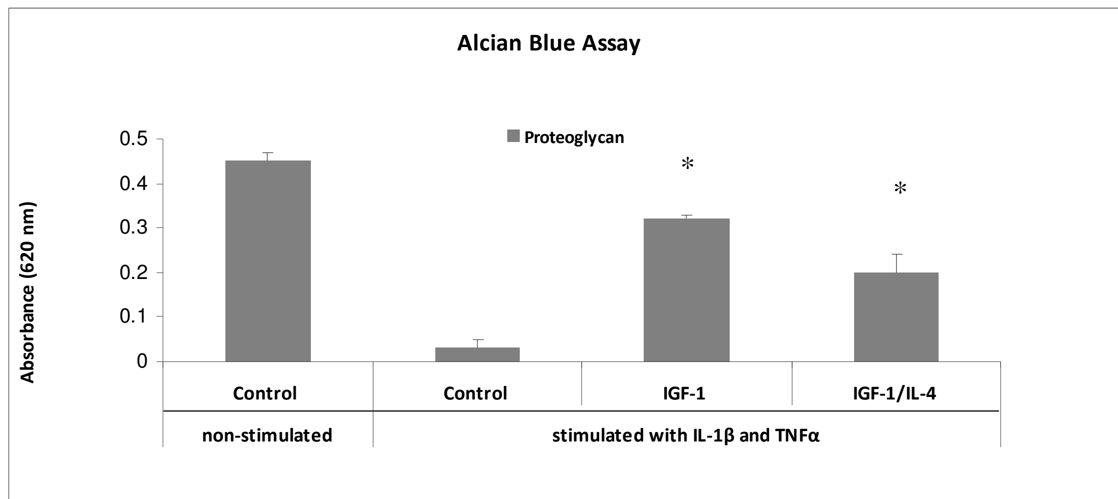


Figure 15: Alcian blue assay of canine chondrocytes. Alcian blue assay was performed on chondrocytes (passage 3–4) transfected with IGF-1 and IGF-1/IL-4 to quantify the amount of proteoglycans in the cells. Non-transfected cells served as control. Analyses were performed following 48 h of stimulation with the pro-inflammatory cytokines measuring the optical density of the cells at 620 nm. Cells transfected with IGF-1 and IGF-1/IL-4 showed an increase in the expression of proteoglycans in comparison to the stimulated control. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the stimulated, non-transfected control ($p<0.05$).

3.2.10 Immunocytochemical analysis of type II collagen in chondrocytes

Above it was shown on the mRNA level that canine chondrocytes transfected with IGF-1 and IGF-1/IL-4 express type II collagen (Fig. 14). In order to analyze the expression of this gene on the protein level, immunocytochemistry was performed. Canine chondrocytes were transfected with IGF-1 or IGF-1/IL-4 to study the regenerative potential of the cells transfected with the respective genes. Non-transfected chondrocytes served as control. Following incubation with pro-inflammatory cytokines for 96 h, cells were trypsinized and cultivated in chamber slides and submitted to staining with type II collagen antibodies using Dakocytomation. IGF-1 as well as IGF-1/IL-4 transfected cells showed an increase in type II collagen protein compared to the stimulated, non-transfected control (Fig. 16 A–D).

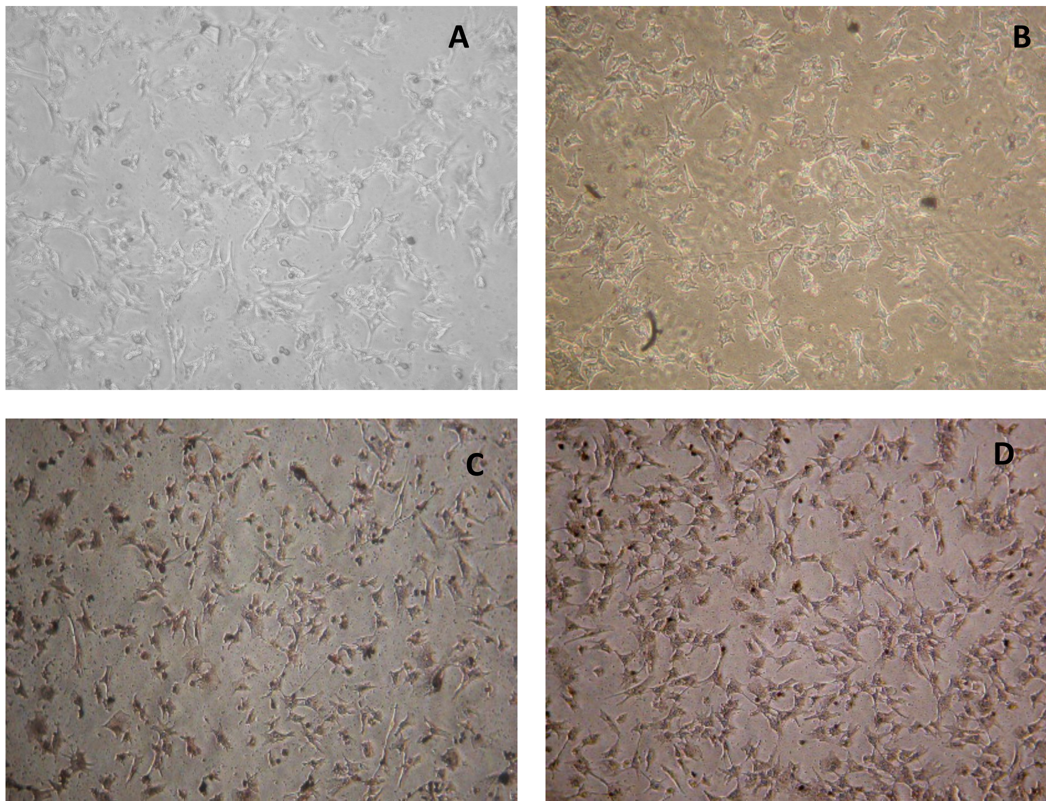


Figure 16: Immunocytochemistry of transfected canine chondrocytes. Canine chondrocytes were transfected with IGF-1 or IGF-1/IL-4 and stimulated with pro-inflammatory cytokines 24 h post-transfection for 96 h. Transfected cells and non-transfected control were seeded in chamber slides and stained using Dakocytomation and type II collagen antibodies. Stimulated cells transfected with IGF-1 and IGF-1/IL-4 expressed type II collagen (C and D, respectively). The non-treated and the stimulated, non-transfected control cells showed no expression of type II collagen (A and B, respectively). The magnification of the cells was x4.

In summary, canine chondrocytes treated with pro-inflammatory cytokines and transfected with both IGF-1 and IGF-1/IL-4 possess the ability to down-regulate the expression of catabolic mediators while stimulating the expression of cartilage matrix proteins. Cells transfected with both therapeutic genes were able to suppress the expression of IL-1β, TNFα, IL-6, MMP-1, MMP-3, MMP-13, iNOS and NO more effectively than IGF-1 alone. Both IGF-1 and IGF-1/IL-4 transfected cells expressed type II collagen and proteoglycans on the mRNA and protein levels. These results indicate that over-expression of IGF-1 alone can initially stimulate the expression of extracellular matrix proteins. However, there is a need for the combined expression with IL-4 to alleviate the effects of pro-inflammatory mediators associated with the disease processes.

3.3 Characterisation of cMSCs

Harming healthy cartilage donor sites to obtain enough cartilage material and retrieving a sufficient number of chondrocytes from cartilage tissue, which is known for its low-cellular nature, are two of the problems associated with using chondrocytes to heal cartilage defects. Usage of mesenchymal stem cells offers an alternative cell source for cartilage therapy. The advantage of using MSCs include that these cells are easily isolated, retain high expansion potential, and play important roles in growth, regeneration, and repair of mesenchymal tissues. Of importance for their use in cartilage repair is that these stem cells are known to have chondrogenic potential.

At the beginning of this study, canine mesenchymal stem cells (cMSCs) had not yet been characterized. Therefore, these cells were first characterized based on their ability to undergo chondrogenesis before utilization in transfection studies involving combined expression of IGF-1 and IL-4. First, optimization of cMSC isolation was performed. Then, the characterization of the cells, both non-differentiated as well as differentiated, was studied. The results obtained are listed and described below.

3.3.4 Isolation and characterization of cMSCs

Canine mesenchymal stem cells (cMSCs) were isolated from bone marrow aspirates utilizing a Percoll gradient with a density of 1.073 g/ml. The number of cells isolated per 2 ml aspirate was between 50 x 10^6 and 100 x 10^6. Cells were plated at a density of approximately 3.5 x 10^6 cells/cm^2. The isolated cells consisted of round shaped erythrocytes, non-adherent haematopoietic cells and cMSCs. Unlike cMSCs, the other cells did not attach to the flask and were removed 72 h post-isolation with the first medium exchange. Three days after seeding of the cells, the first cMSCs were seen under the microscope along with the round shaped erythrocytes. Approximately 7 d following seeding of the cells, densely formed colonies were observed. Once the individual colonies were confluent, the cells were trypsinized and passaged. Thereafter, the cells showed their typical fibroblastic form (Fig. 17 A–D).

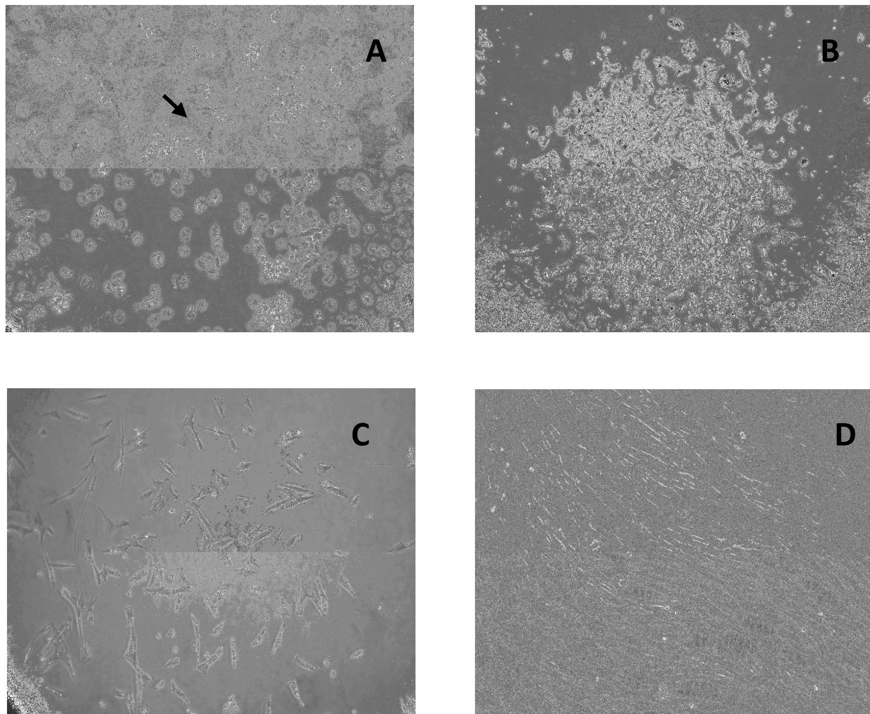


Figure 17: Microscopic analysis of cMSC grown in monolayer cultures. Canine MSCs were isolated from bone marrow aspirates using a Percoll gradient (density: 1.073 g/ml). The cells were seeded 1.5 – 3.5 x 10^5 cells/cm^2 cell culture flask. **A)** Freshly isolated cells consisted of mainly round shaped erythrocytes, non-adherent haematopoietic cells, and cMSCs. Approximately three days following seeding of the cells, the cMSCs attached to the flask (see arrow). **B)** Approximately 7 d following isolation confluent cell colonies were observed and could be passaged. **C)** cMSCs in passage 1 showing typical fibroblastic morphology 2 d after seeding 5000 cells/cm^2. **D)** Confluent cells in passage 1 had the typical fibroblastic morphology associated with MSCs approximately 14 d following isolation of cMSCs. This morphology was continuously seen in the following passages. (Original magnification: x4)

3.3.5 Analysis of non-differentiated cMSCs

Currently, there exists no specific MSC-marker for humans or animals, which could be used to verify that the cells isolated from canine bone marrow aspirates are in fact MSCs. However, there are a number of cell surface markers that are considered to be present or absent on MSCs cultured *in vitro*. For this reason, FACS analysis was performed to test for a few from the number of cell surface markers that are generally accepted to be positive or negative for MSCs. MSCs are known to lack haematopoietic markers like CD45 and CD34,

whereas they do express cell surface markers like CD90, CD29, and CD44. Due to the lack of canine-specific antibodies, human and mouse antibodies were used. As seen in Fig. 18 the isolated cells were found to be negative for CD45- and CD34- and positive for CD29+, CD90+ and CD44+.

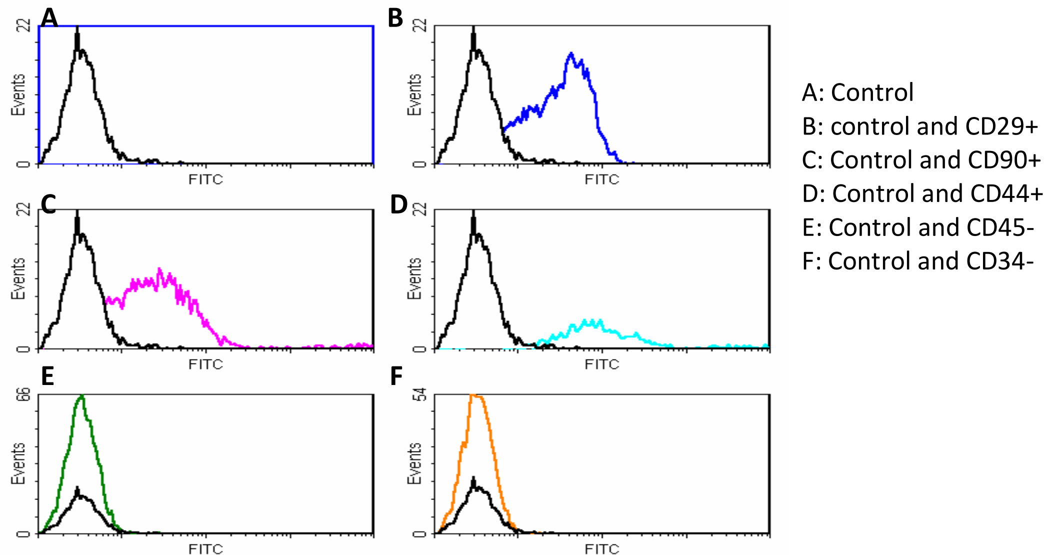


Figure 18: FACS Analysis of Non-differentiated cMSCs. Non-differentiated cMSCs (1x 10^6 cells) in passage 3 were used for FACS analysis. The cells were suspended in 1X PBS along with fluorescein isothiocyanate (FITC) coupled antibodies against CD29, CD45 and CD34. A secondary antibody labeled with FITC was used for CD90 and CD44. No antibody treated cells served as control. Cell fluorescence was measured in FACS instrument. The cells were shown to be positive for surface markers CD90+, CD29+, CD44+ and negative for CD45- and CD34-.

3.3.6 Chondrogenic potential of cMSCs

Important for this study was whether the cMSCs possessed the potential to under go chondrogenesis. It has been reported that the induction of chondrogenesis in human MSCs is possible by using chondrogenic medium containing of TGFβ3. Also, to induce chondrogenesis alginate cultures are normally used due to the ease associated with the cultivation and retrieval of the cells from the matrix. Therefore, cMSCs were cultivated in 1.2% alginate cultures (Fig. 19) for up to 21 days in a defined chondrogenic medium with 10 ng/ml TGFβ3. Cell number per bead was found to be 30,000 and cell vitality approximately 95%.

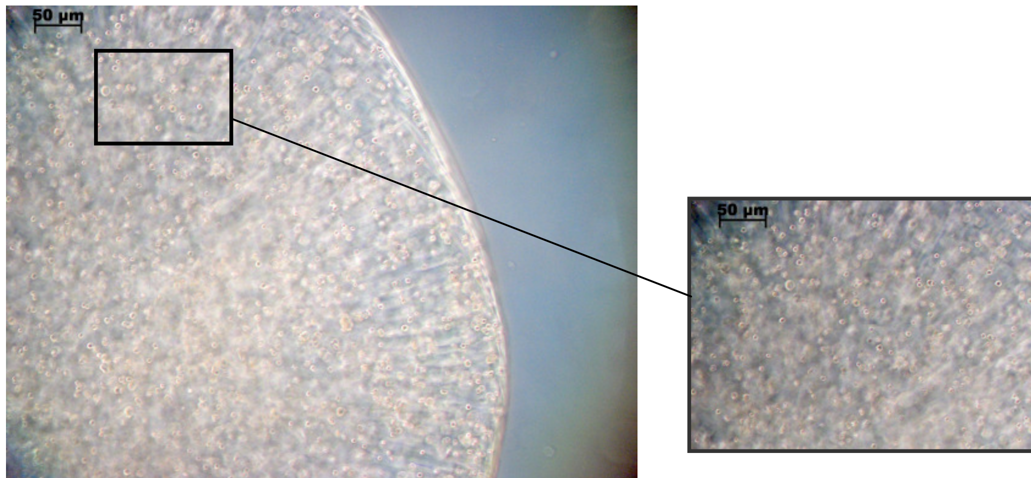
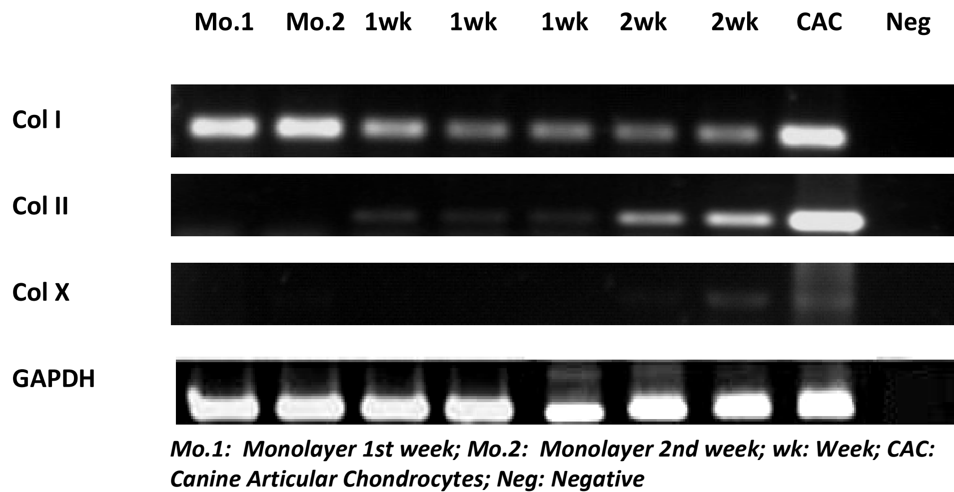


Figure 19: cMSCs cultivated in 1.2% alginate. To analyze the chondrogenic potential of cMSCs, cells in passage 2 were trypsinized and seeded into alginate beads. The beads were incubated with chondrogenic medium containing TGFβ3. Beads that were not treated with TGFβ3 served as control. Each bead was shown to have approximately 30,000 cells.

To analyze the effect of TGFβ3 on the chondrogenic potential of cMSCs, RT-PCR was performed. One marker gene used to determine whether the chondrogenesis of MSCs has is type II collagen. This gene was used to verify the differentiation of cMSCs. Both cells in monolayer as well as in the 3D alginate cultures were shown to express type I collagen (Fig. 20). As shown in this figure, cells began to express type II collagen in the 1st week of exposure to TGFβ3. Also, the expression of type X collagen was first seen in one of the 2nd week chondrogenic samples. GAPDH was used as a positive control and was expressed in all samples. The negative control was free of contaminants.



Mo.1: Monolayer 1st week; Mo.2: Monolayer 2nd week; wk: Week; CAC: Canine Articular Chondrocytes; Neg: Negative

Figure 20: RT-PCR of cMSC matrix proteins. cMSCs in alginate cultures were exposed to differentiating medium consisting of 10 ng/ml TGFβ3 for a total of 2 wks and analyzed for their expression of matrix proteins. Monolayer cultured cMSCs served as control. Cells expressed type I collagen and only TGFβ3 treated cells showed the expression of type II collagen. Type X collagen was expressed in the 2 wk alginate cultures. The negative control was free of contaminants. Passage 1 canine chondrocytes in monolayer served as a positive control. The experiment was repeated in duplicates.

Similar results were seen by real-time qRT-PCR (Fig. 21). The samples were normalized to GAPDH as an internal standard. Type I collagen was expressed in all samples but more so in samples treated for 3 weeks with TGFβ3. In addition, type X collagen expression was first detected in 3rd week samples. Type II collagen and aggrecan were detected in samples treated for 1 week with TGFβ3 but the expression was the greatest in the 3rd week.

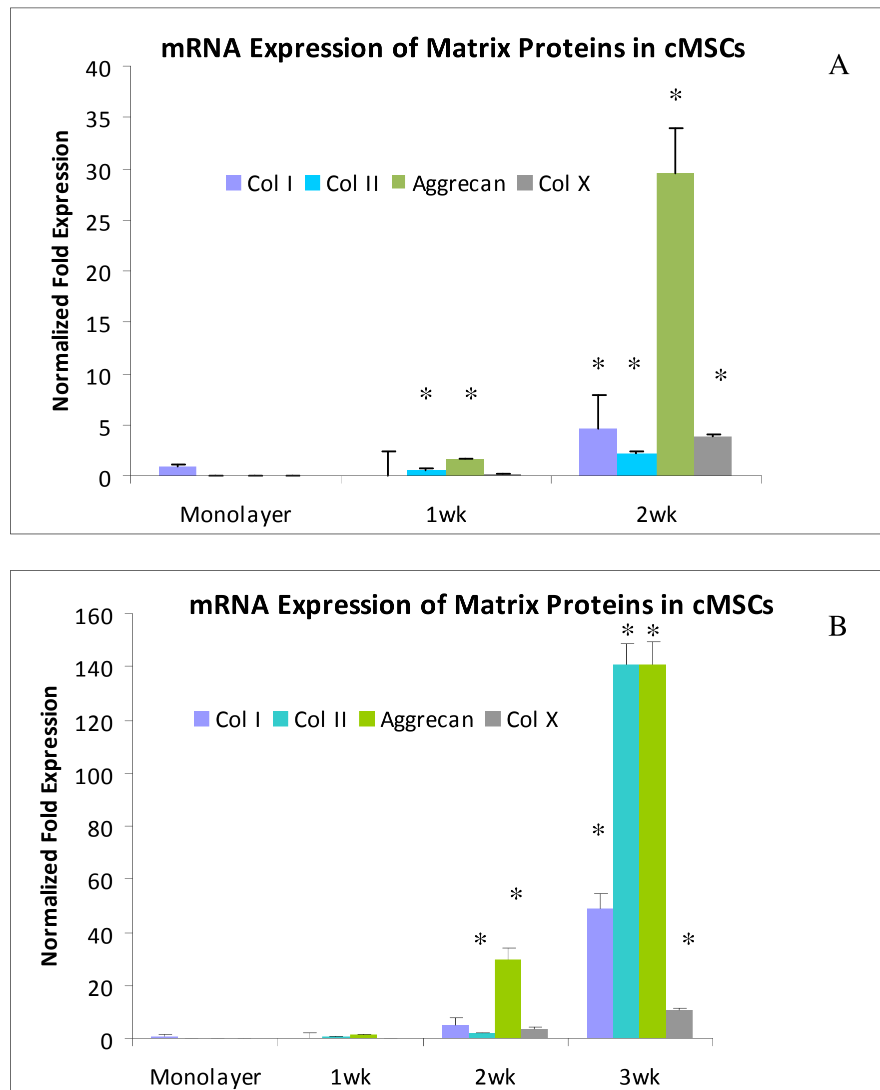


Figure 21: Real-Time qRT-PCR of cMSC matrix proteins. Canine MSCs in passage 3 were used for this experiment. The cMSCs were cultured in alginate and were exposed to 10 ng/ml TGFβ3 for up to 3 weeks. Thereafter, the cells were analyzed for their mRNA expression of matrix proteins by real-time qRT-PCR. The samples were normalized to GAPDH. Cells not exposed to TGFβ3 served as control (monolayer). The mRNA expression levels of all genes were the greatest for in the 3rd week. Upper figure (A) is shown to better depict expression of genes in 2nd week. Negative controls were free of contaminants. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the monolayer, non-differentiatedcontrol cells ($p<0.05$).

3.2.4 Immunocytochemistry of non-differentiated cMSCs

To further examine the expression of type I and type II collagen on the protein level, immunocytochemistry was performed using Dakocytomation. Non-differentiated cMSCs were cultivated in chamber slides and detection of the above mentioned proteins was performed using the antibodies anti-goat collagen type I and anti-rabbit collagen type II (Fig. 22). Non-differentiated cMSCs not treated with primary antibodies served as control. Moreover, the non-differentiated cMSCs are known to express type I collagen and not type II collagen. No staining was seen in these control cells or in non-differentiated cMSCs stained for type II collagen. However, type I collagen was detected in non-differentiated cMSCs. In summary, non-differentiated cMSCs were positive for type I collagen and negative for type II collagen.

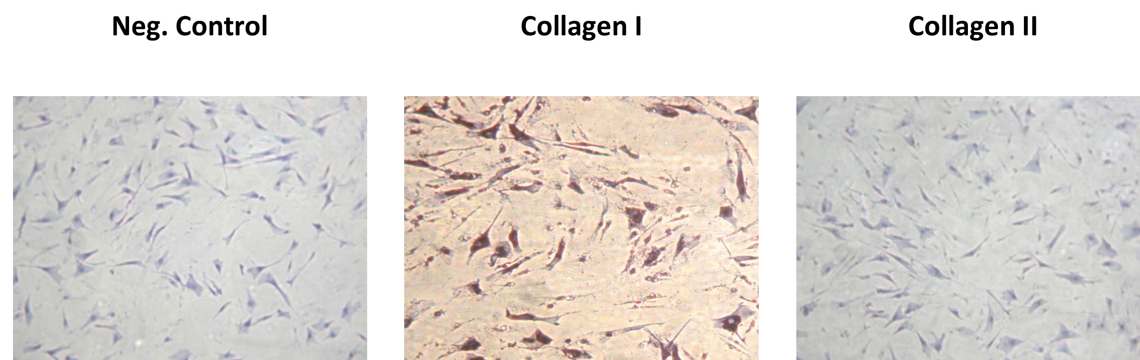


Figure 22: Immunocytochemistry of non-differentiated cMSCs in monolayer. Non-differentiated cMSCs in passage 3 were cultivated in chamber slides and stained using Dakocytomation to analyze the protein expression of type I and type II collagen. Anti-goat collagen type I and anti-rabbit collagen type II antibodies were used 1:800 and 1:300, respectively. Non-differentiated cMSCs were positive for type I collagen and negative for type II collagen. Non-antibody treated cMSCs served as negative control.

After investigating the expression of the extracellular marker protein type II collagen in non-differentiated cMSCs, it was necessary to determine whether differentiated cMSCs expressed type II collagen on the protein level. To assay this differentiated as well as non-differentiated cMSC were cultivated in alginate beads with or without TGFβ3, respectively. These beads were cryo-sectioned and thereafter, stained using Dakocytomation. Non-differentiated cells stained negative for type II collagen where as differentiated cells, which

had been treated with TGFβ3 for 2 weeks, tested postitive for type II collagen (Fig. 23). These results correlate to the real-time qRT-PCR results obtained for differentiated cMSCs (Fig. 21).

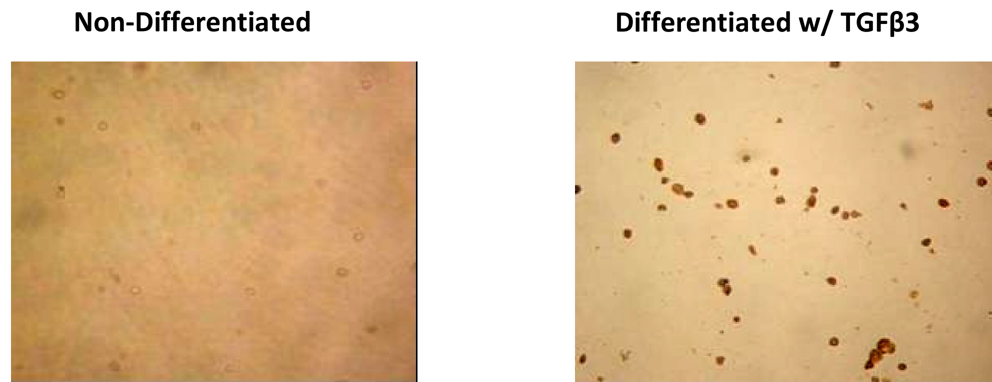


Figure 23: Immunocytochemistry of non-differentiated and differentiated cMSCs in alginate beads. Passage 3 cMSCs were were analyzed for expression of type II collagen. Non-differentiated were seeded in alginate beads and incubated either with non-differentiating medium (no TGFβ3) or differentiating medium (with 10 ng/ml TGFβ3). Both samples were incubated for 2 weeks and subjected to staining using Dakocytomation and the antibody rabbit anti- type II collagen. Non-differentiated cMSCs were negative and differentiated cMSCs positive for type II collagen.

3.2.5 Western blot analysis of cMSC

Anther way to analyze the type II collagen protein expression in differentiated and non-differentiated cMSCs was by Western blot as described under material and methods. For this experiment, non-differentiated cMSCs were cultivated in alginate beads for 3 weeks with or without treatment with TGFβ3. Passage 1 canine chondrocytes cultivated in monolayer were used as a positive control due to their ability to express type II. Each sample was shown to express type I collagen protein (Fig. 24). This was seen on the membrane as a pattern of two bands, of which the lower band (137 kDa) represents type I collagen. However, the origin of the upper band is unknown, but it is thought to represent of unspecific binding. Moreover, differentiated cMSCs and canine chondrocytes were positive for type II collagen, which is depicted on the Western blot as a single band of 141 kDa.

Figure 24: Western-Blot analysis of cMSCs. Cell lysates were collected from passage 3 non-differentiated and differentiated cMSCs, which had been cultivated for 3 weeks in alginate beads. After releasing the cells from the alginate, the cMSCs were lysed by RIPA buffer and proteins were loaded on a 7% SDS gel. The proteins were electroblotted on PVDF membranes and then incubated with either type I or type II collagen antibodies. Detection was made by horse raddish peroxidase conjugate secondary antibodies. Non-differentiated cMSCs were positive for type I collagen and negative for type II collagen. Differentiated cMSCs and positive control were positive for type II collagen. The positive control was canine chondrocytes in passage 1. (ND: non-differentiated cMSCs; D: differentiated cMSCs; CAC: canine articular chondrocytes)

3.2.6 Transfection efficiency

MSCs are not easily transfected with a non-viral vector. To test the transfection efficiency of cMSCs, flow cytometry was performed. The cMSCs were transfected with a DSRed2N1 vector and then the transfection efficiency was measured. These cells showed a transfection efficiency of 24.5% (Fig. 25).

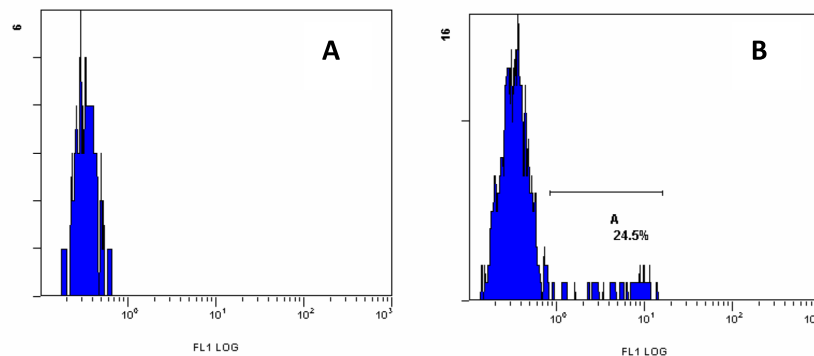


Figure 25: Transfection efficiency of cMSCs. Non-differentiated cMSCs in passage 3 were transfected with the non-viral DSRed2N1 vector utilizing FuGENE6 and analyzed via flow cytometry. Red fluorescence was seen in transfected cMSCs (B) and not in the non-transfected cMSCs (A). In B the horizontal line indicates the window settings in which the fluorescence was measured to give the

percent values for total fluorescence in the window. The y-axis denotes the number of cells measured. The transfection efficiency of the cells was found to be 24.5%.

3.2.7 Type II collagen expression study

A study the chondrogenic effects of IGF-1, cMSCs were transfected with pVitro-2-IGF-1-IL-4 and the expression levels of type II collagen and aggrecan were analyzed. These results were compared to cMSCs cultivated in chondrogenic medium containing TGFβ3. In order to insure the analysis of all cells which had been transfected with the therapeutic genes, the cells were cultivated in high density pellet cultures for 7 d and analyzed by real-time qRT-PCR. The cMSCs transfected with IGF-1/IL-4 were able to stimulate the mRNA expression of type II collagen and aggrecan. As shown in Fig. 26, the mRNA expression levels of type II collagen and aggrecan were greater, however, in cMSCs stimulated with TGFβ3. Non-differentiated cMSC showed no expression of the chondrogenic marker gene, type II collagen.

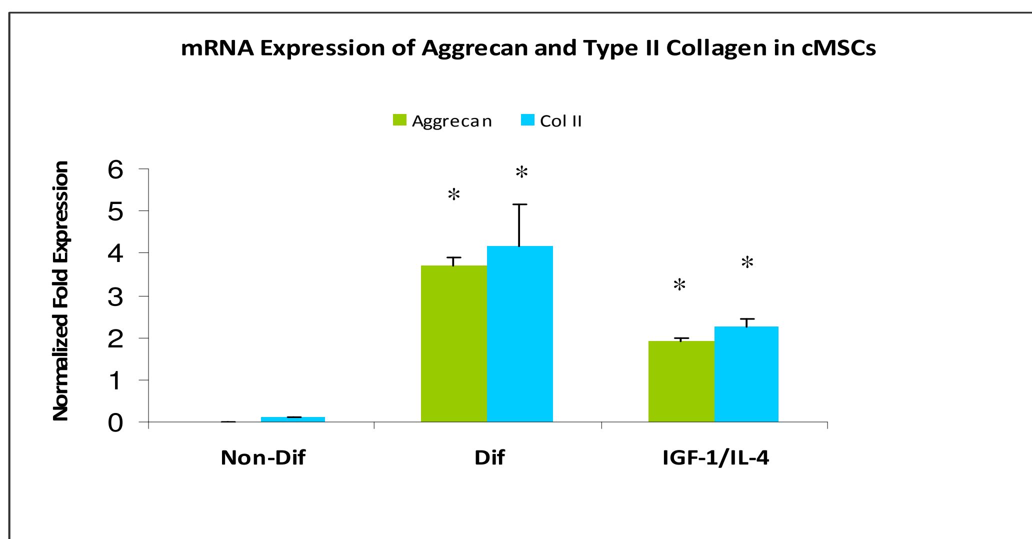


Figure 26: Real-time qRT-PCR of type II collagen expression in transfected cMSCs. The cMSCs in 3–4 passage were transfected with the double expression vector (IGF-1/IL-4) as described in the material and methods. The expression levels of aggrecan and type II collagen in these cells were compared to the expression levels in non-differentiated cMSCs (Non-Dif) and cMSCs which were cultivated with chondrogenesis medium including 10 ng/ml TGFβ3 (Dif). Following incubation in differentiating and non-differentiating media or transfection for 7 d, the samples were harvested, and cDNA was synthesized and analyzed by real-time qRT-PCR. Type II collagen (Col II) and aggrecan expression was up-regulated in samples transfected with IGF-1/IL-4 and samples stimulated with TGFβ3. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to the non-differentiated control ($p<0.05$).

In summary, cMSCs were characterized based on their ability to adhere to cell culture flasks, the presence or lack of chosen cell surface markers, and their ability to differentiate into chondrocytes. The cMSCs were positive for the initially selected cell surface markers CD90, CD29, and CD44. However, they were negative for CD34 and CD45. Their ability to undergo chondrogenesis was based upon the expression of cartilage specific extracellular matrix proteins, namely aggrecan and type II collagen. The mRNA expression of aggrecan and collagen type II was verified in differentiated cMSCs by real-time qRT-PCR. In addition, the protein expression of collagen type II was seen by Western blot and Immunocytochemistry.

3.4 Co-Culture studies

The concluding experiment of this study was to develop a stable chondrocyte cell line which expressed IGF-1 as well as IL-4 under the influence of COX-2 promoter. This promoter construct is disease sensitive allowing for the expression of IL-4 only when inflammation or pro-inflammatory cytokines are present. This stable cell line was then co-cultivated with non-differentiated cMSCs in passage 3 or canine chondrocytes in passages 4–6. Real-time qRT-PCR and the nitrite assay were performed to determine if cMSCs underwent chondrogenesis and if the passaged chondrocytes would show anabolic activity once cultivated with stably transfected chondrocytes.

3.4.4 Stable cell line

The pre-existing stable chondrocyte cell line (unpublished data) stably transfected with pCOX-2-IL-4 was super-transfected with IGF-1/pVitro2. Following selection with antibiotics, colonies were picked and expanded. These super-transfected cells were found to be fast growing and they possessed the spherical shape normally seen in freshly isolated chondrocytes. In addition, these cells maintained the expression of both genes over more than 20 passages. The expression of IGF-1 and IL-4 in these cells were analyzed by real-time qRT-PCR. Stably transfected chondrocytes which were stimulated with both IL-1β and TNFα were found to express both genes but the expression of IL-4 was greater than that of IGF-1 (Fig. 27).

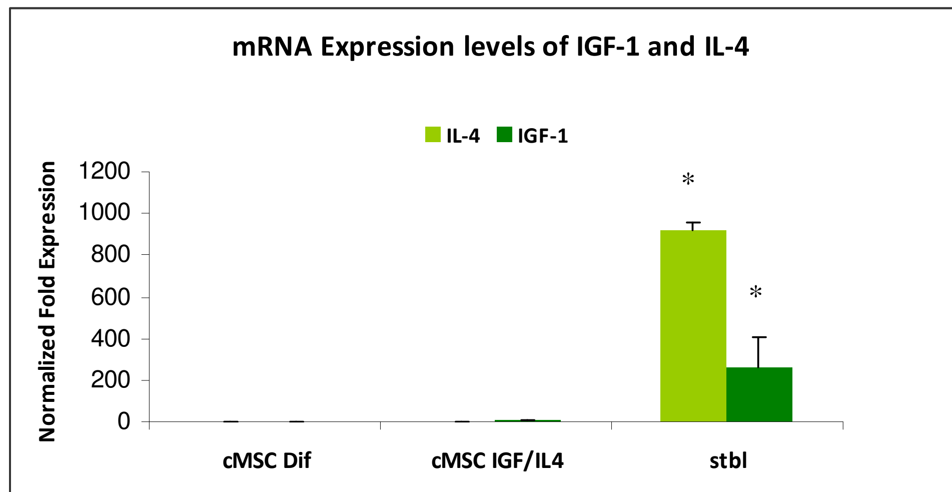


Figure 27: Real-time qRT-PCR analysis of IGF-1 and IL-4 expression in stable cells. Canine chondrocytes which stably express pVitro2-IGF-1 and pCOX2-IL-4 were checked for their ability to express both genes. This was compared to cMSCs transfected with the plasmid DNA IGF-1/IL-4 (cMSC IGF/IL4) and cMSCs stimulated with 10 ng/ml TGFβ3 (cMSC Dif). Stably transfected cells were stimulated with 100 ng/ml IL-1β and 50 ng/ml TNFα to activate the expression of IL-4 from the pCOX-2 vector, a pro-inflammatory sensitive promoter. Stable cells (stbl) express IGF-1 and IL-4 over 200x more than in cMSCs transfected with IGF-1/IL-4 and cMSCs treated with TGFβ3. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to cMSCs differentiated with TGF β3 ($p<0.05$).

3.4.5 Co-culture with cMSCs

In this experiment, cMSCs were co-cultivated with the stably transfected cells, which expressed both pVitro-IGF-1 and pCOX2-IL-4. Of interest was whether IGF-1 from the stably transfected cells would be able to stimulate the differentiation of the cMSCs. Non-differentiated cMSCs in passage 3 were cultivated in pellet cultures with stable cells expressing IGF-1 and IL-4 under the pCOX-2 promoter in different ratios (cMSCs/stable cells), namely 100/0, 0/100, 80/20, 70/30, and 50/50. Pellet cultures were chosen to insure that the cells would be cultivated in the given proportions. Stable cells (100%) and cMSCs (100%) in pellet cultures served as controls. The cMSCs (100%) were stimulated with chondrogenic medium containing TGFβ3.

Following 48 h of cultivation in the pellet cultures, co-cultivated cMSCs showed an up-regulation of type II collagen by real-time qRT-PCR. The ratio 80/20 showed the highest expression level of type II collagen followed by 70/30 and 50/50, in that order. Stable cells alone did not express type II collagen (Fig. 28a). However, cMSCs which were incubated for 1

wk in the pellet cultures and chondrogenic medium demonstrated an increased expression level of type II collagen. The expression level of type II collagen remained relatively constant for the 1 wk co-cultivated samples (Fig. 28b).

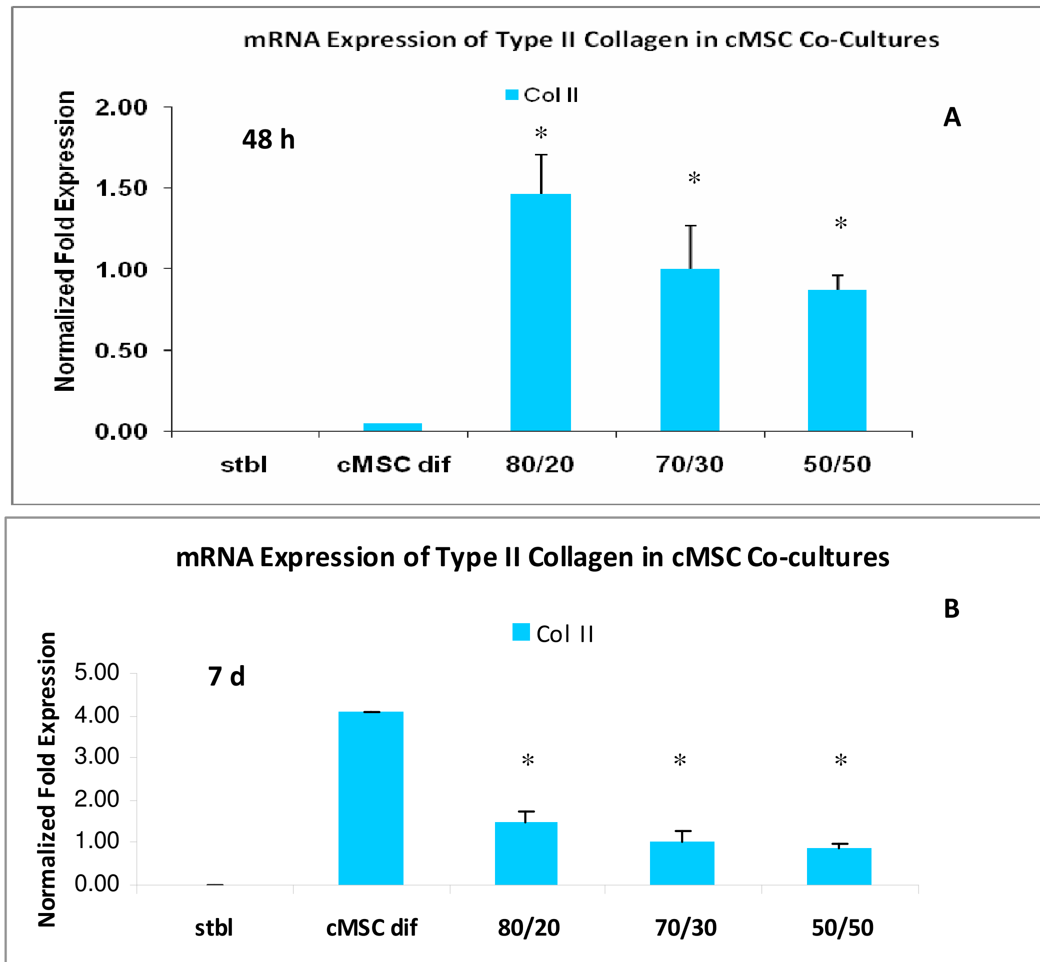


Figure 28: Expression of type II collagen in cMSCs co-cultures. cMSCs were cultured in combination with stable cells (stbl) expressing pVitro2-IGF-1 and pCOX2-IL-4 under in ratios (cMSCs/stable cells) of 100/0, 0/100, 80/20, 70/30, and 50/50 in high-density pellet cultures. Stable cells in monolayer and cMSC in pellet cultures were used as controls. cMSCs alone were cultured in chondrogenic medium containing TGFβ3 (cMSC dif). **A)** Cells were cultured for 48 h in the pellet cultures and then analyzed by real-time qRT-PCR. Cells cultured at a ratio of 80/20 expressed the highest level of type II collagen followed by 70/30 and 50/50. cMSCs in chondrogenic medium expressed the lowest level of type II collagen. Stable cells showed no expression of type II collagen. **B)** Cells were cultured for 7 d in the pellet cultures and then analyzed by real-time qRT-PCR. cMSCs cultured in chondrogenic medium showed the highest level of type II collagen. The collagen levels of the co-culture samples remained approximately the same as in the 48 h samples. Stable cells did not express mRNA levels of type II collagen. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to cMSCs differentiated with TGF β3 ($p<0.05$).

3.4.6 Co-culture with chondrocytes

Passaged canine chondrocytes (passage 4–6) were used in combination with stably transfected chondrocytes expressing pVitro2-IGF-1 and pCOX2-IL4 (Fig. 29). This experiment was done to study the effect of the stably transfected cells on passaged chondrocytes. It was important to analyze the stimulation of the extracellular matrix protein type II collagen and the regulation of one of the main catabolic mediators in OA MMP-13 in co-culture samples under pro-inflammatory conditions. Passaged chondrocytes (passage 6) divided very slowly and took almost 14 d to become confluent. Stable cells on the other hand were fast growing and normally were passaged every 4 days. The cells were cultured in ratios (chondrocytes/stable cells) of 100/0, 0/100, 80/20, 70/30, and 50/50. However, it was not determined if the cells remained in this ratio throughout cultivation.

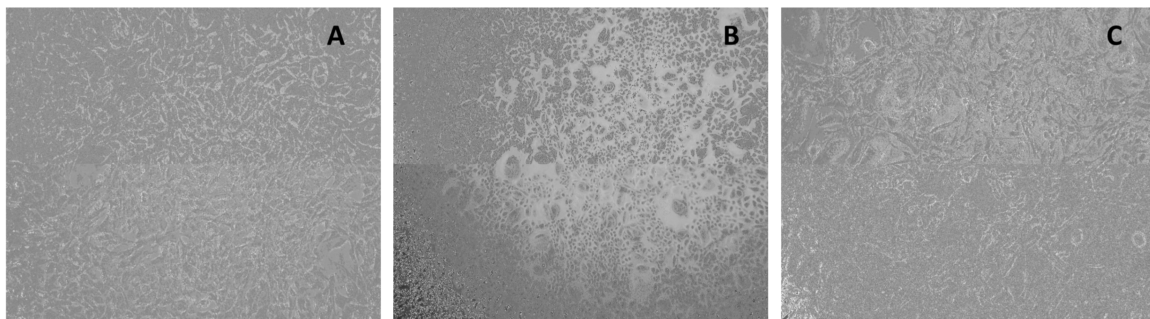


Figure 29: Microscopic depiction of chondrocytes, stable cells and co-culture cells. A) Canine chondrocytes in passage 4. **B)** Stable cells which express IGF-1 and IL-4. **C)** Co-culture cells; chondrocytes and stable cells (80/20). (Magnification: x4)

3.4.6.1 Expression of type II collagen and MMP-13

The mRNA expression levels of canine chondrocytes co-cultivated with stable cells expressing both pVitro-2IGF-1 and pCOX2-IL-4 were analyzed by real-time qRT-PCR. Of interest was whether co-cultured cells could stimulate the expression of type II collagen in combination with passaged chondrocytes while at the same time down-regulating the cartilage degrading enzyme MMP-13. As seen in Fig.30, chondrocytes cultured with stable cells indeed up-regulated the expression of type II collagen and down-regulated the expression of MMP-13 as shown by real-time qRT-PCR. Cells cultured in the ratio 50/50 expressed type II collagen comparable to that in the non-stimulated chondrocyte control

sample. IGF-1 and IL-4 from the stable cells had a positive effect on the expression of type II collagen and were able to regulate the expression of MMP-13.

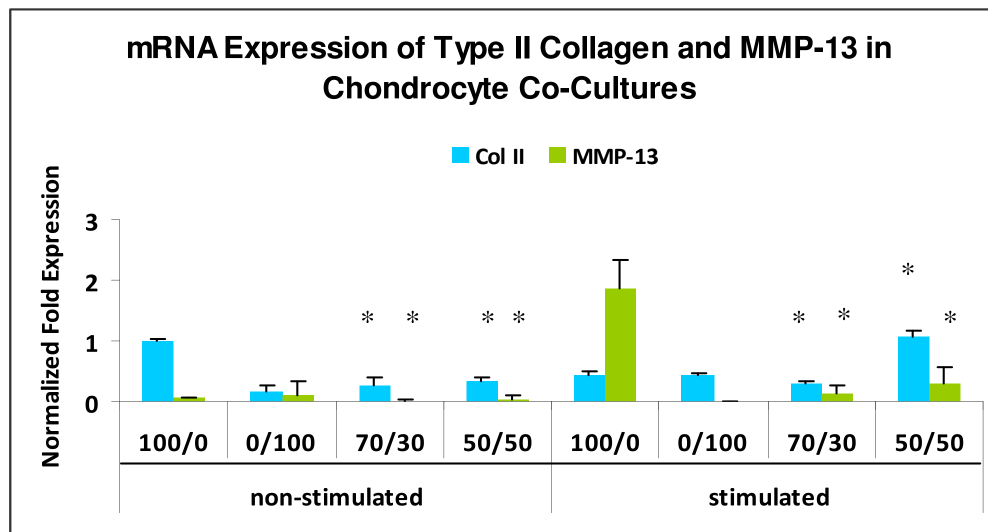


Figure 30: Real-time qRT-PCR of type II collagen and MMP-13 expression in chondrocyte co-cultures. Canine chondrocytes (CAC) in passage 4 were co-cultivated in pellet cultures with stably transfected chondrocytes (stbl), expressing pVitro2-IGF-1/COX-IL-4, in different ratios (primary chondrocytes/stably transfected chondrocytes). The cells were incubated for 72 h in the presence of 100 ng/ml IL-1β and 50 ng/ml TNFα. Thereafter, RNA was isolated, cDNA was synthesized and real-time qRT-PCR was performed. GAPDH served as house-keeping gene. Co-cultivated cells (50/50) showed a down-regulation of MMP-13 and an up-regulation of type II collagen. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to both of the control groups, canine chondrocyte and stably transfected cells ($p<0.05$).

3.4.6.2 Nitrite assay of chondrocyte co-cultures

Nitric Oxide (NO) is up-regulated by IL-1β and TNFα and partakes in the degradative processes of OA. Its expression levels are good indicators of inflammation. One way to measure NO levels is to measure one of its breakdown products, nitrite. IL-4 can down-regulate the expression of NO. This assay, which was performed using the Greiss reagent system, shows the functional activity of IL-4 in co-culture under stimulatory conditions. Fig. 31 shows that non-stimulated stable cells express nitrite at levels comparabe to the stimulated control chondrocytes. However, when IL-β and TNFα are added to the cells, the NO levels decrease. Amongst the co-culture stimulated samples, the 50/50 sample showed the lowest level of NO.

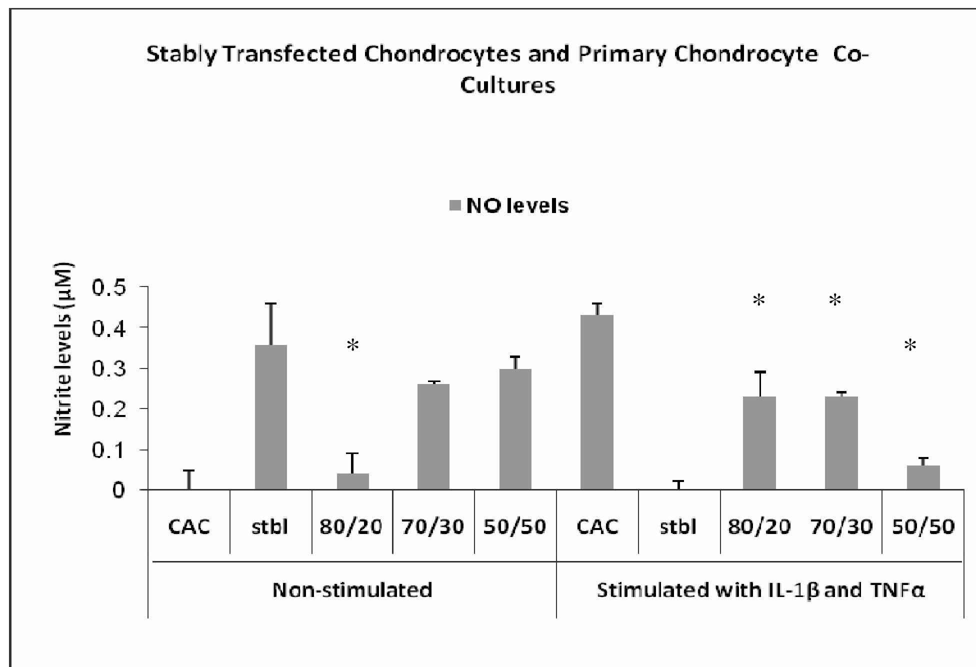


Figure 31: Nitrate assay of chondrocyte co-cultures. Chondrocytes (CAC), stable cells (sbl) and co-cultures were incubated in 1% FCS DMEM for 72 h. Conditioned medium was collected and used for analyses of NO levels in the respective samples. Data represents mean ± SD. Asterisks (*) indicate statistical significance compared to control groups, canine chondrocytes and stably transfected cells ($p<0.05$).

In summary, the co-cultivation of cMSCs and chondrocytes with stably transfected chondrocytes expressing pVitro2-IGF-1 and pCOX2-IL-4 showed positive effects on anabolic mediators. The stably transfected chondrocytes were able to express the therapeutic genes over a number of passages. IGF-1 in combination with IL-4 was able to stimulate the differentiation of the cMSCs denoted by the expression of type II collagen. Also, IGF-1 and IL-4 from the stably transfected cells co-cultured with passaged chondrocytes could down-regulate the expression of MMP-13 and up-regulate the expression of the chondrocyte differentiation marker type II collagen after stimulation with the pro-inflammatory cytokines.

4 Discussion

The current problem associated with the treatment of OA is the inability to reduce pain and inflammation while at the same time increasing the synthesis of extracellular matrix proteins. Available treatments are ineffective for they tend to treat only OA related symptoms for a limited period of time without restoring the degenerated cartilage or reestablishing the balance between the catabolic and anabolic mediators. Under such conditions, the disease worsens and the degeneration of cartilage tissue is enhanced. In extreme cases, partial or total joint replacement is necessary, which results in limited joint movement. A gene-based therapy combining both the effects of chondroregenerative and anti-inflammatory mediators would offer an attractive alternative for the treatment of OA. Taking this as key, the general goal of this study was to analyze the effects of the co-expression of insulin-like growth factor-1 (IGF-1) and the anti-inflammatory cytokine interleukin-4 (IL-4) on canine chondrocytes and mesenchymal stem cells (cMSCs). During the development of this study, IGF-1 and IL-4 were chosen due to their known capability to stimulate the regeneration of extracellular matrix proteins and to down-regulate the expression of pro-inflammatory mediators, respectively, in chondrocytes. Furthermore, the well-researched ability of IGF-1 to stimulate chondrogenesis of cMSCs also rendered IGF-1 a good candidate gene. In order to better understand the advantages of combining the expression of these two genes in an *in vitro* OA model, this research has been divided into three sections. In the first section, IGF-1 and IL-4 were co-expressed in an *in vitro* pro-inflammatory chondrocyte model to analyze their effects on pro-inflammatory mediators and the stimulation of extracellular matrix protein synthesis. In the second section, cMSCs were first characterized, and their ability to undergo chondrogenesis was studied. In order to stimulate chondrogenesis, chondrogenic medium containing TGFβ3 or a non-viral vector with the transgenes IGF-1/IL-4 was used. Lastly, chondrocytes stably expressing IGF-1 from pVitro2 vector and IL-4 from a disease sensitive promoter construct, namely the pCOX-2 vector, were utilized in combination with chondrocytes or cMSCs. Subsequently, catabolic and anabolic mediators were examined following implementation of therapeutic genes in a pro-inflammatory environment.

4.1 Dual expression of anabolic mediators in canine chondrocytes

Despite the progress seen in utilizing single genes to combat OA disease processes, a complete restoration of OA cartilage has not been possible. For this reason, several sources emphasize the importance and necessity of employing multiple genes to combat the different facets of OA (Cucchiarini *et al.*, 2008; Haupt *et al.*, 2005; Loeser *et al.*, 2003). In this research, the ability of a non-viral vector expressing both IGF-1 and IL-4 to stimulate the regeneration of cartilage matrix proteins and to down-regulate pro-inflammatory mediators was studied using an *in vitro* inflammatory model (Rai *et al.*, 2008).

In order to insure that the proteins from the respective therapeutic genes were being produced, the protein expression levels were assayed utilizing immunoassays. Transfection of respective plasmid DNA in the canine chondrocytes resulted in an increased protein expression of IGF-1 and IL-4 in those cells. The IGF-1 immunoassay showed that co-expression of IGF-1/IL-4 caused more IGF-1 production than in cells transfected with IGF-1 alone (Fig. 7). Presence of IL-4 in the cells seems to trigger a signal cascade for IGF-1 production. This is in agreement with a previous observation which showed that the expression of IL-4 enhances IGF-1 production in canine chondrocytes under stimulatory conditions (Rachakonda *et al.*, 2008b). One explaination for this is that IL-4 operates not only in a STAT6-dependent manner (Rachakonda *et al.*, 2008a), but regulation of IL-4-induced proliferative and differentiating responses works by means of insulin receptor substrates (IRS) (Wurster *et al.*, 2002). The major substrates of IGF-1 receptor tyrosine kinases are IRS-1 and IRS-2. Incidentally, IRS-1 and IRS-2 share extensive structural and functional identities and act as critical mediators of not only IGF-1 but also IL-4 mitogenic signaling (Gingras *et al.*, 2000). This implies that IL-4 could indeed exert a positive regulation on the production of IGF-1 due to the similarities in their signal pathways, specifically suiting arthritis.

Further indication of cross-talk between the signaling pathways of IGF-1 and IL-4 was noted by IGF-1 eliciting IL-4 production in the presence of IL-1β and TNFα (Fig.8). This increase in IL-4 protein production was neither seen in control cells with or without stimulation nor in non-stimulated IGF-1 transfected cells. Unexpectedly, the stimulation of the cells with IL-1β and TNFα led to a positive regulation of IL-4 in the canine chondrocytes transfected with IGF-1/IL-4. In stimulated chondrocytes transfected with IGF-1/IL-4, the expression of IL-4 was greater than the levels seen in their non-stimulated counterparts. This once again leads to the conclusion that up-regulation of IL-4 protein occurs as a direct response to the

inflammatory stimulus in cells transfected with the anabolic mediators. On the other hand, no such occurrence could be noted in control cells subjected to similar inflammatory stimulation. Nevertheless, what has to be considered is that both IGF-1 and IL-4 are assumed to act in both an autocrine as well as a paracrine manner. For this reason, it will be necessary to analyze the concentration of each protein not only in the cell lysates but also in the conditioned medium. This could give a better overall view of how the up-regulation of the respective genes affects the cells in culture.

The anabolic effects of IGF-1 are transmitted by the IGFR1, which belongs to the family of receptor tyrosine kinases (Ullrich and Schlessinger, 1990). In OA, the diseased chondrocytes are insensitive to the increase in IGF-1 expression levels, despite an increase in the expression of IGFR1 (Dore *et al.*, 1994). Stimulation with pro-inflammatory cytokines resulted in an increase in the receptor levels compared to the non-stimulated control (Fig. 9). However, the increase in the mRNA expression level of IGFR1 was not due to an increase of IGF-1 expression levels. Transfection of chondrocytes with IGF-1 resulted in a relatively similar mRNA expression level of the receptor compared to the stimulated non-transfected control. This signifies that an increase in the expression level of IGFR1 results from a pro-inflammatory stimulus and not from the up-regulation of IGF-1 mRNA expression. To support this further, the combined usage of IGF-1/IL-4 possibly had an indirect affect on the expression of IGFR1 resulting from the anti-inflammatory effects of IL-4. This is assumed to not be due to a decrease in the expression of IGF-1, because in stimulated samples transfected with IGF-1/IL-4 there is a higher IGF-1 protein level compared to stimulated cells transfected with IGF-1 alone (Fig. 7). It is conceivable that in cases where inflammation is subdued, for example via IL-4, that cells do not find it essential to signalize the up-regulation of IGFR1 expression levels, indicating an IGF-1 independent regulation of IGFR1 expression levels.

In addition, IGF-binding proteins (IGFBPs) have higher affinities for IGF-1 exceeding that of IGFR1, and they possibly play a large role in the suppression of IGF-1 anabolic effects. Because IL-1β and TNFα can trigger the release of IGFBPs (Linkhart and MacCharles, 1992; Matsumoto *et al.*, 1994; Olney *et al.*, 1995), it becomes necessary to co-express IGF-1 with an anti-inflammatory cytokine like IL-4 in order to normalize the expression of IL-1β and TNFα and subsequently the expression of the IGFBPs. As seen in this study, stimulation of chondrocytes with the above mentioned pro-inflammatory cytokines resulted in an increase

in the mRNA expression of IGFBP-2,-3, -5 and -6 (Fig. 9), which have been indicated in arthritis conditions (Olney *et al.*, 1996; Chevalier and Tyler, 1996; Porter *et al.*, 2006). This is also similar with studies showing an increase in IGFBP mRNA expression in chondrocyte monolayer cultures from OA cartilage compared with their normal counterparts (Dore *et al.*, 1994; Tardif *et al.*, 1996). In comparison to the stimulated control, the transfection of chondrocytes with IGF-1 and the stimulation with IL-1β and TNFα caused a further increase in the expression of IGFBP-3, -4, -5, and -6, which is also similar to a previous study (Olney *et al.*, 1993). However, the synergistic effects of both IGF-1 and IL-4 resulted in a decrease in the mRNA expression levels of all IGFBPs except IGFBP1, suggesting that co-expression of IGF-1 with IL-4 results in regulation of the expression levels of binding proteins to levels comparable to the non-stimulated control cells. Nevertheless, the role of IGFBP1 and IGFBP4 in OA is unknown. In this study, IGFBP4 expression levels were quite unaffected by the gene transfer of IGF-1 and IGF-1/IL-4 under inflammatory conditions, and IGFBP1 levels were down-regulated in all stimulated samples compared to the non-stimulated control. These two binding proteins possibly partake in OA-independent processes or they may function solely as carriers rather than inhibitors of IGF-1 in cartilage tissue.

The ability of IGF-1 to stimulate proteoglycan aggrecan and type II collagen expression under arthritis conditions is controversial. Several studies have shown that IGF-1 alone can stimulate the expression of cartilage extracellular matrix proteins (Madry *et al.*, 2001; McQuillan *et al.*, 1986; Tyler, 1989). However, one study showed that transduction of chondrocytes from horses with IGF-1 alone followed by incubation with IL-1β for 6 days was unable to stimulate the expression of proteoglycan aggrecan and type II collagen. Yet, in combination with IL-1 receptor antagonist the effects of IGF-1 were intensified and extracellular matrix proteins were expressed (Haupt *et al.*, 2005). Similarly, another study concluded that exogenous addition of IGF-1 protein to murine cells under inflammatory conditions resulted in desensitization to IGF-1 possibly due to an up-regulation of IGFBPs (Verschure *et al.*, 1995; Schalkwijk *et al.*, 1989). Nevertheless, in this study, canine chondrocytes transfected with both IGF-1 and IGF-1/IL-4 resulted in an increase in the mRNA expression of aggrecan, transcriptional factor SOX9, and type II collagen when compared to the stimulated non-transfected control (Fig. 14). It was observed here that an up-regulation of IGF-1 and IGF-1/IL-4 resulted in significantly higher mRNA expression levels of aggrecan and type II collagen compared with the stimulated control. However, stimulated cells

transfected with both therapeutic genes showed a higher mRNA expression level of aggrecan and type II collagen in relation to IGF-1 transfected cells under the same conditions. Furthermore on the protein level, the regeneration of proteoglycan aggrecan and type II collagen was seen in IGF-1 and IGF-1/IL-4 transfected cells under stimulatory conditions for 48 and 96 h, respectively (Fig. 15 and 16). Alcian blue staining of the chondrocytes, is a simple method of measuring proteoglycan content of in the cells. In order to get a better overview of the effects of IGF-1 and IGF-1/IL-4 on the release of proteoglycans in an inflammatory model, quantification using S-35 incorporation will be necessary. Despite this, in canine chondrocytes, both IGF-1 and IGF-1/IL-4 genetically modified cells were shown to stimulate the synthesis of the extracellular matrix protein, type II collagen, which was shown by immunocytochemistry. Due to the lack of quantification of the staining intensities of the cells in Fig. 16, it is not possible to say if the expression of type II collagen was affected more positively by the combined expression of IGF-1 and IL-4. But what can be assumed is that both IGF-1 and IGF-1/IL-4 lead to the up-regulation of cartilage extracellular matrix proteins. Differences in the literature concerning the effects of IGF-1 alone denote possible dissimilarities in cell types, diversity between organisms, and incubation times of therapeutic genes with of the cells.

Additionally, the anti-inflammatory effects of IL-4 were demonstrated in the cells transfected with IGF-1/IL-4. In those samples, the mRNA expression of the pro-inflammatory cytokines IL-1β, TNFα and IL-6 were down-regulated to levels comparable to the non-transfected, non-stimulated control (Fig. 10). Cells transfected with IGF-1 alone showed also a decrease in the mRNA expression of IL-6 and minimal reduction in that of IL-1β compared to the stimulated control. However, in those same samples an increase in the expression level of TNFα was observed, a trend which was reversed in cells co-expressing IGF-1 and IL-4. The effect of IGF-1 on TNFα was also observed in another study (Renier *et al.*, 1996). Even though IGF-1 alone was shown here to stimulate the regeneration of cartilage matrix proteins, the need for the cumulative expression with an anti-inflammatory mediator is evident by the inability of IGF-1 to effectively regulate the expression of certain catabolic mediators.

Moreover, both IGF-1 and IGF-1/IL-4 can down-regulate the expression of the catabolic mediators MMP-1, -3 and -13, important mediators in the degradation of cartilage (Fig. 11). This is in accord with previous experiments showing the down-regulation of these enzymes

by IL-4 (Rachakonda *et al.*, 2008a; Rachakonda *et al.*, 2008b; van Lent *et al.*, 2002; van Roon *et al.*, 2001). Comparable to the affects of IL-4 on MMPs, IGF-1 also possesses the ability to repress the enzymatic activity of collagenases (Hui *et al.*, 2001; Zhang *et al.*, 2008; Im *et al.*, 2003). The capability of IGF-1 alone to down-regulate the mRNA expression of MMP-1, -3, and -13, proposes one mechanism of action as to how IGF-1 partakes in protecting cartilage from catabolic mediators such as matrix degradative enzymes. Nonetheless, the mRNA expression levels of the enzymes were lower in cells transfected with IGF-1/IL-4 compared to the stimulated control.

The inability of IGF-1 alone to subdue certain inflammatory mediators was further reflected in its disability to suppress NO levels compared to IGF-1/IL-4 transfected cells. Nitrite, being one of the catabolites of the inflammation cascade whose levels are increased via the iNOS pathway, provides a good measure of inflammation within the cell (Amin *et al.*, 1995; Pelletier *et al.*, 1998; Sakurai *et al.*, 1995). The combined expression of IGF-1/IL-4 was needed in order to reduce iNOS mRNA expression levels to a level comparable to the non-stimulated control (Fig. 13). Significant reduction of NO levels was seen also only with IGF-1/IL-4 transfected cells (Fig. 12). These results further highlight the necessity of combined expression of the therapeutic genes rather than usage of IGF-1 alone.

Overall, co-expression of IGF-1/IL-4 by means of a non-viral vector in chondrocytes was effective in hampering the destructive effects induced by IL-1β and TNFα. This was demonstrated by the down-regulation of catabolic mediators and IGFBPs as well as by the up-regulation of matrix proteins, type II collagen and aggrecan. Even though IGF-1 alone was shown to be capable of stimulating the synthesis of extracellular matrix proteins, it was the combined expression of IGF-1/IL-4 demonstrated better regenerative as well as anti-inflammatory potential. Nonetheless, the risks of continuous over-expression of IGF-1 and IL-4 will have to be studied and shown if this could have an effect on the therapeutic potential of the genes. Also, at the beginning of this study, it was believed that IL-4 only behaved as an anti-inflammatory cytokine. It is now known that IL-4 has also regenerative potential (unpublished data). For this reason it will be necessary to further elucidate the effects of IL-4 alone on the regeneration of cartilage extracellular matrix proteins in order to conclude whether the combined expression of IGF-1/IL-4 or possibly the single expression of IL-4 would be more advantageous in cartilage repair.

4.2 Characterization and co-expression of anabolic mediators in cMSCs

One of the main problems associated with autologous cell transplantation is obtaining a sufficient number of differentiated cells. In addition to autologous chondrocytes, MSCs have been suggested as an alternative cell source for cartilage repair due to their ability to differentiate into a number of cell lineages including chondrocytes (Caplan, 1991; Johnstone *et al.*, 1998; Trippel *et al.*, 2004). MSCs are ideal for regenerative therapy for they are isolated relatively easy from various tissues, readily expanded in culture, and differentiate under suitable stimulation.

Although MSCs were originally derived solely from bone marrow (Friedenstein *et al.*, 1966; Pittenger *et al.*, 1999), it is now known that these cells can be retrieved from tissues such as the dermis, synovial membrane, periosteum, adipose (Mimeault and Batra, 2008; Chen *et al.*, 2006; Bianco *et al.*, 2008). Yet, bone marrow derived MSCs are still preferred for the stimulation of chondrogenesis (Bernardo *et al.*, 2007; Koerner *et al.*, 2006). Despite the vast amount of research performed on MSCs there is no uniformly accepted single surface marker that can be used to identify isolated cells. For this reason, certain *in vitro* criteria such as plastic adherence, the expression of surface specific antigens, the absence of hematopoietic stem cell markers, and their differentiation ability have been accepted to prove stem cell identity. In this study, canine MSCs (cMSCs) were isolated from bone marrow using a Percoll gradient. The cells which were isolated consisted initially of a heterogenous population, where the cMSCs adhered to the cell culture dishes and formed colonies after approximately 7 days in culture (Fig. 17). Only a small percentage of the isolated cells of the mononuclear fraction were cMSCs, which is similar to that of other species (Martin *et al.*, 2002; Pittenger *et al.*, 1999). Thereafter, non-adherent cells where removed with a change of medium and the remaining confluent colonies were trypsinized. Those cells grew in confluent monolayers with fibroblastic-like morphology, which is typical for MSCs.

Based on cell-surface antigen profile, cMSCs are similar to rodent and human MSCs (Kamishina *et al.*, 2006). As shown by flow cytometry analyses, cMSCs are positive for CD29, CD90, CD44, and negative for CD45 and CD34 (Fig. 18). This is comparable to another study utilizing cMSCs (Csaki *et al.*, 2007a). These surface markers were chosen as an initial attempt to characterize the cMSCs.

Another defining feature of MSCs is their ability to differentiate when supplied with certain external stimuli. On the mRNA and protein level, non-differentiated cMSCs were devoid of

type II collagen expression and were positive for type I collagen (Fig. 20 and 22). This is typical for non-differentiated MSCs for they are absent of this particular cartilage marker (Bradham *et al.*, 1995; Johnstone *et al.*, 1998). It is only following stimulation of chondrogenesis that the expression levels of chondrocyte marker genes are seen.

The capability of MSCs to undergo chondrogenesis underlines their potential in regenerative therapy. Stimulation of differentiation is possible through the usage of a stimulating medium containing growth factors such as BMPs, TGFβ and IGF-1 (Johnstone *et al.*, 1998; Lennon *et al.*, 1995; Kaps *et al.*, 2002; Reddi, 1994). TGFβ3 was used to characterize the chondrogenic potenetial of cMSCs, for it has been shown to induce a more rapid and representative expression of chondrogenic markers (Barry *et al.*, 2001; Mackay *et al.*, 1998). *In vitro* chondrogenic differentiation of MSCs is normally performed in matrices such as type I collagen or alginate beads or in pellet 3D-cultures (see section 1.5.4). In alginate beads, cMSCs were stimulated with chondrogenic medium, and following 1 week of stimulation with TGFβ3, an increase in the expression levels of type II collagen and aggrecan was observed (Fig. 21). This is similar to previous studies, which showed that the expression of cartilage extracellular matrix proteins is present following stimulation with TGFβ (Park and Na, 2008; Johnstone *et al.*, 1998; Yoo *et al.*, 1998). In the 3rd week following stimulation, the highest expression levels of type II collagen and aggrecan were observed, denoting the increasing capacity of cMSCs to undergo chondrogenesis for extended time periods. Type II collagen expression levels were also seen on the protein level (Fig. 23 and 24) further denoting the ability of cMSCs to undergo chondrogenesis.

Type X collagen is a marker of advanced chondrocytic hypertrophy. *In vitro* studies of MSC-chondrogenesis have shown the expression of type X collagen as early as 3 days following stimulation with TGFβ alone or in combination with IGF-1 (Steinert *et al.*, 2008). In cMSCs signs of hypertrophy were noticeable first in the 2nd week of cultivating (Fig. 20). In order to develop functional hyaline articular cartilage, chondrogenic differentiation will have to be controlled before the stem cells progress to this advance stage of hypertrophy. Further analyses of the interaction between TGFβ3 and cMSCs will have to be performed in order to evaluate the potential of this growth factor for future tissue regeneration techniques.

Another method to induce chondrogenesis in MSCs is by means of genetic modulation. This can be achieved by transfecting the stem cells with DNA constructs encoding certain growth factors that induce chondrogenesis. It is hypothesized that the transfection of MSCs with

IGF-1/IL-4 would be advantageous, because cells would be produced with a dual function, namely to differentiate MSCs and to exert anabolic functions of the therapeutic genes in OA inflicted defects. Viral vectors are able to provide high transduction efficiency, integration in the host genome and high level of gene expression (Blesch, 2004). Transduction efficiencies can reach up to 90% (Marx *et al.,* 1999; Stender *et al.,* 2007; Bosch *et al.,* 2006) Despite this, viral approaches are complicated by immune response, intracellular trafficking, potential mutations, and genetic alterations due to integration (Hacein-Bey-Abina *et al.*, 2003; Li *et al.*, 2002). Although non-viral vectors are not associated with such problems, obtaining high transfection efficiencies in stem cells is challenging. The transfection of cMSCs with IGF-1/IL-4 utilizing FuGENE6 resulted in a transfection efficiency of 24.5% (Fig. 25), showing that the use of lipid-based transfection agents is capable of moderate transfer of plasmid DNA into MSCs.

The potential of IGF-1 to stimulate chondrogenesis in MSCs is poorly understood. Several studies show that IGF-1 alone is unable to stimulate chondrogenesis and that only in combination with TGFβ is the growth factor effective (Fukumoto *et al.*, 2003; Sakimura *et al.*, 2006; Worster *et al.*, 2001). Another study found that IGF-1 is equally potent as TGFβ in stimulating chondrogenesis (Longobardi *et al.*, 2006). In this study, cMSCs were transfected with IGF-1/IL-4 in order to stimulate chondrogenesis. Similar to TGFβ3, the transfection of cMSCs with IGF-1/IL-4 induced the expression of type II collagen and aggrecan (Fig. 26). Even though IGF-1 enhanced the chondrogenic potential of cMSCs, chondrogenesis was more pronounced when cMSCs were stimulated with TGFβ3. This could be a direct result of the moderate transfection efficiency of the cMSCs or imply the need for additional stimulation by TGFβ3. Transfection by nucleofection yielded transfection efficiencies between 40 and 94% (Aluigi *et al.*, 2006). Possibly with an increased gene transfer rate, the effects of IGF-1/IL-4 on chondrogenesis could be comparable to that of chondrogenic medium supplemented with TGFβ3. However, further optimization of the transfection of cMSCs with DNA plasmid vectors will be necessary to assess its feasibility.

4.3 Co-cultivation of stably transfected chondrocytes

Low transfection efficiencies and short-term gene expression are characteristics of non-viral gene transfer. This may be advantageous in clinical settings where gene expression is only needed for short periods, like in wound healing or bone regeneration (Bleiziffer *et al.*, 2007). However, in cases where extended gene expression is desired, selection of a cell line stably

expressing a gene or a combination of genes over a long period of time offers an alternative to viral vectors.

Co-cultivation of cells has been shown to provide a means of cell-cell interaction, which plays an important role in tissue development and influences cell proliferation, differentiation and physiology (Bhatia *et al.*, 1997; Gerstenfeld *et al.*, 2003). Previous experiments have analyzed the effects of co-cultivating MSCs with different types of cells like differentiated chondrocytes (Chen *et al.,* 2009) or osteoblasts (Csaki *et al.,* 2009b) with the purpose of analyzing the synergistic interaction between the two different cell types without the addition of growth factors. This study assays the use of stably transfected chondrocytes in combination with MSCs or passaged chondrocytes. It was hypothesized that stably transfected chondrocytes co-cultivated with passaged chondrocytes or MSCs could mimick the *in vivo* affect of transplanting stable cells in a cartilage defect where dedifferentiated cells are present. This procedure could present a possibility to increase the number of cells available for therapies such as autologous cell transplantation (ACT) as well as to enhance tissue formation of a chondrogenic lineage. In addition, cells which stably express therapeutic genes may increase the half-life of bioactive factors and reduce their loss of concentration in the joints.

First, co-cultivation of chondrocytes stably transfected with pVitro2-IGF-1 and pCOX-2-IL-4 was tested. New to this study was the usage of pCOX-2-IL-4. This is a construct with a disease sensitive promoter, namely the COX-2 promoter, which leads to the expression of IL-4 under pro-inflammatory conditions (Rachakonda *et al.*, 2008b). This is an attractive alternative in OA gene therapy, because extended over-expression of IL-4 could possibly promote this cytokine to shift from being an anti-inflammatory cytokine to promoting the synthesis of pro-inflammatory cytokines (Van *et al.*, 2005). For this reason, the disease-sensitive promoter was generated to allow for the controlled expression of IL-4 only when needed, where IGF-1, on the other hand, is continually expressed. Chondrocytes, which stably express IL-4 under the COX-2 promoter, were super-transfected with pVitro2-IGF-1. Before use, the stably transfected chondrocytes where assayed per real-time RT-PCR to observe if the two genes (IGF-1 and IL-4) were expressed (Fig. 27). Characteristic for these cells was that they showed a spherical morphology, which is similar to that of freshly isolated primary chondrocytes contrasting to the fibroblastic morphology of dedifferentiated chondrocytes (Fig. 29). However, in culture these cells did not express type II collagen and showed an

increase in the expression of NO compared to the non-stimulated control (Fig. 30 & 31), which is more characteristic of dedifferentiated chondrocytes. Yet, these stably transfected cells cultivated with and without passaged chondrocytes under stimulatory conditions showed an increase in the expression of type II collagen and a decrease in the expression of pro-inflammatory mediator, MMP-13 (Fig. 30). The expression of type II collagen was most profoundly increased in samples cultured with 50% stable cells and 50% passaged chondrocytes. There the expression level reached and mildly surpassed the levels of the non-stimulated chondrocytes. Unfortunately, the co-culture samples were not able to increase the type II collagen expression profoundly but only to levels relative to the non-stimulated passaged chondrocytes. Possibly the use of chondrocytes in passages 0–4 in combination with the stably transfected cells would yield more promising results. Despite this discrepancy, the ability of the co-culture samples to stimulate the expression of type II collagen in passaged cells in the presence of IL-1β and TNFα was effective (Fig. 30).

Moreover, noticeable for the non-stimulated stable cells and the co-cultured cells is the increased level of NO, which is, however, apparently not enough to switch on the COX-2 promoter. However, these increased levels could be due to stress induced responses via IL-1β independent mechanisms. Expression of IL-4 from the promoter construct was only activated when IL-1β and TNFα were present in the culture medium, resulting in a decrease in the levels of NO in co-cultures compared to the controls (Fig. 31). In addition, the expression of MMP-13, one of the main enzymes which degrade type II collagen, was inhibited in stimulated cells co-cultured with the stably transfected cells (Fig.30). This underlies the ability of the cells to down-regulate additional catabolic mediators in OA.

Furthermore, the chondroinductive ability of stably transfected chondrocytes was analyzed via co-culture with cMSCs. Co-cultivation of MSCs with chondrocytes has been shown to up-regulate the cartilaginous phenotype of bovine articular chondrocytes (Tsuchiya *et al.*, 2009). In this study, the ability of the chondrocytes stably transfected with pVitro2-IGF-1 and pCOX-2-IL-4 to stimulate the expression of the cartilage marker type II collagen was shown to be greater than that of cMSCs stimulated with TGFβ3 after 48 h of cultivation (Fig. 28a). Thereafter, the ability of the stable cells to stimulate the expression of type II collagen remained constant while that of the cMSCs stimulated with TGFβ3 increased beyond their levels (Fig. 28b). Although it is not known if the ratio of stably transfected chondrocytes and chondrocytes remained the same throughout the incubation time periods, the cells were

placed in pellet cultures in order to at least control the initial number of cells per pellet. Even so, the stably transfected cells are fast growing cells. Therefore, it can not be assumed that the cells remain in the initial ratios. It is possible that the stably transfected cells outnumber the cMSCs, which could possibly affect the expression of cartilage specific proteins. Even though the ability of stable cells to stimulate chondrogenesis *in vitro* seems promising, additional studies will have to be performed to further decode their practicality in OA therapy.

4.4 Future Outlook

Treatment of cartilage loss will require a restoration of the balance between catabolic and anabolic mediators. One effective treatment option that can be used to treat non-OA cartilage defects is autologous cell transplantation (ACT). A major problem associated with the use of ACT in OA is that cells would be replaced in a catabolic environment that would counteract any regenerative activity of the transplants. There is little to no chance for damaged or diseased adult cartilage to heal. Current drugs address the pain associated with OA, but there is still no disease modifying agent available. For this reason gene therapy in combination with ACT (autologous conditioned cell transplantation—ACCT) offers an alternative avenue that would introduce anabolic mediators which could possibly regulate the catabolic activity present in the diseased joint. Autologous chondrocytes or MSCs, the two prime candidate cell types for genetic modification, would be placed within a matrix and then be used to fill partial cartilage defects. In most cases, the use of a single gene is not sufficient to counteract the catabolic mediators (Haupt *et al.*, 2005; Loeser *et al.*, 2003; Cucchiarini *et al.*, 2008). This is why the use of multiple genes has been suggested in order to tackle the pathological processes in OA cartilage. There are a number of genes which have been suggested to be used in a combination therapy to control the degradation and to allow for regeneration of cartilage loss. In this study, the co-expression of IGF-1 and IL-4 led to an up-regulation of cartilage matrix proteins and a down-regulation of IL-1β, TNFα, IL-6, MMPs, and NO. *In vivo* testing of the ability of the combined genes to combat the symptoms associated with OA will have to be performed in order to clarify the effectivity of this therapy approach.

Nonetheless, one disadvantage associated with genetic modulation of cells is the risk associated with using recombinant DNA technology in clinical therapy. Constitutive overexpression of any gene transfected in stem cells or chondrocytes could have unpredictable physiological effects *in vivo*. One solution to this problem could be placing recombinant expression of a particular gene under the control of a disease-sensitive promoter. Co-expression of anabolic mediators under a promoter devoid of viral sequences which would be switched on when needed would be the ideal therapy of the future.

Lastly, a vast amount of information has been gained from *in vitro* experimentation and animal model studies relating to the degenerative and inflammatory processes of OA. Difficulty has been found in defining single events within the regulatory pathways. More

concentration will have to be placed on singling out particular events in the signal cascades in order to define early biomarkers, which could be used in detecting the disease in its early phases. Alternatively, in later stages of the disease process it would be necessary to further develop and explore the option ACCT to implement the gene transfer of anabolic mediators in autologous chondrocytes or MSCs to regenerate cartilage tissue and alleviate the pain associated with the OA.

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Curriculum Vitae

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