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Reviewing Challenges in Osteoarthritis Gene Therapy and Introduction of a Molecular Therapeutic Approach in an Equine Model System

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Meiner Familie -

insbesondere meinen Eltern, meiner Schwester

und meinem Großvater Joachim

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AAV	Adeno-associated virus
ACLT	Anterior cruciate ligament transection
ACT	Autologous chondrocyte transplantation
ACR	American College of Rheumatology
ADAMTS	A disintegrin and metalloproteinase with a thrombospondin motif
ANOVA	Analysis of variance
AP-1	Activating protein 1
BLAST	Local alignment and search tool
BMP	Bone morphogenic protein
bp	base pairs
BSA	Bovine serum albumin
CAIS	Cartilage autograft implantation system
CAIA	Collagen-antibody induced arthritis
CD	Cluster of differentiation
cDNA	Complementary DNA
C/EBP	CAAT-Box/Enhancer binding protein element
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CINOD	Cyclooxygenase-inhibiting nitric oxide donor
CMV	Cytomegalovirus
Col I/II	Collagen Type I/II
COMP	Cartilage oligomeric matrix protein
Cox-2	Cyclooxygenase-2
CRE	Camp response elements
Ct	Cycle threshold
CT	Computed tomography
CTX-II	C-terminal telopeptide of collagen type II
DLT	Dose-limiting toxicity
DMEM	Dulbecco’s modified Eagle medium
DMOAD	Disease-modifying osteoarthritis drugs
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease dNTP 2´-deoxynucleoside 5´-triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EULAR	European League against Rheumatism
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FRZB	Frizzled-related protein
GAPDH	Glycerinaldehyde 3-phosphate dehydrogenase
GDF5	Growth differentiation factor
GFP	Green-fluorescent protein
HA	Hyaluronic acid
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunglobulin
IGF	Insulin-growth factor
IL	Interleukin
IL-1Ra	IL-1 Receptor antagonist
iNOS	Inducible nitric oxid synthase
IRES	Internal ribisomal entry site
i.v.	intravenous
LacZ	cDNA encoding β-galaktosidase from *E.coli*
LB	Luria-Bertani medium
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
mAB	Monoclonal antibody
MACT	Matrix-associated chondrocyte transplantation
MHC	Major histocompatibility complex
MIP-1γ	Macrophage inflammatory protein
MMLV	Moloney murine leukemia oncoretrovirus
MMP	Matrix-metalloproteinase

MRT	Magnetic resonance tomography
mRNA	Messenger RNA
MSC	Mesenchymal stromal cell
MTD	Maximum tolerated dose
NCBI	National center for Biotechnology Information
neo	Neomycin phosphotransferase
NF-1	Nuclear factor 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxid
NSAID	Non-steroidal antiinflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OC	Osteochondrosis
OD	Optical density
P	Passage
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGA	Polylactic acid
PGE	Protaglandine
PGIA	Proteoglycan-induced arthritis
p.os	per os
PPAR	Peroxisome proliferators-activated receptors
PPRE	Peroxisome proliferator response element
qPCR	qualitative PCR
qRT-PCR	qualitative Real-Time PCR
RA	Rheumatoid arthritis
req	recombinant equine
rhu	recombinant human
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
s.c.	subcutaneous
SD	Standard deviation

SDHA	Succinate dehydrogenase complex
SDS- Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SNP	Single-nucleotide polymorphism
Sox-9	Sex determining region Y-box 9
SRF	Serum response factor
TFB	Standard transformation buffer
TGF	Tissue-growth factor
Th-cell	T-helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor
TRAP	TNF-related activation protein
UV	Ultraviolet
VCAM	Vascular cell adhesion protein
WHO	World Health Organisation

CHAPTER 1: General Introduction

Hyaline cartilage is one of the most fascinating and unique complex structures that our body provides in the form of tissue. The horizontal superficial cell layer (10-20%) passes into the tangential and vertical zone (middle zone 40-60%) which ends in chondrocyte lacunas (deep zone) and crossovers to the subchondral bone (*Figure 1*). This unique structure facilitates the optimal impact absorption during every step we take or movement we do. Cartilage in the joints allows us to walk for a long time, to jump, and just to be flexible and agile.

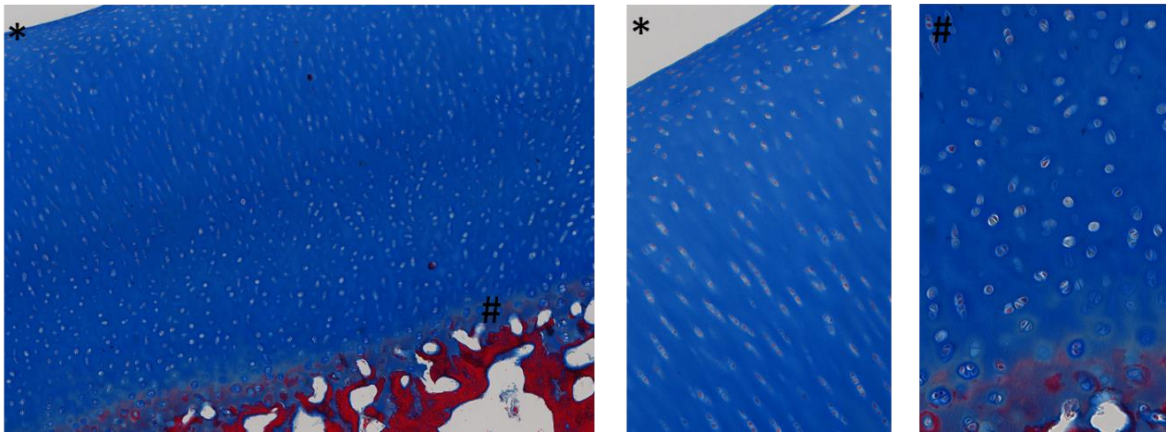

Figure 1: Azan blue stained equine cartilage of the fetlock joint.
*Survey on hyaline cartilage and its crossover to the subchondral bone. *The superficial layer passes over to the tangential layer where chondrocytes show the classic arcades building. #The cross-over to the subchondral bone is characterized by chondrocyte lacunas. Slice was kindly provided by Gabriela Korus (Julius-Wolff Institute, Charité-Universitätsmedizin Berlin).*

The importance of hyaline cartilage becomes clear when cartilage is destroyed such as in osteoarthritis (OA). By 2020, OA will be the fourth leading cause of the world´s most common disabilities induced by increasing life expectancy and an aging population [1]. Cartilage destruction is accompanied by a tremendous loss of life quality due to deprivation of agility and flexibility, morning stiffness, and pain during every step. The progress of OA is slow and takes several years. That is why mainly elderly people are affected. Over many years, it was accepted that OA is only a degenerative disease characterized by biomechanically caused cartilage attrition and abrasion. This assumption was disapproved during the last years and a heated debate started between clinicians and researchers. The central question was, whether OA is inflammation-driven or not. Researchers showed the complex interplay between cartilage degradation and increased inflammatory catabolic mediators such as cytokines, matrix-degrading enzymes or bioenergetic- relevant proteins. However, classic symptoms for inflammation (heat, flush, swelling) can not be observed. In the meantime, it is accepted that the pathogenesis of OA is characterized by a cartilage degradation process

which is driven by inflammatory processes on the molecular level. Additionally, there are presently assumptions that the inflammatory development of OA can originate from the synovial membrane (classic starting point), the cartilage or even the subchondral bone.

The main problem is that hyaline cartilage is not able to regenerate. Comparable to the scar formation, primitive fibrocartilagenous tissue takes unattainable functions. To the date, there is no researcher who accomplished to rebuild hyaline cartilage *in vitro* or *in vivo*. Moreover, many approaches have been tested to properly treat OA, but beside pain medication and protective substances for the remaining cartilage, there is no opportunity for the prospect of cure. In particular, the complete joint replacement is the last expedient. New tissue engineering approaches are e.g. autologous cartilage transplantation (ACT; only indicated for young patients) or collagen scaffold transplantation to restore cartilage tissue. In contrast, pharmaceutical developments mainly focus on inhibition of inflammation and pain blockade. Based on the grown and more detailed knowledge on signaling pathways which are involved in OA, specialized inhibitors or natural inhibiting cytokines can be generated and investigated.

At the beginning of the 1990s, gene therapy was introduced to treat rheumatic disorders of the joint. The aim was to develop a local therapy which has an anti-inflammatory or regenerative effect and only concerns the affected joint. Gene therapy is defined as an introduction of genetic information encoding biological agents with therapeutic potential into the host tissue. The vision is to combine gene therapy with ACT to inhibit the inflammation and to restore the cartilage in erosion areas, respectively. Nevertheless, big challenges of gene therapy are safe gene delivery strategies, the uncontrolled and mainly continuous expression of the therapeutic gene after transduction in the target cell, as well as the choice of the effective transgene. Unfortunate setbacks such as the death of Jesse Gelsinger during a trial in 1999 [2] or the first death of a patient during an arthritis gene therapy trial [3] delayed the advancement of the gene therapeutic approaches and raised much wariness in the population and society. During recent years, prejudices could be reduced by the development of higher safety standards and thus gene therapy can be considered suitable as future-oriented and promising approach for effective OA treatment.

For further developments, especially the question of the right animal model is of great importance. Due to the progress in genetic modification strategies to produce knock-out mouse lines and therefore new disease models, the number of animals used in experiments exploded. Between 1995 and 2012 the number was doubled from less than 1.5 million to more than 3 million animals in Germany [4]. Compared to the few breakthroughs in the clinical research as well as the moderate number of new pharmaceuticals which are introduced to the market every year, the number of used animals is not justifiable. The mouse

as animal model for human health research is probably suitable for basic research questions on signaling pathways but in case of translational approaches even larger models should be considered to increase the translation rate. In OA research, large animal models such as dogs and horses are naturally occurring models and offer the possibility to test the approach after the experimental phase in the animal patient before translating it to humans. This new way of thinking is also applied in zoonosis research and is called “ONE HEALTH” [5, 6]. Poole et al. stated the sentence: *”[…] we should always remember that animals are stakeholders and not just research tools”* [7].

The aim of this thesis is to transfer and validate a new inflammation-controlled and self-limiting gene therapeutic approach in an *in vitro* equine cell culture model. The strategy and results of the experimental work are described in detail in Chapter 4 and 5 followed by the discussion in Chapter 6.

During the work on this project, several questions arose in discussion and conversations with other researchers. The first question is on the feasibility and safety of OA gene therapy and the second question focuses on the possible risk and toxicity of Interleukin-4 (IL-4) used as effective transgene. In addition, the third question includes the applicability of large animal models in OA research – Does size matter? Thus, in Chapter 2, each question is addressed in detail, and therefore, comprising a substantial part of this thesis. Moreover, the risk and toxicity analysis on IL-4 is also discussed particularly at the end of the thesis.

The detailed aims and objectives including the detailed work packages of this thesis are explained in Chapter 3.

Parts of this thesis were published in Lang et al. 2014 [8].

CHAPTER 2: Literature Review

2.1 Osteoarthritis

2.1.1 Prevalence and Risk Factors

Osteoarthritis (OA), a degenerative joint disease, ranks among the most common musculoskeletal disorders around the globe including rheumatoid arthritis, osteoporosis, back pain and musculoskeletal trauma accounting for considerable disabilities and a financial burden on society. According to the world health organization (WHO), 9.6% of men and 18% of women aged 60 or older suffer from OA worldwide [1].

In 2010 around 27.1% of women and 17.9% of men suffered from OA in Germany [9]. Additionally, OA is listed as the third (Coxarthrosis) and fourth (Gonarthrosis) (11,5% of all recognized diagnosis) leading diagnosis of men in prevention and rehabilitation facilities in Germany in 2012 [10]. OA caused 7.1 billion Euro of direct costs with upward tendency according to the increasing patients number in 2006 [11]. In Europe hip and knee replacement have increased during the past years across the countries whereby Germany has the highest rate with 295 (per 100,000 population) surgeries and 213 (per 100,000 population) in 2010, respectively [12]. Moreover, in other European countries the number of these interventions increases apparently. The estimated costs for a knee and hip replacement were 6.300 and 7,600€ in 2009, respectively. In the U.S. OA was the fourth leading cause for hospitalization in 2009 leading to around 905,000 knee and hip replacements at a cost of approximately $42.3 billion [13].

Also in veterinary medicine, OA is becoming more and more common among dogs and horses [14]. Approximately 20% of the canine population aged 1 or older suffers from OA and 33.7% of treated horses in the clinics were affected by joint disease and caused high costs for the owner [15]. In race horses OA is one of the leading cause for lameness and therefore mainly responsible for lost training days [16].

OA is believed to originate from a variety of factors such as age, obesity, gender, genetics, anatomical incongruences and trauma. Meanwhile it is a fact that the prevalence of OA rises with age as a lot of studies have shown during the last decade, but the underlying mechanisms are still unclear [17]. The age-related phenotype switch of chondrocytes as well as the decreasing metabolic activity and the declining sensitivity to growth factors are supposed to play a key role beside the matrix changes (degradation of aggrecans and hydration) and different pathways such as the WNT-, Transforming growth factor (TGF)-β- and Reactive oxygen species (ROS)-signaling that are involved [18-20].

Obesity increases the risk of knee OA due to different mechanisms such as the change in mechanical loading and the loss of muscle activity [21, 22]. A higher BMI leads to an increased risk of OA [23]. Interestingly, the prevalence of hand OA increases too, leading to the hypothesis that other factors such as adipokines further the progression of OA [24].

The higher prevalence of OA in women is controversially discussed. A few studies indicate that sex is a potential risk factor by reporting a reduced risk of knee and hand OA in males aged 55 or younger as well as a higher risk of females aged 55 or older for knee OA [25, 26]. In contrast, several other studies falsified the hypothesis showing no relationship between OA and sex [27, 28].

Genetic epidemiology has been arisen during the last decades impelled by the development of new techniques for genotyping and single-nucleotide polymorphism (SNP) identification [29]. Patient studies revealed evidence for genetic predisposition for OA. However, identification of risk related genes is complicated due to the complex pathogenesis of the disease. Several genes such as Frizzled-related protein (FRZB) and Growth differentiation factor (GDF)-5 have been suggested to raise the risk [30-32]. Furthermore, heritability estimates of radiological outcomes for knee, hip and hand OA amount to 39, 60 and 59%, respectively [33].

Anatomical incongruences e.g. limb-length inequality and misalignment cause load changes in the load-bearing areas of the cartilage and consequently increase the OA risk in the knee [34-36].

Traumatic events such as anterior cruciate ligament and meniscal injuries are known to increase the prevalence of OA because they cause small cartilage defects that are supposed to initiate progression of the disease [37-39]. However, discussions are conducted answering the question whether exercise is damaging or essential for the articular cartilage. In fact there is some evidence that moderate exercise strengthens the cartilage by activating chondrocytes and enhancing the diffusion of nutrients into the matrix [40]. That is why many recommendations for the management of OA include a moderate level exercise [41]. Nevertheless, the high level of sporting activity in professional athletes is linked to a higher risk of OA development even at young age [42]. Therefore, the localization of the affected joint depends on the discipline; football players are more affected by knee OA, whereas dancers suffer from hip OA due to their higher flexibility strain in that joint [42, 43]. In addition, some studies evaluated the relationship between the type of daily work and the prevalence of OA. For example heavy lifting as well as farming lead to a higher risk [44].

2.1.2 Symptoms and Diagnosis

The first obvious symptom is intermittent pain in the affected joint mostly during or after activities. Furthermore, the patients recognize stiffness in the morning, after long term of inactivity or in the evening that can be resolved by motion. In some cases swelling can be observed and in the majority of cases the knee, hip or hand joints are involved. Later stages include the loss of mobility and limitation in the daily activities. Subsequently, the life quality is restricted. The first step in diagnosis is the physical examination to exclude any other causes. Therefore, attention has to be paid on joint enlargement, crepitus or restricted passive movement. In addition, neural and spine examination is needed. The American College of Rheumatology (ACR) as well as the European League against Rheumatism (EULAR) have published recommendations for diagnosis, classification as well as management of rheumatic disorders. The different features for the diagnosis of OA are listed and compared in *Table 1*. Thereby, the clinical evaluation is of great importance beside the radiographic and laboratory examinations [45].

Imaging methods can confirm the diagnosis, enable determination of the clinical stage and monitor the progress of the disease. The “gold standard” is the plain radiography providing high-resolution images quickly and cost-effectively, whereas disadvantages are the radiation exposure, the incapability to display synovial tissue and the lack of standardisation [46-48]. Parameters that can be assessed are the cartilage thickness, the joint space, cartilage roughening as well as the presence of loose bodies (cartilage fragments). Ultrasound, in contrast, is able to provide information about the inflammation status, vascularisation, synovial tissue (synovial villi) and general features of the synovial fluid [49, 50]. Newer techniques including Computed tomography (CT) and Magnetic resonance tomography (MRT) have the advantage to access three-dimensional images and yield semi-quantitative evidence according to the cartilage quality [51, 52].

In 2006 the OA Research Society International (OARSI) published a histopathology grading system for clinical and experimental application especially for imaging methods [53]. Grade I and II comprise cartilage oedema, condensation of collagen fibres, whereas the superficial zone is nearly intact despite some microscopic fibrillation and fissuring that can only be detected via physiological MRT. Grade III can be evaluated by morphological MRT showing vertical fissures extending into the middle zone without significant cartilage loss. Grade I to III are termed as early OA where therapeutic measures can be applied successfully. Plain radiography is applicable for late stage OA, characterized by ongoing (Grade IV) and complete cartilage erosion (Grade V and VI) [53].

Table 1: Comparing different recommendations established by the ACR or EULAR.

	American College of Rheumatology (ACR)			The European League Against Rheumatism (EULAR)
Hand OA	Hand pain, aching, or stiffness and 1. Hard tissue enlargement of 2 or more of 10 selected joints 2. Hard tissue enlargement of 2 or more DIP joints 3. Fewer than 3 swollen MCP joints 4. Deformity of at least 1 of 10 selected joints			1. Presence of Heberden's nodes 2. Age more than 40 years 3. Family history of nodes 4. Joint space narrowing in any finger joint (radiographic)
Diagnosis *Sensitivity* *Specificity*	*3 of the 4 features* *92%* *98%*			*all* *88%*
Hip OA	1. ESR<20 mm/hour 2. Radiographic femoral or acetabular osteophytes 3. Radiographic joint space narrowing (superior, axial, and/or medial)			-
Diagnosis *Sensitivity* *Specificity*	*2 of the 3 features* *89%* *91%*			-
Knee OA	Clinical and laboratory	Clinical	Clinical and radiographic*	- age >40 years - usage-related joint pain - short-lived morning stiffness - functional limitation and 1≥ of the following signs are present 1. crepitus 2. restricted movement, and 3. bony enlargement
	Knee pain and 1. Age > 50 years 2. Stiffness < 30 min 3. Crepitus + (Osteophytes)* 4. Bony Tenderness 5. Bony enlargement 6. No palpable warmth			
	7. ESR <40 mm/h 8. RF <1:40 9. SF OA			
Diagnosis *Sensitivity* *Specificity*	*5 of 9* *92%* *75%*	*3 of 6* *95%* *69%*	*1 of 3* *91%* *86%*	*diagnosis can be made without radiographic*

References that were used: Hand OA [54, 55], Hip OA [56], Knee OA [41, 57]; Abbreviations: DIP, distal interphalangeal; MCP, metacarpophalangeal; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor; SF, synovial fluid signs of OA (clear, viscous, or white blood cell count <2,000/mm^3)

However, the diagnosis of especially early OA as well as the prognosis for the patient is still a big challenge. Therefore, scientists have focused on the detection and establishment of biomarkers to either identify the presence and stage of the disease or determine the prognosis and efficacy of the therapy. Biomarkers have to show validity, reproducibility and a cross-link to the disease, they should be preferably detectable in blood, urine or synovial samples. In OA there are different candidates known which all relate to the aggrecan, collagen or cartilage metabolism (consequence of joint destruction). Other markers relate to

the early inflammatory process in the joint [58]. The diagnostic markers and their origin are depicted in the schematic *Figure 2*. Promising biomarker candidates are e.g. C-terminal telopeptide of collagen type II (CTX-II) detected in urine and serum cartilage oligomeric protein (COMP) isolated from serum [59, 60]. Nevertheless, there is no single candidate which could fulfil all requirements and consequently new techniques such as genomics, metabolomics, proteomics and lipidomics are used to design and investigate different marker profiles and their relationship to the severity of the disease [61, 62].

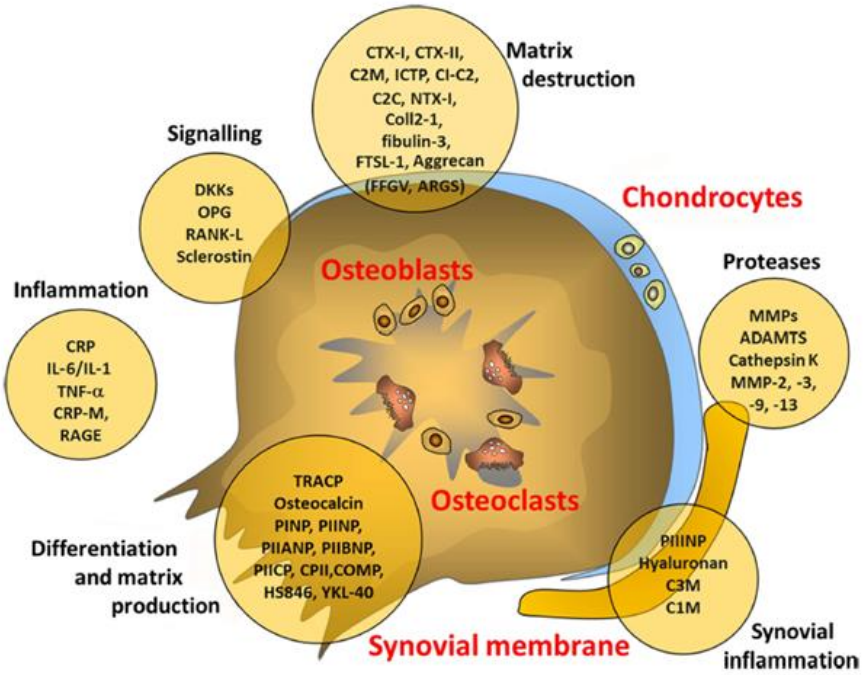

Figure 2: Sources of possible biomarkers in osteoarthritis.

Figure with legend reproduced from Lotz et al. 2013 [63]. Abbreviations: C2C,cleavage of type II collagen; C2M, collagen type II-specific neoepitope; C3M, collagen type III-specific neoepitope; Coll 2-1, 9-amino acid peptide of type II collagen (nitrated form Coll 2-1 NO2); COMP, cartilage oligomeric protein; CPII, type II collagen propeptide; CRP, C reactive protein; CTX, C-terminal telopeptide of collagen; DKK, wnt inhibitor; FSTL-1, follistatin-like protein 1; ICTP, type I collagen-derived cross-linked carboxy-terminal telopeptide; IL, interleukin; MMP, matrix metalloproteinase; NTX, N-terminal telopeptide of collagen OPG, osteoprotegerin; PIIANP, N-propeptide IIA of type II collagen; PIIBNP, N-propeptide IIB of type II collagen; PIICP, C-propeptide of collagen type II; PINP, N-propeptide of type I collagen; PIINP, N-propeptide of type II collagen; RANK-L, receptor activator of nuclear factor κB ligand; RAGE, receptor for advanced glycation endproducts; TNF, tumor necrosis factor.

2.1.3 The Pathogenesis of Osteoarthritis - Inflammation-driven Cartilage Breakdown

In mammalians, three types of cartilage are distinguished: hyaline, elastic and fibrocartilage. The articular surface of joints is covered by hyaline cartilage that is characterized by a

unique structure of layers, allowing the compensation of shear-stress and loadbearing compression as well as frictionless articulation. Articular cartilage is an aneural and avascular tissue and therefore only has restricted regeneration potential. It is composed of 60-80% water, 20% proteoglycans/aggrecans, 5% Collagen (Col) type II and 1-5% chondrocytes [64]. Nutrients for the chondrocytes embedded in extra-cellular matrix (ECM) are supplied from the capillaries of the synovium followed by diffusion through the synovial fluid and uptake by the cells fostered by mechanotransduction [65]. The zonal structure of hyaline cartilage features a superficial, middle and deep zone [66]. Chondrocytes from the superficial zone show a small, flattened phenotype and are arranged in a horizontal alignment that parallels the Col II fibrils and the surface. The middle zone includes chondrocytes that are rounded and the Col II fibrils pass into a vertical route being responsible for the arcade-like structure of hyaline cartilage. Deep zone chondrocytes are grouped in clusters [64]. Chondrocytes represent the cellular key elements for cartilage. They are of utmost importance for the cartilage metabolism, building and synthesizing new extracellular matrix as well as producing metabolic active enzymes [67]. Furthermore, they retain cartilage integrity and react to mechanical response on multiple levels [68]. Intermittent elongation e.g. leads to interaction between the extracellular matrix and membrane-bound integrin, activating messenger Ribonucleic acid (mRNA expression) of aggrecans by modulating the cytosolic Ca^{2+}-concentration [69]. Chondrocytes are terminally differentiated cells. The Sex- determining region Y-box 9 (Sox-9) is an important transcription factor of chondrogenesis and responsible for the maintenance of the phenotype. Sox-9 was shown to activate Col II production in chondrocytes and is decreased during *in vitro* cultivation [70]. With age, chondrocytes lose their ability to respond to anabolic stimuli and growth factors [71]. Proteoglycans trap and hold water and therefore control the hydration of matrix leading to the characteristic stiffness of the tissue [66]. In addition, aggrecans are large proteoglycans that comprise glycosaminoglycan subunits and a large number of chondroitin and keratin sulfate.

Early observations on OA indicate a mild raveling of peripheral non-weight bearing regions devolving into erosive exposure to the subchondral bone [72]. However, the initiating event and further etiological factors are not yet well characterized. Early OA is a non-purulent inflammation where the classical inflammatory signs such as infiltration of immune cells especially neutrophils are absent [73]. That is why experts constantly discuss whether OA can be considered as an inflammatory process or not [74]. It is a fact, however, that OA is not a non-inflammatory process since it is well accepted that inflammatory processes at the molecular level lead eventually to the corresponding clinical signs such as joint pain, swelling and stiffness [75, 76].

Apart from genetic components and anatomical incongruence, the development of high biomechanical forces within the joint is of great importance in the pathogenesis of OA that can occur due to acute (e.g. cruciate ligament rupture) or chronic trauma (obesity, overuse or aging). Thus, smallest defects are placed into the cartilage surface, where cartilage particles and cell debris cause an inflammatory reaction in the synovial membrane [77]. Despite the general acceptance that the synovitis in OA is caused as a secondary phenomenon, it furthers inflammation and cartilage breakdown [78]. T-helper (Th)1 cells migrate into the synovial membrane releasing Interleukin (IL)-1β and Tumor necrosis factor (TNF)-α into the synovial fluid, whereas Th2-cells producing anti-inflammatory mediators (IL-4, IL-10, IL-13) are scarce [79]. Consequently the physiological balance between anabolic and catabolic factors shifts in favor of the catabolic factors in the affected joint [80, 81]. The complex interplay of key factors in OA is summarized schematically in *Figure 3* according to Abramson et al. [77].

IL-1β as major propagator of inflammation up-regulates Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) activating the expression of further pro-inflammatory factors such as Matrix-Metalloproteinases (MMPs), Nitric oxid (NO), Prostaglandin (PGE) 2, IL-6, IL-8 and IL-2 that are also able to amplify the initiation step [82, 83]. Furthermore, the pro-inflammatory cytokines can enable their self-propagation due to the expression activation in chondrocytes or synoviocytes [84]. New insights imply that chondrocytes do not produce active IL-1β, but they can be stimulated to express MMPs by applying external Lipopolysaccharides (LPS) or IL-1α [85]. Moreover, Bougault et al. postulate that OA cartilage degradation is independent on inflammasome activity which completely differs from many stated hypotheses before [85].

Nevertheless, chondrocytes undergo a “vicious cycle” trying to repair the damaged and softened tissue by enhancing the matrix synthesis. This is accompanied by an increased production of pro-inflammatory mediators. As consequence, chondrocytes change to a hypertrophic fate and enhance the production of Col X that usually is only found in embryonic development and therefore regarded as a hypertrophic marker [86]. The active form of IL-1β interacts with the type I IL- 1 Receptor Antagonist (Ra) that is used as a target for OA gene therapeutic approaches. Hashimoto et al. report the activation of IL-1β can be initiated due to the Deoxyribonucleic acid (DNA) methylation status at key CpG sites by pro-inflammatory cytokines, and leads to long-term expression of IL-1 β in chondrocytes [87]. Although TNF is only found in low levels in OA tissue, it enrolls multiple biological actions. TNFα increases the expression of pro-inflammatory cytokines, inhibits the matrix synthesis and enhances the bone resorption *in vitro* [88]. IL-6 modulates immune cell function, proliferation and differentiation while stimulating an osteoblast-like development [89]. High expression

levels of IL-6 in OA leads to cartilage damage and accumulation of inflammatory cells in the synovial membrane [90]. Chondrocytes as well as fibroblasts and other cells are capable of producing IL-8 that can commonly be found in acute inflammatory processes [91]. In addition, IL-8 is a chemotactic cytokine for polymorphonuclear neutrophils that are sparsely found in the synovial membrane of OA patients [92].

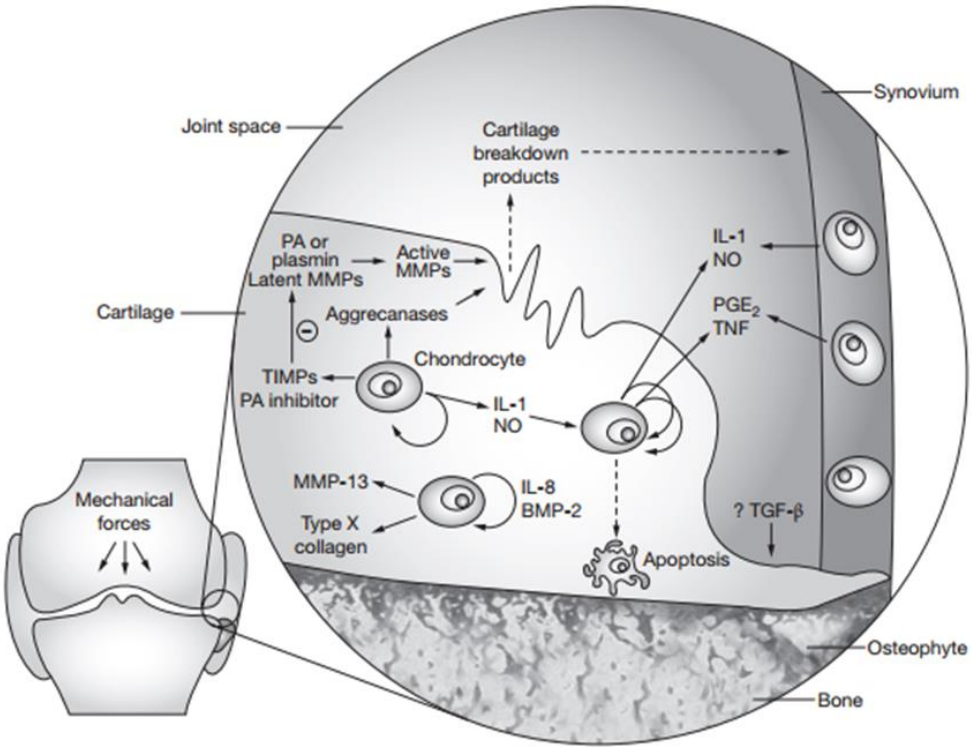

Figure 3: Schematic overview of key pathological events and interaction between anabolic and catabolic factors during OA progression.

Figure with legend reproduced from Abramson et al. 2006 [77]. Abbreviations: BMP, bone morphogenic protein; IL, interleukin; MMP, matrix metalloproteinase; NO, nitric oxide; PA, plasminogen activator; PGE2, prostaglandin E2; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor

NO and PGE2 are released from isolated chondrocytes and cartilage explants [93, 94]. NO is produced by the inducible NOS (iNOS) and plays a major role in IL-1β driven joint pathology such as OA [95]. Besides the inhibitory effect on the Col II and proteoglycan synthesis NO furthers the dedifferentiation of chondrocytes as well as their apoptosis rate and modulates MMP activity [96-99]. Large amounts of PGE2 have been found in arthritic joints where it exerts anabolic as well as catabolic effects and pain [100]. Wang et al. hypothesized the important role of the complement system in OA due to the high levels of complement in osteoarthritic joints demonstrated by proteomic and transcriptomic analyses [101]. Accordingly, the matrix degradation MMPs are of great importance in the pathogenesis of OA. MMPs are zinc-dependent proteolytic enzymes acting as collagenases and degrade extracellular matrix [102]. During the normal cartilage metabolism they are secreted as inactive

form into the ECM by chondrocytes and need proteolytic cleavage for activation. Tissue inhibitor of metalloproteinases (TIMPs), therefore, act as opponents in order to control MMP activity. This balance shifts during the OA progression, whereas the production of MMPs rises and TIMPs are inhibited [103]. MMP-3 is the most common isoform also called stromelysin and activates MMP-1 (collagenase 1) [84]. Additionally, the up-regulated expression of MMP-13 having the ability to more effectively degrade Col II and MMP-9 has been described in the literature [104]. Further enzymes that degrade especially aggrecans are a disintegrin and metalloproteinase with a thrombospondin motif (ADAMTS), whereas ADAMTS-4 (aggrecanase I) and ADAMTS-5 (aggrecanase II) have been described as crucial for the cartilage breakdown during OA. Novel investigations have shown high expression of syndecan-4 in osteoarthritic cartilage activating the MMP-3 expression and exposing ADAMTS-5 to the chondrocyte surface [105].

Anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 are significantly down-regulated in OA patients [106, 107]. The application of these anti-inflammatory cytokines *in vitro* and *in vivo* has been shown to be successful in inhibiting matrix degradation and inflammation [108, 109]. Bone morphogenic protein (BMP) is related to the TGFβ superfamily and can stimulate the proteoglycan synthesis of chondrocytes (BMP-7). Especially, BMP-2 and BMP-9 are known to maintain the phenotype of chondrocytes in culture and even prevent the hypertrophic phenotype switch [110]. TGFβ is unique among growth factors, enhancing the production of enzyme inhibitors such as TIMP and the aggrecan synthesis in activated chondrocytes as well as exerting immunosuppressive activity [111, 112]. A high level of TGFβ was found in synovia and cartilage of osteoarthritic joints and is supposed to enhance growing of osteophytes leading to a new bone formation at the edge of the articular cartilage surface [113].

As a consequence of the chronic inflammation, the synovial membrane begins to hypertroph by building synovial villi [86]. Furthermore, the progressive erosion of cartilage as well as the complete breakdown of the tissue lead to an increasing pressure load on the subchondral bone. The subchondral bone counteracts with compaction of the bone (sclerosis of the subchondral bone). Therefore, the involvement of the subchondral bone has been postulated in early stage of OA [114].

2.1.4 An Update on Current Therapeutic Interventions

At the moment the therapeutic inventions for OA can be divided into non-pharmacological, pharmacological and surgical treatment. Thereby, the combination of these strategies according to the individual needs is beneficial to improve life quality of patients. Symptomatic therapy (stiffness and pain) and delay of disease progression over a long time are currently

the only choice to treat affected patients since an effective therapy to provide a long-term remedy has not been found yet.

The Osteoarthritis Research Society International (OARSI) and the EULAR have published evidence-based guidelines for the management of OA that are routinely applied in clinical practice [115-120]. At the early stage of OA, non-pharmacological interventions such as self-management (life style change), educating the patient followed by exercising and weight reduction if necessary are strongly recommended. In addition, acupuncture, electro-magnetic therapy, ultrasound and lasers can be used to improve the wellbeing of the patient which of course is hard to rate accurately [121, 122].

The guidelines mentioned above recommend for the pharmacological therapy paracetamol as the first-line therapy followed by Non-steroidal anti-inflammatory drugs (NSAIDs) that should be applied in patients who are unresponsive to Paracetamol. However, the application of NSAIDs is often accompanied by adverse effects such as gastrointestinal, renal or cardiovascular disorders and is therefore restricted to short-term treatments. In case of patients with a higher risk for gastrointestinal side effects (perforation, ulcers, bleeding) the combination with gastro-protective agents or the use of cyclooxygenase-2 (Cox-2) selective inhibitors is indicated. Opioids are only used when acetaminophen, NSAIDs and Cox-2 inhibitors have failed. In particular, the combination of weaker opioids and paracetamol/NSAIDs or the administration of strong opioids were found to be beneficial for the pain treatment. But nevertheless, the intake of opioids is often accompanied with adverse effects and involves the danger of drug dependence [123-125].

Currently, different groups of therapeutic drugs are tested in clinical trials, e.g., Naproxcinod a Cyclooxygenase-inhibiting nitric oxide donor (CINOD) [126] or Cindunistat a specific inducible nitric oxide synthase inhibitor [127]. Furthermore, Disease-modifying osteoarthritis drugs (DMOADs), e.g. monoclonal antibodies or soluble cytokine receptors, are under development and evaluation for OA since they have been applied in rheumatoid arthritis [128]. They have the overall goal of blocking dominant cytokines such as IL-1β and TNFα which are mainly responsible for cartilage degradation during OA.

For local therapy, corticosteroids or hyaluronic acid are administered directly into the affected joint by injection. Corticosteroids effectively cause pain relief, particularly in OA of the knee. However, this pain relief lasts only for about three weeks without any significant positive effect on the physical function of the joint [129]. Hyaluronic acid (HA) is an important component of synovia serving functions such as viscoelasticity to the joint fluid and boundary lubrication of the intra-articular soft tissues. Inflammatory processes related to OA decrease the concentration and molecular weight of HA in the synovial fluid which can lead to

joint dysfunction. Therefore, the intra-articular injection of high molecular weight HA can support the restoration of the joint [130]. Nutritional supplement, especially glucosamine and chondroitin sulfate, also called symptomatic slow-acting drugs, are known as an additional, safe and evidence-based method for the management of OA [116, 117].

Surgery conducting lavage debridement, osteotomy, total joint replacement or arthrodesis are indicated for patients who are unresponsive to drug therapy and suffer from outlasting pain and functional limitations. Lavage debridement is only indicated in cases of superimposed structural lesions in the affected joint such as meniscal tear [131]. Osteotomy is performed by trimming the joint which leads to an even distribution of the mechanical load and therefore relieves the defect area [132]. Total joint replacement and arthrodesis are both defined as a stiffening of the joint after surgery, whereas arthrodesis results in complete immobility; the total joint replacement only restricts joint mobility and can be more cost effective compared to conservative therapy [118].

2.1.5 Regenerative Therapies - From Cell Therapy to Biomaterials

Besides chondrocytes even mesenchymal stromal cells (MSC) have been identified as potential tools to treat OA. They have been examined extensively and found to be able to differentiate into chondrocytes as well as into osteoblasts and to enable an unlimited growth potential without changing their phenotype [133-136]. MSC can be isolated from bone-marrow, adipose or other tissues. These cells showed anti-inflammatory properties in co-culture systems and are mostly injected into the affected joint [137-141]. Searching the ClinicalTrials.gov website for the keywords “osteoarthritis” and “mesenchymal stem cells”, 13 clinical trials testing bone-marrow derived MSC (11 autologous and 2 allogeneic) could be shortlisted. Of these, 6 studies have been completed and 7 are still active [142]. Furthermore, two studies examining the potential of autologous adipose-derived MSC could be identified, whereby one study has been completed and the other is still open. One clinical trial investigates the potential of umbilical cord blood-derived MSC to treat OA of the knee (Cartistem, Dong-A Pharmaceuticals Co., Ltd., Korea). Consequently, MSC represent a promising cell type for OA therapy and further clinical trials will unveil the optimal source and application method.

The restricted potential of articular cartilage to regenerate led to the intensive research on cell and/or biomaterial based approaches to fill the cartilage defect area and consequently restore the physiological shape. Therefore, incipient stages in surgical treatment for fresh cartilage defects include the osteochondral (multiple) autograft transfer and mosaic autografts [143-145]. In 1994 Brittberg et al. introduced the autologous chondrocyte transplantation (ACT) [146]. Herein, a cartilage biopsy is taken from a non-weight bearing area of the

joint; the isolated chondrocytes are expanded *in vitro* and afterwards injected onto the defect under a periostal flap. This method has proven to repair efficiently small fresh lesions in the articular cartilage and has been applied to thousands of patients worldwide. But it has a restricted medical indication according to the patient, resulting in the exclusion of patients with weak and advanced OA [147-149].

The ACT technique was further improved by embedding the chondrocytes into a matrix (also named scaffold) before transplantation - the matrix-associated autologous chondrocyte transplantation (MACT). Therefore, a wide range of suitable materials have been produced and tested to satisfy the following requirements [150, 151]: non-toxic and biodegradable following a physiological mechanism, enabling attachment, migration, proliferation and differentiation of tissue-forming cells, suitable protection for the newly inserted cells, biomechanical stability comparable to the native tissue, non-immunogenic, easy handling and application. In general, materials for cartilage tissue engineering can be divided into three categories: natural, biosynthetic and synthetic.

Natural materials used for reconstruction consist of biocompounds mimic the natural architecture. They are mostly biodegradable. As an example, collagen-based (Col I or III) as well as hyaluron-based scaffolds are widely used in the clinic [152-154]. Biosynthetic materials combine bio- (silk, cellulose, hydroxyapatite, chondroitin-sulfate) with synthetic materials (Polylactic acid (PLA) or Polyether ester copolymer scaffolds) to design new scaffolds by utilizing the optimal properties of each material. Synthetic materials offer optimal properties with regard to fiber diameter and pore size. The production is performed automatically and highly reproducible. Such materials are often composed of PLA and Polyglutamic acid (PGA) [155, 156] and polyethylene glycol (PEG) macromeres, especially for hydrogels [157]. Scaffold-less tissue engineering has become more and more attractive allowing the cells to maintain their rounded morphology as well as producing their own matrix. Different techniques can be distinguished; non-adherent surfaces lead to self-assembly of chondrocytes as well as applying defined strain on the cells in e.g. a bioreactor [158]. After 4 weeks the self-assembled, neocartilage is biochemically, histologically and biomechanically comparable to native cartilage and provides many advantages that have been reported in preclinical studies [159]. There are two clinical studies going on for the subsequent products: DeNovo NT (Zimmer, Inc., Indiana, United States) and Chondrospheres (co.don AG, Berlin, Germany). All described tissue engineering techniques have one thing in common, they are developed to fill cartilage defects. Patients with osteoarthritic changes in the joint are excluded.

2.2 Gene Therapy in Osteoarthritis

2.2.1 Definition and Delivery Strategies

Gene therapy is defined as an introduction of genetic information encoding biological agents into the host tissue for therapeutic purposes [160]. In general, three strategies of gene therapy following different aims can be distinguished [161]. The first strategy, the gene replacement, involves the surrogate of the defective gene by a new functional copy of the required gene to retain the production of the respective protein and is mainly used for hereditary diseases [162, 163]. Gene addition instead covers the introduction of genes that are not normally produced by the cells, but targets therapeutic purposes. Furthermore, genes can be introduced to control the expression of target genes (knock-down) [161]. Although there are some genetic mutations known to enroll OA (e.g. Type II collagen gene mutations), gene therapy on OA principally focuses on the addition of anti-inflammatory genes or growth factors.

Especially, synovial cells and chondrocytes as well as recently MSC are of interest as target cells for gene therapeutic approaches. The application of gene therapy can be carried out either *in vivo* by injecting the transgenic plasmid (including the target gene) directly into the joint or *ex vivo* by transfecting autologous cells and transplanting them, embedded conceivably in a matrix, into the joint [164, 165] (*Figure 4*). However, the strong cartilage matrix comprising only a low cell density hampers *in vivo* gene transfers into chondrocytes. Synovial cells are readily accessible for *in vivo* gene therapy since they have been shown to take up particles injected into the synovial fluid. *In vivo* gene transfer is much simpler and quicker but needs suitable plasmids without risk concerns [166]. In contrast, *ex vivo* gene transfer provides a larger range of plasmids that can be used but is much more expensive due to the need of a Good Manufacturing Practice (GMP)-facility.

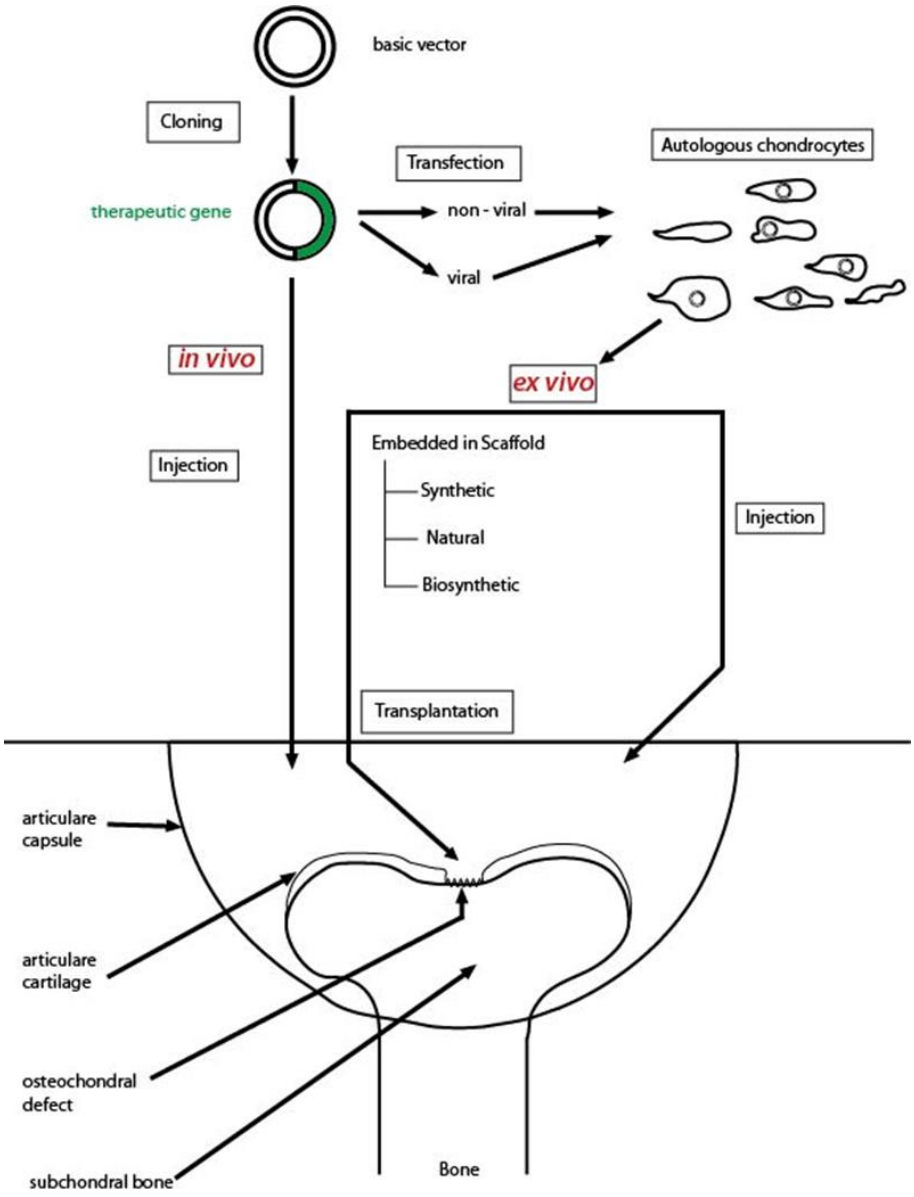

Figure 4: Overview on different gene transfer strategies to the affected joint.
The in vivo strategy involves the direct injection of plasmids, whereas the ex vivo strategy includes the intermediate step with cell isolation and modification. Afterwards, the modified cells can be directly injected into the joint or embedded in a scaffold transplanted into the cartilage defect.

However, to successfully perform gene therapy, an effective gene transfer into target cells is required. Therefore many methods have been investigated for transfection of mammalian cells, but not all of them are suitable for OA gene therapy. There are two main methods to transfect cells: by either using viral or non-viral plasmids. Viral strategies usually provide a higher efficiency of gene transfer but, owing to their potential immunogenicity of viral products and to mutagenicity, they are supposed to be less safe. In contrast, non-viral methods are widely accepted since they are considered to be safe, but they are clearly less efficient [167].

Non-viral gene transfer delivers plasmid DNA into the cells with selected chemical and physical methods and only achieves transient transfection. Most common methods base on liposomal or non-liposomal agents (lipofection) to form DNA-lipid complexes which are taken up by the cell via endocytosis and directly translocated to the nucleus by endosomal-liposomal vesicular transport [168]. Liposomal agents build cationic liposome-DNA complexes, whereas non-liposomal agents comprise lipids that envelop the DNA in micelle-like structures and can be combined to non-ionic surfactants [142, 169]. Several reagents, transfecting chondrocytes with differing efficiencies, are commercially available [170]. Different protocols for *ex vivo* gene transfer by lipofection are established, but the transfection rate is low and due to cell cytotoxicity of most compounds *in vivo* transfection lacks feasibility. Sant et al. reported the possibility of intra-articular injection of naked DNA plasmids without addition of liposomal agents to transfect synoviocytes [171].

Electroporation is widely used in the laboratory routine achieving high transfection rates of different cell lines and does not need cytotoxic chemicals. To achieve high transfection efficiencies in chondrocytes, specific protocols using radiographic electroporation are needed, and therefore the equipment demand is higher [172, 173]. Grossin et al. achieved *in vivo* transfection of articular chondrocytes by applying external electric pulses to knee joints after intra-articular DNA injection [174]. Magnetofection includes the binding of plasmid DNA to magnetic microparticles to be dragged into cells by strong directional magnetic fields. This method does not require cytotoxic transfection reagents, but can be combined to raise the transfection rate and has also been applied *in vitro* to articular chondrocytes [175].

Viral gene transfer takes advantage of the viral ability for horizontal gene transfer into a variety of target cells. Several virus types are known to infect mammalian cells and have been investigated as well as optimized to even transfect chondrocytes and synovial cells. Retroviral transfection systems are derived from the well characterized Moloney murine leukemia oncoretrovirus (MMLV) [176]. MMLV only infects dividing cells and is therefore restricted to *ex vivo* transfection, where cell propagation can be achieved [177]. Retroviruses integrate persistently into the genome but nonspecifically, enhancing the risk of insertional mutagenesis. Lentiviruses, derived from the human immunodeficiency virus (HIV), are able to infect resting and dividing cells persistently and with high efficiency. They show low immunogenicity because they do not produce viral proteins in the cell. In addition, lentiviruses carry a risk of insertional mutagenesis [167].

Adenoviruses readily transduce dividing and non-dividing cells containing large transgenes and are an often employed system [178]. The integration is still transient and transduced

cells show immunogenicity even *in vivo*. To circumvent their immunogenicity, either adeno-virus strains which normally infect different host species are used, or immunogenic viral proteins are deleted from the constructs [179]. Adeno-associated viruses (AAV) have a low immunogenicity, are small in size and have been engineered to not insert their genome into the host DNA for biosafety reasons. Nevertheless, AAV are difficult to manufacture and generate moderate transfection rates. They possibly penetrate the cartilage matrix and could therefore transduce chondrocytes *in vivo* [180, 181]. In general, viral gene transfer is highly efficient transducing synovial cells *in vivo*, and chondrocytes *ex vivo*. Disadvantages of viral systems are biosafety issues, the need of specific cell lines for virus generation and propagation, and the risk of insertional mutagenesis accompanied with the advantage of persistent insertion that is required for a chronic, long lasting disease such as OA.

2.2.2 Candidates for Gene Delivery

OA is characterized by a progressive degeneration of articular cartilage, caused by an imbalance of catabolic and anabolic processes. Therefore, targets of gene therapy are either inhibitors of the catabolic factors or inducers of cartilage regeneration. To counteract inflammatory matrix degeneration and cell apoptosis induced by pro-inflammatory cytokines such as IL-1β, and TNFα specific small interfering RNA (siRNA) can be beneficial. In particular, siRNA can target the mRNA for signaling molecules, the overexpression of IL-1 receptor antagonist (IL-1Ra) or soluble receptors for IL-1 (sIL- 1R) and TNFα (sTNFR) [182, 183]. Gene transfer and overexpression of both, IL-1Ra and a sTNFR-immunglobulin FC fusion construct (Etanercept), have been already tested in clinical trials to treat arthritis disorders [177, 184, 185]. Furthermore, the expression of anti-inflammatory cytokines can lead to the inhibition of IL-1 and TNF-α. Therefore, candidate genes are IL-4 [107, 186, 187], IL-10 [188], or IL-13 [189] which reduce the infiltration of activated immune cells, decrease the level of pro-inflammatory cytokines and downregulate the expression of iNOS, MMP-1, -3 and -13 and IL-6. Viral overexpression of MMP inhibitors such as TIMP-1 [190], -3 [191] or -4 [192] diminishes the cartilage degradation and consequently reduces OA progression. The described inhibition of catabolic pathways reduces OA damage, but cannot activate or further the cartilage regeneration. Therefore, the activation of matrix synthesis can be enhanced by expressing enzymes required for synthesis of precursors of matrix compounds [193-195]. In addition, the cartilage phenotype is maintained by overexpression of transcription factors such as SOX-9. So far, promising candidates of the TGF-β superfamily are TGF-β 1, 2 and 3 which stimulate proteoglycan synthesis and repair of cartilage lesions [196]. Other promising growth factors are insulin-like growth factor (IGF)-1, BMPs, fibroblast growth factors (FGFs) and epidermal growth factor (EGF) [197-200].

2.2.4 Preclinical and Clinical Studies - Recent Achievements towards OA Therapy

Between 1989 and 1998 the number of gene transfers towards various diseases in humans has increased [201]. The introduction of gene therapy to rheumatic diseases was designated to address RA. However, the first clinical trial on Rheumatoid Arthritis (RA) patients was performed in 1996 by Evans and colleagues to examine the feasibility, safety and efficacy of gene transfer to human metacarpophalangeal joints [202]. Thereafter, some other studies were successfully accomplished whereby one trial reached Phase II. Despite unfortunate setbacks such as the death of Jesse Gelsinger during a trial in 1999 [2] or the first death of a patient during an arthritis gene therapy trial [3], researchers continued to slowly translate gene therapy into the clinics. However, several preclinical studies share the efficiency of OA gene therapy *in vivo* and in experimental animal OA models (*Table 2* and *Table 3*).

Table 2: Ex vivo gene delivery to different types of cells using experimental induced OA in animal models.

Cells		Transgene	Plasmid	Animal model	Reference
Chondrocytes		Lac Z neo	Retrovirus	Rabbit cartilage defects	[203]
		BMP-7	Adenovirus	Horse cartilage defects	[204]
		IGF-1	Lipofection, Alginate beads	Rabbit cartilage defects	[205]
		FGF-2	rAAV	Rabbit cartilage defects	[200]
		FGF-2	Plasmid	Rabbit cartilage defects	[197]
		IGF-1/ FGF-2	Lipofection	Rabbit osteochondral defects	[206]
Synovial cells		Lac Z neo	Retrovirus	Rabbit normal joints	[160]
		IL-1 Ra IL-10	Retrovirus	Rabbit normal joints	[207]
		IL-1 Ra	Plasmid	Rabbit ACLT	[208]
Mesenchymal stem cells	Perichondrium-derived	BMP-1 IGF-1	Adenovirus	Rat cartilage defects	[209]
	Bone-marrow derived	CDMP1	Plasmid	Rabbit cartilage defects	[210]
Other cells	Fibroblasts	TGFβ	Retrovirus	Rabbit cartilage defects	[211]
	Muscle-derived cells	LacZ	Retrovirus	Rabbit osteochondral defects	[212]

Table modified and reproduced from ® 2011 M.F. Rai, A. Lang, M. Sieber, M.F.G. Schmidt. Adapted from [213]; originally published under CC BY-NC-SA 3.0 license. Available from: 10.5772/1013; Abbreviations: LacZ= β-galaktosidase E.coli; neo= neomycin phosphotransferase; CDMP1= Cartilage-derived morphogenetic protein 1; ACLT = anterior cruciate ligament transection

Table 3: In vivo gene delivery with different transgenes using experimental induced OA in animal models.

Transgene		Plasmid	Animal model	Reference
Cytokine Inhibitors	IL-1 Ra	Adenovirus	Dog ACLT	[183]
		Plasmid	Rabbit meniscectomy	[161]
		Adenovirus	Horse osteochondral defects	[214]
	IL-1Ra/IGF-1	Adenovirus	Horse osteochondral defects	[215]
	IL-1Ra, IGF-1, FGF-2	Adenovirus	Rabbit ACLT	[216]
Other therapeutic genes	FGF-2	rAAV	Rabbit osteochondral defects	[217]
	BMP-2	Plasmid	Rabbit cartilage defects	[218]
	HSP-70	Plasmid	Rat MIA injection	[219]
	TSP-1	Adenovirus	Rat ACLT	[220]
	BMP-2/-6	Adenovirus	Horse osteochondral defects	[221]
	shRNA MIP-1γ	Lentivirus	Mice ACLT	[222]

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The first *in vivo* study using gene therapy in an OA animal model was performed in 1992 by Bandara and colleagues [160]. Thereby, allogeneic synovial cells were transfected with cDNA encoding β-galactosidase from *Escherichia coli* (LacZ) and neomycin phosphotransferase (neo) in order to examine the feasibility of gene therapy in the joint *in situ* and *in vivo*. Similar feasibility studies were carried out by Kang et al. 1997 transducing allogeneic chondrocytes by retroviral gene delivery [203, 223]. Ikeda et al. 1998 tested the *in vivo* strategy in Hartley guinea pigs [263]. *Table 2* and *Table 3* depict the enrolled gene therapeutic studies in animal models using *in vivo* or *ex vivo* strategies to the date. Recently, Madry et al. observed the formation of new cartilage tissue and a significantly better integration of the transplant in the surrounding cartilage driven by IGF-1 overexpression in an *ex vivo* chondrocyte system and *in vivo* [205]. Several studies revealed an intensified effect of IGF-1 in combination with other therapeutic transgenes such as IL-4 that resulted in a strong protective effect for the cells [206, 224]. In addition, Chen et al. suggested that the multiple gene transfer is far more effective than applying only FGF-2 alone [216]. But so far, FGF-2 was also shown to significantly improve histological parameters and chondrocytic mitotic activity

[225]. Furthermore, TGFβ is the first therapeutic transgene that had been applied in a clinical trial for OA gene therapy [211]. A new approach was published by Shen et al. using small hairpin RNA (shRNA) to suppress MIP-1γ (macrophage inflammatory protein-1γ) expression resulting in the inhibition of MMP-13 and matrix degradation [211, 222]. Another promising approach is now applied to animal patients in Germany, using Proteoglycan 4 under the control of the Col II promoter [226]. The first clinical study (Phase I) on OA gene therapy was completed in 2010 and used retroviral transduced allogeneic chondrocytes expressing TGFβ that were intra-articularly injected [227]. After isolation, the cells were irradiated, eliminating the proliferation potential in order to avoid adverse reactions due to uncontrolled propagation. The therapy was applied to 13 patients suffering from OA in the knee and no adverse reactions despite some effusion and warming were reported. Importantly, no virus DNA could be detected in the blood. Further examinations indicate clinical improvements and a clinical phase II study has been initiated. In addition, a clinical phase I study is planned, investigating the efficiency of intra-articular injection of AAV plasmids overexpressing IL-1Ra [228].

One of the first and widely investigated potential transgene represents the IL-1Ra. The IL-1Ra has been revealed to reduce *in vitro* and *in vivo* cartilage degradation, MMP production and the progression of OA lesions [183, 229]. Preclinical studies applying the IL-1Ra in small as well as large animal models proved the potential to reduce the progression of structural changes of OA by enhancing the proteoglycan production [183, 215]. Due to the suppressed anabolic activity during OA, some approaches try to reinstate the balance between synthesis and degradation by supplying IGF-1 or other growth/differentiation factors that is also of mutual interest for the transduction of MSC. IGF-1 in chondrocytes serves as paracrine and autocrine stimulator of matrix synthesis [230].

2.3 Interleukin-4 as Potential Transgene for Osteoarthritis Gene Therapy

2.3.1 A Multifunctional and Essential Cytokine

Interleukin (IL)-4 is a multifunctional pleiotropic cytokine type 1 and is produced by i.a. T-cells, mast cells, basophils, macrophages and eosinophils [231]. It is a strict species-specific cytokine. The size of IL-4 differs between 15 and 24 kDa (mice: 19-24 kDa; human: 15-19 kDa) depending on the type of splicing variants that can induce different immunological functions [232]. However, the full-length variant predominates and is therefore commonly examined. Furthermore, IL-4 resembles IL-13 in its sequence, receptor usage and signaling [233, 234]. Genes encoding IL-4 and IL-13 lie separately on chromosome 5 (human). Several major regulatory elements on the promoter explain the restricted secretion pattern by only activated T cells and mast cells [233].

The central function of IL-4 is to promote the maturation of Th2-cells which release IL-4, IL-5, IL-10, IL-13. Their cytokines down-regulate Th1-cells and their cytokines [235]. In addition, IL-4 regulates cell proliferation, apoptosis and the expression rate in various cell types [236, 237]. Furthermore, it promotes the immunoglobulin class switch on human B-cells from Immunoglobulin (Ig) E to IgG4 and on mouse B-cells to IgG1. In addition, it enhances the production of Major histocompatibility complex (MHC) class II [238-240]. As co-stimulator with Cluster of differentiation (CD) 40 monoclonal antibodies, IL-4 is able to activate the long term proliferation of B-cells and is also known to play an important role in the development of the protective immune response against parasites, especially helminthes [241, 242]. Together with TNFα, it leads to an increased expression of Vascular cell adhesion protein (VCAM)-1 by decreased expression of E-selectin on endothelial cell surface to effectively recruit T-cells to the site of inflammation [243, 244]. However, IL-4 inhibits osteoclast activity and survival and therefore blocks bone resorption *in vitro* [245]. The IL-4R comprises an IL-4R α-chain that mediates biochemical signals within the cell and a second γ-chain that is needed for heterodimerization to transfer physiological signals [237]. Additionally, the IL-4R α-chain can be a part of the IL-13R complex and the IL-4R γ-chain can in parts be replaced by the IL-13Rα1 [231].

IL-4 is known to be involved in the development of allergic reactions evoked by an overproduction together with IL-5 and IL-13 [246]. Moreover, IL-4 has been shown to induce allergen-induced airway leucocyte recruitment [247]. Thus, several approaches focus on the anti-IL-4 therapy to overcome allergic reactions [248]. IL-4 is involved in other diseases too, where controversal effects have frequently been observed as e.g. in tumor immunology [249]. On the one hand, IL-4 is suggested to further the disease progression and on the other hand the therapeutic application to some tumor diseases (e.g. Non-Hodgkin lymphoma, renal carcinoma, chronic lymphocytic leukemia, malignant melanoma) has shown partially beneficial outcomes during clinical trials [250-253]. Furthermore, the role of IL-4 in liver disease has been controversially discussed [254]. In contrast, the administration of IL-4 in autoimmune disease has shown promising results. A study treating 20 patients suffering from psoriasis demonstrated significantly better clinical outcomes after application of IL-4 which was explained by the shift of auto-reactive Interferon (IFN) γ-producing Th-cells to IL-4 releasing (positive) Th2-cells [255]. Such IL-4^{+}Th2-cells have been reported to alleviate autoimmunity since it was investigated in animal models before [256]. In an experimental mouse model of autoimmune encephalomyelitis, IL-4-overexpressing MSC were shown to prevent the autoimmune demyelination by increasing anti-inflammatory cytokines which were able to attenuate the clinical disease [257].

2.3.2 Physiological Role in Cartilage - Interleukin-4 mediates Mechanotransduction

The articular surface of joints is covered by hyaline cartilage that is characterized by an unique structure of layers and allows the compensation of shear-stress and loadbearing compression as well as frictionless articulation. Mechanical loading is of great importance for the structure development and maintenance as well as the function of musculoskeletal tissue. Additionally, mechanical stimuli are able to counteract catabolic signals such as in OA. However, an intact cytoskeleton is needed to mediate secretion of IL-4 that is further involved in the integrin mechanotransduction pathway [258]. Studies on chondrocytes embedded in alginate beads showed an increased expression of IL-4 as well as IL-1β after regular compression for 14 days [259]. In detail, stretch-sensitive Ca^{2+}-channels encounter the transport of IL-4 through the cell [258]. The mechanotransduction involves the integrin-regulated secretion of IL-4 that subsequently interacts with the α5β1 integrin in the cell membrane [260]. Moreover, the IL-4 signaling is mediated in an autocrine or paracrine manner by type II IL-4R leading to a membrane hyperpolarization through K^+ and Ca^{2+}-channels [261]. Furthermore, the hyperpolarization promotes the aggrecan production and inhibits the MMP synthesis [261]. In case of OA, an abnormal mechanotransduction process can be observed caused by the absence of the α5β1 integrin mediated IL-4 release and the enhanced release of IL-1β [261, 262].

2.3.3 Anti-inflammatory Potential of IL-4 towards OA

The main advantage of IL-4 is to effectively inhibit IL-1β and TNFα production that was reported in synovial tissue obtained from OA patients [263, 264]. Therefore, IL-4 interacts with both forms of the TNF receptor and consequently modulates the TNF receptor mediated cellular response [265]. Moreover, the induction of potent inhibitors and receptor antagonists such as IL-1Ra and soluble IL-1R additionally reduces the synthesis and action of IL-1β and TNFα [266-269]. Canine IL-4 was shown to repress NFκB and mediated the anti-inflammatory activity through STAT-6 [107, 270].

However, IL-4 inhibits IFNγ, IL-1β and TNFα induced release of NO by hampering the NO synthase and is known to act anti-inflammatory on chondrocytes by suppressing the NO release as well as the IL-1β induced PGE2 release that could be enhanced by parallel moderate implemented compression on the cells [271-274]. In addition, IL-4 suppresses the TNFα mediated PGE_2 production in OA synovial fibroblasts and furthers the proliferation of chondrocytes [273]. IL-4 and IL-13 were shown to have anti-apoptotic effects on human synoviocytes [108].

Moreover, IL-4 is able to suppress the MMP release of macrophages, monocytes and fibroblasts and promotes the production of TIMPs by chondrocytes [275-277]. IL-4 was shown

to prevent cyclic stress induced of MMP-13, cathepsin B and IL-1β expression [278]. Oncostatin M and TGFβ induced response of arthritic chondrocytes can be antagonized by IL-4 and leads to the inhibition of MMP-13, ADAMTS-4 and TIMP-3 [279]. Furthermore, IL-4 was shown to have the capability to induce 15-lipooxygenase whose metabolites are known to inhibit inflammation [280]. This induction was reported to also occur as a synergistic effect with IL-1β and TNFα.

A study, focusing on the serum levels of sIL-4R, indicated significant higher levels of sIL-4R in OA patients allowing the hypothesis that the serum level increase reduces the availability in the joint and consequently the intervention of IL-4 in the disease progression [281]. Furthermore, another study reported higher levels of IL-4 producing $CD8^+$ cells in arthritis patients compared to controls, indicating the potential of IL-4 to suppress IFNγ release [282]. Additionally, van Meergen et al. demonstrated the prevention of blood induced cartilage damage by application of IL-4 and IL-10 on human cartilage explants [109].

2.3.4 Anti-inflammatory Potential of IL-4 towards Rheumatic Diseases – Lessons from Animal Models

Several studies on the therapeutic potential of IL-4 in rodents have been conducted during the last two decades starting in 1999. Unfortunately, studies in large animal models as well as relevant OA models are lacking. However, there is only one study investigating the effect of IL-4 against OA in a mouse model [283]. Therefore, IL-4 was shown to down-regulate iNOS mRNA expression as well as NO production in chondrocytes in an osteoarthritic ACLT mice model.

The most frequently used model in rodents has been the collagen induced arthritis (CIA) model, where mice are immunized with an emulsion of complete Freund's adjuvant (CFA: oil-water-emulsion including immortalized *Mycobacteria tuberculosis*) and Col II [284]. This model is used to simulate partly the pathogenesis of rheumatoid arthritis (RA). In contrast to OA, RA is an autoimmune disease that is characterized by the massive invasion of immune cells to the joint and synovial membrane e.g. of macrophages and dendritic cells producing high amounts of pro-inflammatory cytokines causes the typical rheumatic features such as deep cartilage destruction, subchondral bone erosion, synovitis and pannus development. $CD4^+$ T-cells are supposed to play a major role in the progression of RA and are supported by the occurrence of autoantibodies-producing B-cells.

RA animal models differ in the underlying immune response, leading to a variety of models representing only some aspects of the disease and providing different outcomes according

to the therapeutic testing. Therefore, most of the models are called “arthritis” model simulating different inflammatory processes within the joint. Findings in these models are also of interest for OA research.

First studies in a rat arthritis model (CIA), intra-articular injecting retroviral plasmids containing IL-4, reported a high amount of IL-4 in the treated joint after 8 days whereas no plasmid could be found via qualitative Polymerase chain reaction (qPCR) in the contralateral joint [285]. A reduction in paw swelling and radiographic evidence for bone destruction was observed, caused by the supposed IL-4 increase of Th2-cells. Moreover, adenoviral transfer of IL-4 to CIA mice resulted in a decrease of IL-17, IL-12, IL-6, IL-1β, TNFα, cathepsin K and osteoprotegerin ligand, leading to an impressive reduction of joint erosion as well as a more compact subchondral bone structure [187, 285, 286]. The latter is explainable due to the suppressed TNF-related activation protein (TRAP) and subsequently inhibition of osteoclast-like cells. Similar results were obtained by Watanbe et al. reporting the detectable protection of IL-4 encoding adenoviral plasmids against CIA [287]. An alternative transfer system was tested by Cottard et al. using an adeno-associated plasmid system to transfer IL-4 before inducing arthritis in mice [288]. Thereby, the plasmids were injected into the muscle and an expression over 129 days could be observed and therapeutic benefit revealed in reduction of paw swelling and histological synovitis as well as a delayed onset (10 days) of arthritis.

Another way of IL-4 transfer to affected joints was tested by Tarner et al. who administered T-cell hybridomas, that were retrovirally transduced to overexpress IL-4, to the joints of CIA mice before the day of induction, leading to a reduced number of inflamed joints whereas the application after induction of CIA showed no therapeutic effect [289]. Kageyama et al. observed IL-4 driven amelioration of murine CIA after applying IL-4 DNA via gene gun or intradermal injection whereas the gene gun delivery showed higher immunosuppressive effects since the incidence and severity of the disease were reduced [290]. A really innovative gene transfer system was employed in 2004 using *in vivo* electrotransfer to deliver IL-4 to CIA mice [291]. The transduction lasted for at least 17 days and synovitis and cartilage erosion as well as IL-1β expression were significantly reduced whereas the TIMP-2 expression was increased. Furthermore, also the combination of IL-4 with other anti-inflammatory cytokines or substances showed promising results [224]. Hemmerle et al. recently published a study that showed the anti-inflammatory action of IL-4 in a CIA mouse model connecting IL-4 to a fusion antibody that recognizes the alternatively spliced extra domain A of fibronectin. This domain is strongly overexpressed in arthritic synovial membranes and therefore facilitates the systemic application of the fusion antibody [292].

In contrast, collagen-antibody induced arthritis (CAIA) in mice showed reduced incidence of arthritis when combining it to IL-4 knockout [293]. CAIA was, therefore, induced by injecting Col II-specific monoclonal antibodies (mAb) and is suited for studying the inflammatory phase. The underlying mechanism involves Fc receptor and complement as well as neutrophils and macrophages as the major mediators, but no B or T cells, explaining the immune activating function of IL-4 in this model. Cao et al. reported a reduced immune response in a proteoglycan-induced arthritis (PGIA) model after treatment with IL-4 [294]. This effect could not be confirmed in IL-4 knockout mice. Additionally, Finnegan et al. suggested that IL-4 controls the severity of arthritis in a PGIA model by IL-12 and reported more severe arthritis in IL-4 knockout mice [295]. PGIA is $CD4^+$ T-cell and B-cell driven that explains the effect of IL-4 leading presumably to a Th-2-cell switch [296]. According the question of the association between the chosen RA model and the IL-4 outcome in a K/BxN model, IL-4 was shown to be required for full development of arthritis [297].

2.3.5 Possible Risks of IL-4 as Transgene in Gene Therapeutic Approaches - Dosage and Toxicity Studies

Interleukin-4 is a strict species-specific cytokine and therefore requires suitable models to effectively study the toxicity [298]. The safety of IL-4 has been tested in non-human primate models [299-301]. In these studies cynomolgus monkeys were treated subcutaneously or intravenously with human IL-4 using dosages up to 100 µg/kg for maximal 6 months. However, the incidence and severity of toxicological effects depended on dosage, treatment duration and route of administration. The intravenous application leads to severe adverse reactions compared to the subcutaneous injection. In particular, the highest not lethal doses were 2 µg/kg/d i.v. for one month, 25 µk/kg/d s.c. for one month and 5 µg/kg/d s.c. for 6 months. Observed adverse reactions include chronic focal hepatitis, hepatic necrosis and chronic end- and periarteritis. However, the application 5 µg/kg/d IL-4 s.c. for 6 months was the highest tolerated dose showing negligible adverse effects such as minimal increase granulopoiesis, weak hepatitis and arteritis [302]. Furthermore, IL-4 acted highly immunogenic in monkeys (intravenous > subcutaneous) leading to antibody titers over 8000. In contrast, the immunogenicity was classified as slight in clinical studies [302].

Several clinical phase I dose escalation and phase II studies have been conducted during the last years focusing on the tolerance and safety of recombinant human IL-4 in patients suffering from different kinds of malignant neoplasia or autoimmune disease such as psoriasis (*Table 4* and *Table 5*). During a literature search on Pubmed (http://www.ncbi.nlm.nih.gov; Effective May 2015), using the terms “IL-4 toxicity” (1986-

2015; 1291 hits) and "IL-4 clinical study (1990-2015; 4012 hits), 19 studies could be identified which evaluated the toxicity of rhuIL-4 in cancer or psoriasis patients. Therefore, 9 studies were clinical phase I dose escalation studies (*Table 4*) and 10 studies were either clinical phase I/II or phase II studies (*Table 5*).

Table 4: Clinical phase I dose escalation studies on IL-4 toxicity between 1986 - 2015

Author & Year	Disease	Patient Number	Application	Dosage	Treatment plan	Maximum tolerated dose (MTD)	Reference
Atkins et al. 1992	Several malignancies	10	i.v. bolus over 5 min	10, 15, 22, 30 µg/kg	every 8 h from day 1-5 and 15-19	15 µg/kg* =45 µg/kg/d	[303]
Gilleece et al. 1992	Several malignancies	9	s.c.	0.5, 1.0, 5.0 µg/kg/d	day 1 and 8-17 and (29-57)	1.0 µg/kg/d*	[304]
Prendiville et al. 1993	Several malignancies	19	i.v. bolus/ infusion	40, 120, 280 and 400 µg/m^2/d	i.v. bolus (d1) i.v. infusion (d4) s.c. (d8-21)	400 µg/m^2/d	[305]
Margolin et al. 1994	Several malignancies	27	i.v. bolus	600, 800 µg/m^2	2 times in 5d with a 9d break	600 µg/m^2	[251]
Davis et al. 2000	Several malignancies	14	s.c.	0.25, 1.0, 5.0 µg/kg/d	day 1, 8-17, 28-57	5.0 µg/kg/d*	[306]
Majhail et al. 2004	Several malignancies	26	i.v. over 24h	0.25, 0.5, 1.0, 2.0, 4.0 µg/kg	day 1-5 and 15-19	2 µg/kg/d	[307]
Ghoreschi et al. 2002	Psoriasis	22	s.c.	0.05, 0.1, 0.2, 0.3, 0.5 µg/kg/d	6 weeks	0.5 µg/kg/d*	[255]
Miles et al. 2002	AIDS-related Kaposi's sarcoma	17	s.c.	0.5, 1.5 µg/kg/d	16 weeks	0.5 µg/kg/d*	[308]
Werkmeister et al. 2004	Oral squamous cell carcinom	7	in/beside tumor	1, 3, 5 µg/kg	3 times a weeks for 4 weeks	5 µg/kg*	[309]

**MTD not clearly stated in the publication, assumed according to the toxicity description; Patient number corresponds to treated or enrolled patients, it shows not how many patients finished the study.*

As application routes most often subcutaneous injections were used beside intravenous bolus or infusion. In one study rhu IL-4 was applied in drops per os [310]. Doses of rhIL-4 ranked between 20 fg/d (p.os.) and 45 µg/kg/d (i.v.) [303, 310]. In all studies, patients had not received any chemotherapy or radiation for at least three weeks before the treatment starts. Clinical parameters were defined and baseline parameters were determined according to patient disease history. Subsequently, it was possible to relate adverse effects to IL-4 treatment. In addition, the trial was stopped in case of severe toxicities and patients were observed for at least two weeks or until the effects disappeared. The aim of the phase I

dose escalation studies was to find the maximum tolerated dose (MTD). Additionally, the dose limiting toxicity (DLT) was specified in each protocol.

Table 5: Clinical phase I/II or II studies on IL-4 toxicity and efficacy between 1986 - 2015

Author & Year	Disease	Patient Number	Application	Dosage	Treatment plan	Maximum tolerated dose (MTD)/ applied dose	Reference
Sosman et al. 1994	Several malignancies	17	i.v. over 24h	40, 120, 360, 600 µg/m^2/d	7d	360 µg/m^2/d	[311]
Stadler et al. 1995	Renal cell carcinoma	18	s.c.	1 µg/kg/d	3 times a week	1 µg/kg/d*	[312]
Tulpule et al. 1997	AIDS-related Kaposi's sarcoma	18	s.c.	1 µg/kg/d	6 months	1 µg/kg/d*	[313]
Vokes et al. 1997	Non-Small cell lung cancer	63	s.c.	0.25, 1.0 µg/kg/d	3d/week for 8 weeks	1.0 µg/kg/d*	[314]
Taylor et al. 2000	NHL	41	s.c	3 µg/kg/d	3d/week for 13-15 month	3 µg/kg/d*	[315]
Lundin et al. 2000	B-CCL	14	s.c	2, 4, 6 µg/kg/d	3d/week for 8 weeks 4 weeks rest 3 cycles	6 µg/kg/d*	[316]
Whitehead et al. 2002	Renal cell carcinoma cancer	49	s.c.	5 µg/kg/d	21d 7d rest >1 cycle	5 µg/kg/d*	[317]
Kurtz et al. 2007	NHL	41	s.c.	2.5, 5 µg/kg/d	28 d 14 d rest >1 cycle	5 µg/kg/d*	[250]
Wiernik et al. 2010	B-CCL, NHL	37	s.c	5 µg/kg/d	3d/week for 3 weeks 2 weeks rest >1 cycle	5 µg/kg/d*	[318]
Roberti et al. 2014	Psoriasis vulgaris	48	p.os.	10 fg/ml 20 drops twice/day = 20fg/d	3 months daily (cross-over)	20fg/d*	[310]

**MTD not clearly stated in the publication, assumed according to the toxicity description; Patient number corresponds to treated or enrolled patients, it shows not how many patients finished the study.*

The clinical phase I/II and II trials focused on the response rate and partially on the MTD. Therefore, the DLT as well as the primary endpoint were stated in the protocols. Toxicological effects were classified in 4 Grades according the WHO. Most frequent toxic effects were headache, fever, vomitus, fatigue, diarrhea, edema, nausea, anorexia, arthralgia as well as increased hepatic enzymes which are often described for Grade 1 and 2. Kurtz et al. observed in 25 Grade 3 or higher toxicities in the high dosed group (5 µg/kg) compared to the low dosed group (5 µg/kg) with 9 Grade 3 or higher toxicities [250]. In this study with Non-Hodgkin lymphoma patients the most common adverse effects (>40%) were edema (66%),

malaise (56%), elevated liver enzymes (56%) and headache (41%). The toxicities in the study of Whitehead et al. were slightly comparable and described as Grade 3 nausea, vomiting, diarrhea (88%), headache (82%) and malaise/fatigue/lethargy (78%) [317]. Additionally, there were a few patients with either Grade 4 diarrhea, elevated liver transaminase, pulmonary, neurologic or cardiac toxicity. The highest dose was used by Atkins et al. (15 µg/kg every 8h) in patients with several malignancies [303]. Therefore, nasal congestion, diarrhea, nausea, vomiting, fatigue, anorexia, headache, dyspnea and edema were the most frequent toxic effects (Grade 3 and higher). Low level dose caused no adverse effects Grade 3 and higher. Stadler et al. delineated that fever was mostly experienced after the first dose and fatigue became more progressive after 30 days of treatment [312]. In the study of Werkmeister et al., where rhuIL-4 was directly injected in oral squamous cell carcinomas, no toxicities Grade 3 or higher expected injection pain that could be solved by local anesthesia were observed [309]. Furthermore, both studies with psoriasis patients showed few adverse effects or only slight effects in few patients [255, 310]. In the study of Ghoreschi et al. 20 psoriasis patients were included and administered with different doses of IL-4 (5 groups: 0.05, 0.1, 0.2, 0.3 and 0.5 µg/kg) subcutaneously injected three times a day, 5 days a week for 6 weeks [255]. After 3 weeks the dose was increased to the next concentration, but not higher than 0.5 µg/kg. The clinical improvement achieved more than 50% in 19 patients and more than 68% in 15 patients whereas a significant increase in Th2-cells was verified. Only a few side effects occurred. In the highest dosage group one Grade 2 side effect of fever and headache was observed. Furthermore, 9 patients named intermitted Grade 1 side effects and two patients showed slightly edema.

In general, hepatic beside elevated enzymes and renal toxicity was only observed in some cases in patients which had a disease history in the organs before [304, 313]. In most of the studies cardiac toxicity was strictly monitored due to described cases in the literature [303, 315, 319].

2.3.6 Half-life and Stability of IL-4 - Why direct IL-4 Application would be difficult

Several publications can be found, suggesting a short half-life of IL-4 without concretization and absolute numbers [283, 285, 320, 321]. A clinical phase I study from Prendiville et al. investigated the pharmacokinetic of IL-4 in 12 patients suffering from several malignancies [305]. The patients were divided into 4 groups (n=3) and received different concentrations of IL-4: 40, 120, 280 and 400 µg IL-4/m^2/d (converted: 1, 3, 7 and 10 µg/kg/d). In detail, the patients were treated with an intravenous bolus on day 1 whereas blood samples were gathered 0, 5, 10, 15, 20, 30 and 45 min after injection. On day 4 the patients received an intravenous infusion for 24h and blood samples were taken 0, 6 and 24h during the infusion.

After day 8 a daily subcutaneous injection for two weeks was conducted whereby 1, 2, 3, 4 and 8h blood samples were examined. The administration was stopped on after 21 days and serum levels of IL-4 were measured via Enzyme Linked Immunosorbent Assay (ELISA). The median half-life of IL-4 was 19 +/- 8.7 min and differed between the groups as depicted in *Table 6*.

Table 6: The half-time of IL-4

IL-4 concentration in µg/m²/d	T1/2 in the serum in min
40	15.69
120	20.04
280	19.39
400	22.13

Modified table and results reproduced from Prendiville et al. 1993 [304]. Abbreviation: T1/2 = Half-life

Another study investigated the half-life of IL-4 in the plasma and ascites in a pancreatitis model using rats [322]. Plasma and ascetic fluid was gained after induction of pancreatitis and *in vitro* treated with 25 pg/ml IL-4. Subsequently, the samples were incubated under 37°C and IL-4 concentrations were determined after 15 and 30 min as well as one and two hours. The results showed a half-life of 16.4 min in the ascetic fluid and 51.1 min in the plasma. Another study in the cynomolgus monkeys reported a half-life between 10 and 30 min after i.v. injection whereas the half-life increased up to 3.5 und 7 h when injecting IL-4 subcutaneously [298]. Several clinical studies suggest the half-life of IL-4 less than one hour and a serum peak could be observed after 2-6 h. Collectively, the IL-4 was detectable in the serum for 8 -12h after s.c. injection [253, 323].

2.4 Controlled Expression of Transgenes

2.4.1 Recent Approaches

Controlled expression of therapeutic genes in a disease-regulated fashion is of great importance in order to avoid adverse reactions. Most gene therapeutic approaches applied so far strong genetic promoters such as the normally used Cytomegalovirus (CMV) promoter causing high levels of gene expression. For instance, constitutive expression of growth factors (e.g. IGF-1) may lead to uncontrolled cell divisions and subsequent neoplasia [324]. Furthermore, TGF-β has the potential to induce arthrofibrosis [325] and auto-reactive T cells, while IL-4 enhances the activity and proliferation of B-cells, possibly causing allergic reactions and autoimmunity [326]. Hence, transient transfection can provide safety, but is unrewarding. Consequently, inducible promoters present a promising alternative for stable

or long term transfection. The classical tet-on system, using doxycycline administration for controlling gene expression, was shown to efficiently regulate BMP-2 expression in a leporine *in vitro* chondrocyte system [327]. *In vivo* application of this system would necessitate regular monitoring and repeated doxycycline injection. More elegant approaches have taken disease-regulated promoters such as elements of IL-1 and IL-6 promoter, promoters of acute phase proteins, the Saa3 and MMP13 promoter or the coupled C3-tat/HIV promoter [328-331]. All these approaches aim at the expression activation under inflammatory conditions due to several cytokines, the upregulated transcription of transgenes during acute OA and the inhibition of expression rates after eliminating the inflammation [186, 332]. Ruan et al. recently published a study on preventing age-related OA development by applying Proteoglycan-4 under the control of the Col II promoter [226].

2.4.2 The Cox-2 Promoter

The cyclooxygenase (Cox) is the key enzyme in the prostaglandin synthesis. Two isoforms of the enzyme (Cox-1, Cox-2) have been identified. The main difference between them is the production time while the Cox-1 is continuously expressed in most mammalian tissues and cells the inducible Cox-2 is produced by the presence of various cytokines and other factors [333].

Cox catalyzes the conversion of arachidonic acid to prostaglandin H2 (PGH2) and consists of two enzymatic active compounds: the cyclooxygenase and the peroxidase. PGH2 is enzymatically transformed via the prostacyclin synthase, thromboxane-A synthase or prostaglandin D2/E synthase in either prostacyclin, thromboxane A2, and prostaglandin D2 or E2, respectively. These products play a paramount role in physiological processes such as the renal function, blood clotting, embryonic implantation, pain and fever. According to OA, the expression of Cox-2 is enhanced by several factors such as IL-1β. Additionally, the iNOS activates Cox-2 by directed binding and followed nitrosylation. Consequently, the increased amount of prostaglandin E2 leads to pain based on a hyperalgesia [334, 335].

A promoter is defined as the nucleotide sequence, upstream of a gene, to which the RNA polymerase attaches in order to initiate the transcription. These regions are also called cis-acting elements and control the transcription activity of the upstream gene [336]. The eukaryotic promoter contains the core-promoter, where the initiation complex is assembled. At these core promoters, the TATA-Box is located near the base pair -30 and the initiation sequence. Furthermore, the promoter interacts with proteins known as transcription factors. Therefore, the promoter is equipped with various specific receptors called regulatory elements. Transcription factors can activate or repress the expression of the target gene.

The Cox-2 promoter contains a TATA-box that is essential for the polymerase 2 to recognize the gene sequence (*Figure 5*). In addition, the Cox-2 promoter provides a peroxisome proliferator response element (PPRE) as binding site for peroxisome proliferators-activated receptors (PPARs) and two CAMP response elements (CRE). The promoter includes a serum response element binding to the serum response factor (SRF), a ubiquitous 67 Da protein, whose binding appears to be essential for the response of the promoter to serum stimulation [337, 338]. Furthermore, there are two NF-κB binding sites (NRE) playing a major role in cellular responses to stress, cytokines, free radicals, ultraviolet irradiation, oxidized Low-density lipoprotein (LDL), and bacterial or viral antigens [339]. Moreover, incorrect regulation of NF-κB is related to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. However, the number of specificity protein (SP1)-binding site varies between different publications, whereas the SP1 transcription factor is involved in basic cellular processes [337, 340]. The following further regulatory elements have been described: C/EBP (CAAT-Box/Enhancer binding protein element), Activation protein (AP)-2 binding sites as well as the E-box that overlaps the CRE in human and mice [337, 339]. Yang et al. reported a Nuclear factor (NF) -1 (-410/-386) binding element that is regulated by TGF-β and Tazawa et al. further mentioned a polyomavirus enhancer activator 3 [341, 342].

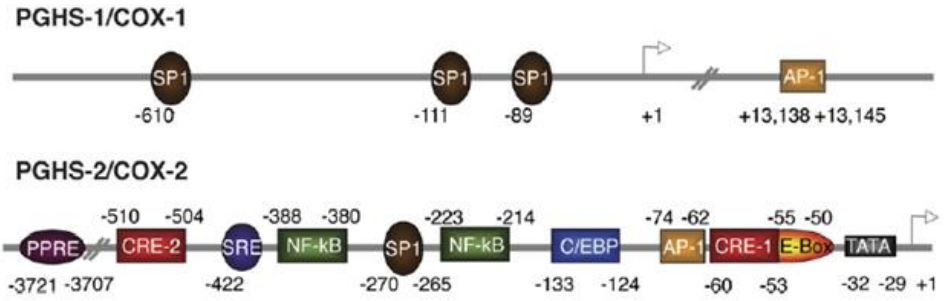

Figure 5: Comparison of regulatory elements on the Cox-1 or Cox-2 promoter.
Figure with legend reproduced from Kang et al. 2007 [337]

Moreover, a large number of signal transduction pathways and transcription factors are involved in the transcriptional regulation of Cox-2 and they vary depending on stimulus and cell type. The induction of Cox-2 in RAW 264.7 cells is mediated by various inflammatory cytokines, catalases, peptidoglycans, double-stranded RNA, viral infection and LPS [343, 344]. In LPS-stimulated RAW 264.7 cells a two phasic Cox-2 production could be reported [343]. Therefore, NF-κB, C/EBP sites and the CRE-1 play an important role in the regulation and supposable lead to persistent gene activation. The transcriptional regulation is schematically depicted in *Figure 6*.

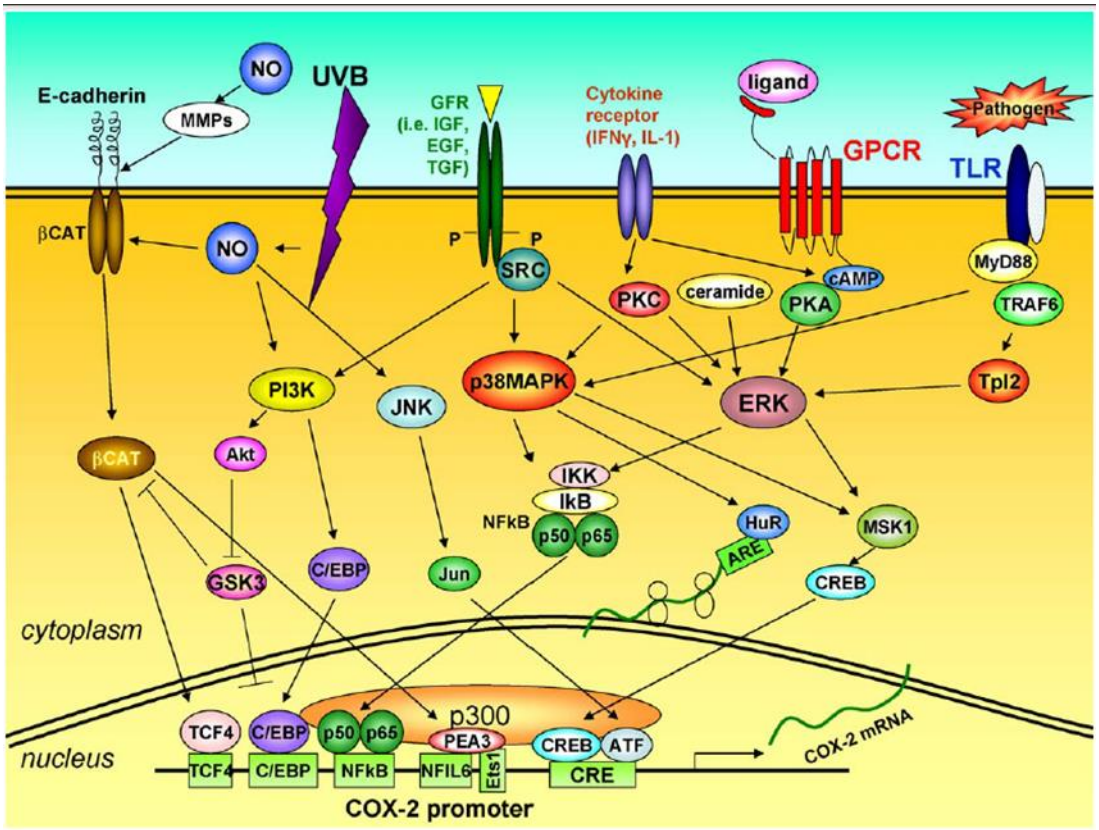

Figure 6: Transcriptional regulation of the Cox-2 promoter.
Figure with legend reproduced from Tsatsanis et al. 2006 [339]

The regulation of the Cox-2 expression is also controlled by complex mechanisms on the post-transcriptional and post-translational level as well as the Cox-2 protein degradation, which will be not discussed in any greater detail here.

In 1994 the entire human Cox-2 gene was isolated from a human bacteriophage P1 genomic library and the promoter was firstly characterized [345]. A second study on the human Cox-2 gene was published in 1997, where they cloned a human lung fibroblast genomic library in a plasmid and screened it by plaque hybridization using mouse Cox-2 cDNA. Four different clones which include the 5'-flanking region were identified, whereas one of them was generated and sequenced. Furthermore, they examined the promoter activity via luciferase reporter assay, demonstrating a basal promoter activity between -77bp and +99bp and a maximum promoter activity from -1838 bp to +99bp [342]. The equine Cox-2 gene was first described in 1998 by Boerboom et al. and the sequence was submitted to the GenBank [346].

2.4.3 Regulated Expression of IL-4 under the Control of the Cox-2 Promoter

During the last years, several studies at the Institute for Immunology (FU-Berlin) under the guidance of Prof. Schmidt have been performed in a canine OA *in vitro* model and reported

effective anti-inflammatory features of IL-4 [107, 224, 347, 348]. Therefore, the investigations include the development and optimization of a safe method to control the transgene expression. To avoid adverse reaction by the introduced transgene products, diffusing into the blood circulation, the expression should be self-limiting and only applied in case of inflammation. Thus, a promoter sequence was placed in front of the therapeutic gene to be regulated [347]. Therefore, the Cox-2 promoter has been chosen as suitable because the expression of the cyclooxygenase (Cox-2) is induced by various cytokines especially IL-1β or other factors such as LPS. Consequently, the Cox-2 is only expressed when mediators of inflammation are present. Thus, the highlight of the approach is to amplify the Cox-2 promoter and clone it upstream of the therapeutic gene IL-4 (*Figure 7*). Thereby, the production of the therapeutic gene is controlled by the severity of the disease. That means, if inflammation increases and typical mediators act on the transfected cells, the Cox-2 promoter is activated and the therapeutic protein produced. Hence, inflammation will be down-regulated and the expression of the therapeutic gene stops via feedback-loop [348]. This approach has never been transferred to other species and was only evaluated for the canine system.

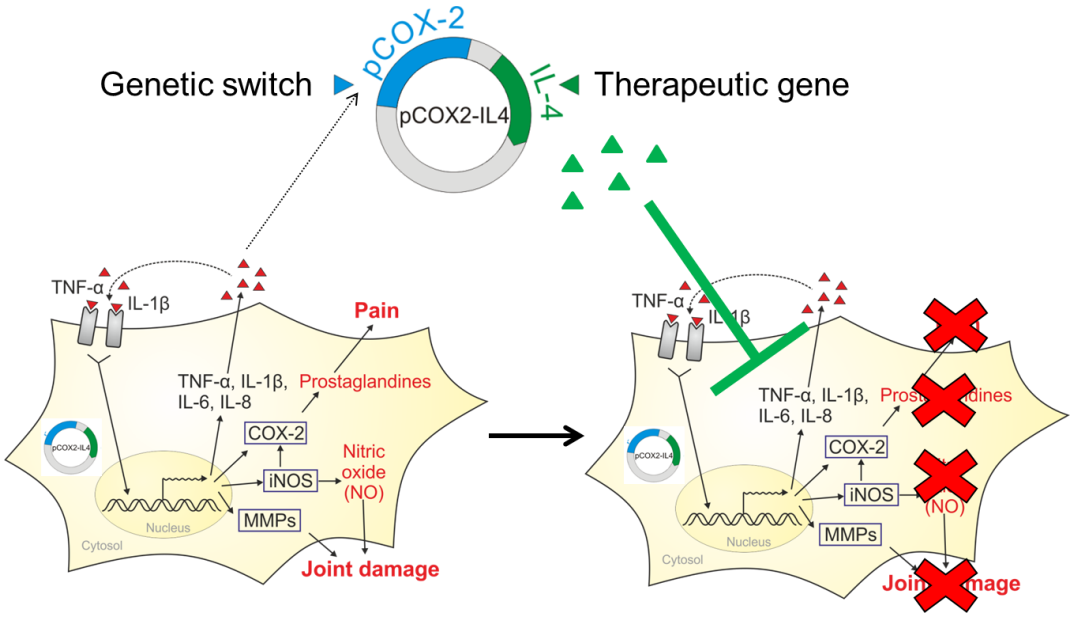

Figure 7: Mechanism of action of the new gene therapeutic approach.

The presence of IL-1β in osteoarthritic cartilage activates the Cox-2 promoter and subsequently IL-4 is produced that inhibits the IL-1β expression as well as the consequences. Figure modified and reproduced from Schmidt et al. 2010 [349].

2.5 The Horse as Animal Model

2.5.1 Overview of Different Animal Models - State of the Art in Osteoarthritis Research

Animal models serve as model systems for simplifying, studying and understanding complex courses of human diseases [350]. *In vitro* data can only help to get a first idea of interactions between different factors or effects of possible treatments. But to examine the processes in the physiological acting organism, animal models are needed.

Over the past years many animal models for OA have been developed and investigated. They can be divided into following underlying mechanisms: spontaneous OA, chemically-induced or physically-induced OA [351]. The spontaneous development of OA in different animals can be induced by using collagen mutation in mice, genetic predisposition such as hip dysplasia in dogs or the natural occurring parameters age and obesity in Hartley guinea pigs. In addition, a naturally occurring model in rabbits has been described and characterized by Arzi et al. [352]. To generate a chemically-induced OA, degrading enzymes (papain, iodioacetate, and collagenase) are injected into the joint to destabilize it by damaging articular cartilage and other relevant structures. Mostly mice or guinea pigs are taken for such a model which parallels rather the post-traumatic acute OA than the primary (idiopathic) chronic and slow progressive OA in humans. These kind of models are widely used in rheumatoid arthritis research, because of the very progressive destruction and the induced immune response [353]. Some of the physically-induced OA models are relevant to prove the efficiency of gene therapy combined with tissue engineering. The destabilization of the joint is achieved by surgical procedures where the anterior cruciate ligament (ACLT) is transected or a meniscectomy is undertaken. Especially rats, rabbits and dogs have been frequently utilized for these models. Boguszewski et al. developed a porcine ACL model that can be used for developing reconstructions strategies and Kon et al. performed a meniscectomy in a sheep model for tissue engineering approaches [354, 355]. Another strategy is to create osteochondral/full-thickness defects of a defined size into the articular cartilage removing the cartilage up to the subchondral bone plate. For this latter procedure large animals such as dogs or horses have been utilized because of the size of the defects and the resulting improved practicability [356]. Nevertheless, it is very common to take rabbits for this kind of modelling [203, 212].

One problem of OA animal models is the lack of standardization. Therefore, Little et al. stated that it is inalienable to define sub-type models and to precise the extrapolation to the human conditions during the designing process [357]. The OARSI published different guidelines for the evaluation of histopathology parameters in each OA animal models [358-363] to standardize and render outcomes comparable. But the histomorphometric parameters

vary between the species [364]. Consequently, the decision of the used OA model has to be made careful in order to choose the appropriate model for the specific research question. An important key word that has to be mentioned at this point is “translatability” of an animal model, playing a crucial role in the drug development, where lots of drawbacks during the last decade have shown the importance [365]. In OA research, for example, the dog is an ideal species for translational investigations on biomarkers [366]. In contrast, according biomarkers or the finding of the initial events mice are mostly inappropriate due to their short lifespan and fast metabolism. Furthermore, Ma et al. described a species difference in the WNT signaling that is important in cartilage [367]. But most data according the pathway are investigated in mice, therefore, the extrapolation to the human is challenging.

The use of gene modified mice has been exponentially grown during the last decade, enforced by the development of new breeding techniques and knowledge about the genome. In OA, several GM mice were developed to examine the molecular pathways of the disease progression. But there are large differences to the human resulting in a slower translation process [368]. To overcome such hurdles, researchers started to develop so called “humanized animal models” meaning the breeding of mice including a human hematopoietic system or genetic components from human that are of interest [369]. This technique prompts the question of functionality and similarity because of the different environment and far more active metabolism of mice.

Different standards are demanded for animal models [350]. The animals have to show a genetic homogeneity or heterogeneity and the environment should have similar conditions. Additionally, the life span of the animals might parallel the time span of the disease as well as the physiological and anatomical attributes should be similar to the human characteristics. Especially animal age has to be considered in surgical OA models due to the age driven changes leading to the specific pathogenesis [370]. Furthermore, standards such as the availability of sufficient joint tissue for the studies, the ease of handling and secured funding are all essential considerations. However, until present many animal models are utilized which reproduce only some key aspects of human OA. Ideal models should parallel the history, pathogenesis and symptoms of the human disease in almost all facets [7]. Furthermore, these model sub-types have to be defined according their field of application. Some researches supposed to optimize animal models by going the other way around, in detail, applying medications that are administered successful in the human to the animal [365]. The ARRIVE-guidelines were published in 2010 and are related to OA research [371]. Nevertheless, it is important to read these guidelines before the animal study and not even before writing the publication [357].

2.5.2 The Horse as Animal Model for OA Research

In Germany approximately 33.7% of treated horses in the clinics suffer from diseases affecting the musculoskeletal-system and are incident to high costs. In North America the costs reach the total of $1 billion annually and the incidence for lameness lies between 8,5 up to 13.7% [372, 373]. Especially racing thoroughbreds are susceptible to fetlock pain and lameness (25%) leading to the early retirement of equine athletes and a high economic loss to the equine industry [372, 374, 375]. Therefore, up to 60% of lameness are caused by osteoarthritic changes of the joints [376]. Particularly in juvenile horses specific osteochondral diseases can be observed comprising osteochondrosis (OC) with an incidence of 30-40% in certain breeds leading to OA when untreated [374, 377]. OC is supposed to result from interplay of damage to the microvasculature with subsequent chondronecrosis and biomechanical forces resulting in osteochondral fragmentation.

The horse is a naturally occurring model which parallels the human disease in many aspects [378]. Frisbie et al. investigated the cartilage thickness in the stifle joint of different species and showed that especially the stifle joint of horses provides the closest approximation to the human thickness of articular cartilage. Five locations within the joint were compared, whereas the thickness of human averages 2.2–2.5 mm, of rabbit 0.3 mm, of sheep 0.4–0.5 mm, of dogs 0.6–1.3 mm, of goats 0.7–1.5 mm and of horses 1.5–2 mm [379]. In rabbits the defect diameter amounts 3.2 - 5 mm with an depth up to 3 mm [200], whereas the diameter circular of the defects in the horse can achieve up to 15 mm [204]. The load conditions in weight-bearing areas of the cartilage differ between horse and human [380]. But it allows the comparative examination of postoperative parameters immediately after surgery [356]. Therefore, special scores indicate the grade of lameness as well as joint effusion that can be recognized by palpating [214]. Imaging techniques are widely available and applicable such as radiographs (portable versions) or CT and MRT [381]. Similarly, especially MRT techniques have been optimized for small animals in order to visualize cartilage changes and synovial membrane alterations [382]. Nevertheless, the horse provides the opportunity for arthroscopic interventions to obtain synovial fluid, to assess the cartilage shape macroscopically and to harvest small pieces of cartilage for molecularbiological investigations [383]. In contrast, using rabbits as animal models, histological parameter are mostly compiled, leading to less comprehensive examinations and requiring more animals that can be euthanized on different days to evaluate the progression/course of the disease or treatment, respectively [200, 205]. Another point is that even more small animals are needed for the same research question in order to obtain enough material at the end, to gather more outcome parameters or even to harvest cells for ACT [197]. That means using the horse as animal model can lead to a reduction of animal testing. A systemic review

listed the average number of used animals amount for the murine model 30, for the lapine model 18.86 and for the equine model 9 [380].

Nevertheless, there are a few disadvantages which have to be mentioned and assessed. Particularly, the cost of the horse model is much higher compared to small animal models, even though the number of animals can in some cases be reduced by taking more joints for the examination [384]. However, each year the equine industry registers high losses by euthanizing many racehorses after leaving the racetrack. Through cooperation between researchers, equine industry and community, research costs can be significantly reduced [356]. Further problems are the high anesthetic risk and the first attempts of the animals to stand up after surgery. These attempts are difficult to control and can stress the joint, whereby enormously, e.g. in transplantation studies, the cartilage transplant can be shifted.

There are different types of modelling OA in the horse. The commonly used model is based on creation of full-thickness defects with an average diameter size of 15 mm [379, 385]. These defects can be immediately filled with autologous cells, transplants or stay free as control. Moreover, the joint can be treated via intra-articular injection of gene therapeutic viral plasmids. The cells for producing the transplants can be obtained by arthroscopic removal from a non-weight bearing area of another joint or from the direct defect creation step and isolating the removed chondrocytes [386]. In contrast to the methods where two surgeries are needed, a one-step surgical procedure was invented, called CAIS (cartilage autograft implantation system) [387]. Frisbie et al. developed a model comprising osteochondral fragments in the carpal joint due to the similar loading conditions comparable to human and includes regular exercise in the study design to further osteoarthritic changes in the affected joint [388]. In contrast, many other groups focus on the femoropatellar joint [386]. Furthermore, there have been different approaches based on intra-articular injection of chemicals, simulating rather a septic arthritis including a large immune response than OA. Therefore, filipin, sodium monoiodoacetate, amphotecerin, *E.coli* LPS, IL-1 as well as polyvinylalcohol and carrageenan were applied [389-392]. Two studies were performed, utilizing joint instability via carpal fractures or cutting collateral sesamoidean ligaments in the MCP joint [393, 394].

Concerning gene therapy, there are a lot of *in vitro* studies which use equine cells, but only a few preclinical studies have employed the horse as an animal model so far. The effect of IL-1 Ra injected directly into an experimentally induced osteoarthritic joint was examined in 2000 [388]. Clinical symptoms were improved and also the content of proteoglycans increased. Two similar studies [204, 215, 385] used an *ex vivo* approach instead of a direct injection of naked DNA. Chondrocytes were transfected with IGF-1 or BMP-7 and showed an increase of a hyaline-like repair tissue without significant differences between the treated

and control groups. Furthermore, viral plasmids were extensively tested for their ability to transfect chondrocytes and synoviocytes *in vivo*, the timespan of transfection as well as systemic effects, the existences of viral/plasmids proteins and immunological responses [388, 395-397]. Frisbie et al. recognized lymphatic infiltration and edema in the synovial membrane after intra-articular injection of ADEqIL-1Ra [388]. Similar results were obtained by Ishihara et al. testing different viral plasmids indicating that the application of AAV is much safer than adenoviral plasmids leading to an increased immune response [396]. Additionally, new developed self-complementary AAV have successfully been tested for high levels of inserted protein production [395].

CHAPTER 3: Aims and Objectives of the Thesis

Many approaches have been developed and validated to treat OA properly, but beside pain medication and protective substances for the remaining cartilage, a complete restoration of the cartilage still remains elusive. Therefore, the combination of tissue engineering with gene therapeutic approaches seems to be a promising strategy and has been further optimized during the last years.

Interleukin-4 (IL-4) provides expedient properties to act as an effective transgene for gene therapeutic purposes towards OA therapy. This has been shown in many animal studies on RA. Only a few animal studies exist on the efficacy of IL-4 in OA. Furthermore, the potential toxicity of IL-4 encoded in a gene therapeutic plasmid and administered into the joint is still unclear as it is also for other potential transgenes. Current gene therapeutic approaches comprise the constitutive expression of transgenes driven by the CMV-promoter. Therefore, new approaches aim at the controlled, disease-driven expression by use of inducible promoters. One of these approaches was developed and tested in a canine OA *in vitro* model in the group of Prof. Michael F.G. Schmidt (Institute for Immunology, FU-Berlin). They used the Cox-2 promoter as genetic switch and cloned it upstream of the therapeutic IL-4. The results showed that an inflammation-regulated expression of IL-4 could be achieved. This approach has never been transferred to other species beside the canine system.

The horse as naturally occurring model is a suitable animal model for OA since it parallels the human form of OA in many aspects. Moreover, OA is becoming more and more common in horses and is responsible for large financial losses in the horse industry.

The aim of this thesis was to transfer and verify the previously developed gene therapeutic approach (described in 2.4.3) in an *in vitro* equine cell culture model. The first and main part includes the experimental work which aims to verify the transferability of the gene therapeutic approach to the equine system as well as the establishment and optimization of the equine cell culture system and the transfection procedure. The second part of this thesis includes a copious literature search/review on the state of the art in OA gene therapeutic research (feasibility and safety), the possible risk and toxicity of IL-4 as effective transgene, and the question of suitable animal models for OA research.

The work packages are the following (see also *Figure 8*):

Part 1: Experimental Work

a. ***Development of an equine inflammatory in vitro 2D chondrocyte model:*** This work package includes the cell isolation and cultivation as well as the optimization

of possible inflammatory stimulants that can be used *in vitro*. Therefore, recombinant equine (req) IL-1β and LPS are tested for their ability to create a proper inflammatory environment to test the therapeutic approach.

b. ***Generation of an equine inflammatory-sensitive plasmid system comprising the Cox-2 promoter and IL-4:*** This work package covers the assessment of the transferability of the canine Cox-2 promoter to the equine system (alignment study) and the following cloning of the equine Cox-2 promoter regulated plasmids expressing IL-4 in an inflammation-dependent manner. Furthermore, the optimization of the transfection protocols and plasmid design is addressed.
c. ***Test of the functionality of a "genetic switch" (Cox-2 promoter):*** The equine Cox-2 promoter is tested for its functionality to activate the expression of IL-4 in inflammatory stimulated chondrocytes.
d. ***Evaluation of the anti-inflammatory potential of IL-4:*** The last work package focuses on the anti-inflammatory potential of IL-4 that has been expressed under the control of the Cox-2 promoter and is able to inhibit inflammatory mediators.

Part 2: Literature Review

e. State of the Art in OA Gene Therapeutic Research (Chapter 2.2)
f. Interleukin-4 – Possible Risk and Toxicity (Chapter 2.3 and 6.5)
g. Animal Models in OA Research (Chapter 2.5)

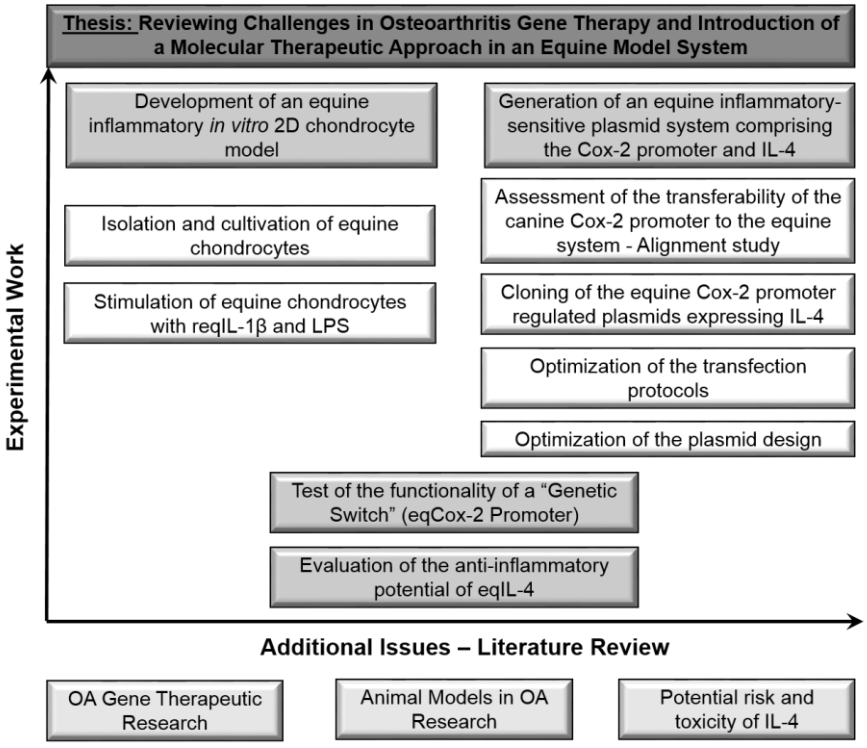

Figure 8: Structure and work packages of the thesis

CHAPTER 4: Materials and Methods

4.1 Materials

4.1.1 Animal Samples

A total of 5 horses aged 5-15 were included in this study. They were euthanized and had no history of joint disease. Two cartilage samples were harvested from horses that had been provided to the Institute of Veterinary Pathology, Freie Universität Berlin for further investigations. Three chondrocyte samples were provided from Dr. Igor Ponomarev, Clinic for Large Animals, Research Center of Medical Technology and Biotechnology, Bad Langensalza. Whole blood for DNA isolation was provided from the Equine Clinic, Freie Universität Berlin.

Number	Age	Sex	Provider
Horse 1	7 years	male	Institute of Veterinary Pathology, Freie Universität Berlin
Horse 2	5 years	male	
Horse 3	15 years	female	Clinic for Large Animals, Research Center of Medical Technology and Biotechnology, Bad Langensalza
Horse 4	11 years	female	
Horse 5	13 years	male	

4.1.2 Cell Culture

Description	Manufacturer	City, State
DMEM, FCS, PBS, HANKS	Biochrom AG	Berlin, Germany
Collagenase CLS II		
Collagenase P	Boehringer	Mannheim, Germany
Hyaluronidase	Sigma-Aldrich Biochemie GmbH	Hamburg, Germany
Accutase		
DMSO		

4.1.3 Plastic-ware/Miscellaneous

Plastic-ware was purchased from PEQLAB Biotechnologie (Erlangen), Greiner Bio One (Frickenhausen), Sarstedt (Nümbrecht) or Eppendorf (Hamburg) unless stated otherwise.

Description	Manufacturer	City, State
Falcon Cell Strainer (100 µm)	Becton Dickinson Labware	Claix, France
ELISA Plates 96-well flat bottom	NUNC	Wiesbaden, Germany
Real-Time PCR 96-well plates	Bio-Rad Laboratories	Munich, Germany
Sterile Filter (0.22 µm)	Merck Millipore	Billerica, MA, USA
Spinner flasks	Wheaton, Science Products	Millville, NJ, USA

4.1.4 Antibiotics

Description	Manufacturer	City, State
Kanamycin	Carl Roth GmbH	Karlsruhe, Germany
Penicillin/ Streptomycin	Pan Biotech	Aidenbach, Germany

4.1.5 Cell Staining – Cell Viability

Description	Manufacturer	City, State
Trypan Blue Solution, 0.4%, Propidium iodide	Thermo Scientific (Life Technologies)	Karlsruhe, Germany

4.1.6 Recombinant Proteins and LPS

Description	Manufacturer	City, State
eqIL-1β	R&D Systems	Minneapolis, MN, USA
LPS (*E.coli* 055:B5)	Sigma-Aldrich Biochemie GmbH	Hamburg, Germany

4.1.7 Plasmids

Description	Manufacturer	City, State
pIRES2-EGFP, pEGFP-N3	Clontech Laboratories	Mountain View, CA, USA

4.1.8 Enzymes

Description	Manufacturer	City, State
Phusion High-Fidelity DNA Polymerase, T4 DNA Ligase, AseI, BglII, Bam HI, XhoI	New England Biolabs	Frankfurt am Main, Germany

4.1.9 Agarose Gel Electrophoresis

All reagents were purchased from Sigma-Aldrich Biochemie GmbH (Hamburg) or Carl Roth (Karlsruhe) if not stated otherwise.

Description	Manufacturer	City, State
Agarose	Invitrogen	Karlsruhe, Germany
UltraPure™ Ethidium Bromide	Thermo Scientific (Life Technologies)	Karlsruhe, Germany
GeneRuler DNA Ladder Mix	Thermo Scientific	Karlsruhe, Germany

4.1.10 Preparation of Competent Cells

All reagents were purchased from Sigma-Aldrich Biochemie GmbH (Hamburg) or Carl Roth (Karlsruhe) if not stated otherwise.

Description	Manufacturer	City, State
XL1-Blue Cells	Stratagene	Santa Clara, CA, USA
Bacto Trypton	BD Bioscience	San Jose, CA, USA
Bacto Yeast Extract		

4.1.11 Transfection Reagents

Description	Manufacturer	City, State
Fugene 6 and HD	Promega	Mannheim, Germany
XtremeGene 9 and HD	Roche Diagnostics	Mannheim, Germany
NanoJuice	Merck Millipore	Billerica, MA, USA
TurboFect	Thermo Scientific	Karlsruhe, Germany
Trans IT 2020	Mirus Bio LLC	Madison, WI, USA

4.1.12 IL-4 Enzyme Linked Immunosorbent Assay (ELISA)

Description	Manufacturer	City, State
Bovine serum albumin	Sigma-Aldrich Biochemie GmbH	Hamburg, Germany
Tween20		
Normal goat serum		
3,3',5,5' tetramethylbenzidine		
Citric acid	Mirus Bio LLC	Madison, WI, USA
H_2O_2	Merck Millipore	Billerica, MA, USA
2N H2SO4		
PBS	Biochrom AG	Berlin, Germany

4.1.13 Western Blot Analysis

All reagents were purchased from Sigma-Aldrich Biochemie GmbH (Hamburg) or Carl Roth (Karlsruhe) if not stated otherwise.

Description	Manufacturer	City, State
Equine IL-4 antibody	R&D Systems	Minneapolis, MN, USA
β-Actin antibody	Abcam	Cambridge, United Kingdom
anti-mouse-HRP	Promega	Madison, WI, USA
anti-goat-HRP		
PageRuler Prestained Protein Ladder	Thermo Scientific (Fermentas)	Karlsruhe, Germany
Coomassie blue G-250	SERVA Electrophoresis GmbH	Heidelberg, Germany
Immun-Star HRP (Luminol)	Bio-Rad Laboratories	Munich, Germany

4.1.14 Kits

Use	Description	Manufacturer	City, State
DNA isolation	Nucleo Spin Blood Kit	Macherey-Nagel GmbH & Co. KG	Düren, Germany
DNA Isolation PCR	Invisorb Fragment Cleanup	STRATEC Biomedical AG	Birkenfeld, Germany
Isolation of plasmid DNA	Invisorb Spin Plasmid Mini Two	Invitek	Berlin, Germany
	NucleoBond Xtra Midi EF	Macherey-Nagel GmbH & Co. KG	Düren, Germany
Transfection	Amaxa® Human Chondrocyte Nucleofector®	Lonza	Basel, Switzerland
	PolyMAG	chemicell GmbH	Berlin, Germany
RNA isolation	Universal RNA purification Kit	Roboklon	Berlin, Germany
	RNeasy Mini Kit	Qiagen	Hilden, Germany
Bioanalyzer	Agilent RNA 6000 Nano	Agilent Technologies	Santa Clara, CA, US
RNA storage	RNA*later*	Qiagen	Hilden, Germany
cDNA synthesis	Revert Aid First Strand cDNA Synthesis Kit	Thermo Scientific	Karlsruhe, Germany
	Sensiscript Reverse Transcription Kit	Qiagen	Hilden, Germany
	Rnase-Free Dnase Set	Qiagen	Hilden, Germany
qRT-PCR	LightCycler Fast Start DNA Master SYBR Green I	Roche Diagnostics	Mannheim, Germany
ELISA	Equine IL-4 DuoSet	R&D Systems	Minneapolis, MN, USA
Protein isolation	Nuclear Extract Kit	Active Motif	La Hulpe, Belgium

4.1.15 Instruments

Description	Manufacturer	City, State
Beckmann Coulter Counter Epics XL-MCL	Beckmann Coulter GmbH	Krefeld, Germany
Biofuge Stratos	Heraeus Instruments GmbH	Hanau, Germany
Feinwaage MP-300	ASA	Schermbeck, Germany
Hettich Zentrifuge Mikro 120	Hettich Zentrifugen	Tuttlingen, Germany
Labofuge 400R	Heraeus Instruments GmbH	Hanau, Germany
Fresco 17 Centrifuge	Thermo Scientific	Karlsruhe, Germany
Nano-Drop Spektrophotometer ND-1000	PeqLab Biotechnologie GmbH	Erlangen, Germany
iCycler iQ-5	Bio-Rad Laboratories	Munich, Germany
Stratagene Mx3000P qPCR	SABiosciences (Qiagen)	Hilden, Germany
SpectraMax Plus 384 Microplate Reader	Molecular Devices	Sunnyvale, CA, USA
2100 Electrophoresis Bioanalyzer Instrument	Agilent Technologies	Santa Clara, CA, USA
Amaxa Nucleofector	Lonza	Basel, Switzerland
Thermomixer 5436	Eppendorf	Hamburg, Germany
PCR-Mastercycler Gradient	Eppendorf	Hamburg, Germany
Bio-Rad Basic Power Supply	Bio-Rad Laboratories	Munich, Germany
Bio-Rad Mini Protean II		
Bio-Rad Trans-blot SD transfer cell		
ImageQuant LAS 4000	GE Healthcare Life Sciences	Freiburg, Germany

4.1.16 Software and Online Services

Description	Manufacturer	City, State
GraphPad Prism V.5 software	GraphPad	San Diego, CA, USA
Coulter Epics XL MCL	Beckmann Coulter GmbH	Krefeld, Germany
Expo 32 ADC software		
FlowJo software	Tree Star	Ashland, OR, USA
Optical system software version 2.0	Bio-Rad Laboratories	Munich, Germany
Stratagene Mx3000P qPCR	SABiosciences (Qiagen)	Hilden, Germany
SoftMax Pro Data Acquisition and Analysis Software	Molecular Devices	Sunnyvale, CA, USA
VerctorNTI Advance	Thermo Scientific (Life Technologies)	Karlsruhe, Germany

Description	URL
National Centre for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/
Ensembl equine genome data base	http://www.ensembl.org
A plasmid editor	http://biologylabs.utah.edu/jorgensen/wayned/ape/
Basic Local Alignment Search Tool (BLAST)	http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome
OligoCalc	http://www.basic.northwestern.edu/biotools/oligocalc.html
Serial Cloner	http://serialbasics.free.fr/Serial_Cloner.html

4.1.17 Solutions and Compositions

Use	Description	Composition
Cell culture	Growth medium	DMEM 500 ml, FCS 50 ml and Pen/Strep stock 5 ml
	Pen/Strep stock	10,000 U/ml Penicillin, 10 mg/ml Streptomycin
Agarose gel electrophoresis	TAE buffer (1L)	40 mM Tris·HCl, 20 mM acetic acid, 1 mM EDTA, pH: 8.0 (from 50× stock solution)
ELISA	Reagent diluent	1% (w/v) BSA in PBS, pH 7.2-7.4
	Substrate solution	TMB solution: 240 µg/ml 3,3',5,5' tetramethylbenzidine Gallati buffer: 42 µg/ml citric acid, pH: 3.95 0.01% (v/v) H_2O_2
	Wash buffer	PBS, 0.05% (v/v) Tween
Bacteria growth medium and agar	SOC medium	LB-medium + 20 mM $MgCl_2$ + 20 mM $MgSO_4$ + 20 mM Glucose and autoclave for 15 min at 121°C (100 kPa)
	LB-agar	12 g select agar/l of LB medium and autoclave for 15 min at 121°C
Preparation of competent cells	TYM medium	2.0% (w/v) Bacto Trypton, 0.5% (w/v) Bacto Yeast Extract, 0.1 M NaCl, 10 mM $MgSO_4$, with dest H_2O ad 1000 ml
	TFB I (pH: 6.2)	30 mM K-acetat, 10 mM $CaCl_2$, 100 mM RbCl, 15% (v/v) Glycerin, 50 mM $MnCl_2 \cdot 4H_2O$, with dest H_2O ad 100 ml; steril filtration (0.22 µm)
	TFB II (pH: 7.0)	10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) Glycerin, pH modulation with 1N KOH, with dest H_2O ad 50 ml; sterile filtration (0.22 µm)
Western Blot	AB-3	48 g acrylamide, 1.5 g of bisacrylamide in 100 ml H_2O
	Stacking gel (4%)	1 ml AB-3, 3 ml 3x Gel buffer, H_2O ad 12 ml; Polymerization: 90 µl APS (10%), 9 µl TEMED
	Separating gel (16%)	10 ml AB-3, 10 ml 3x Gel buffer, 3 g Glycerol, H_2O ad 30 ml; Polymerization: 100 µl APS (10%), 10 µl TEMED
	Anode buffer (10x)	1 M Tris, 0.225 M HCL; pH: 8.9
	Cathode buffer (10x)	1 M Tris, 1 M Tricine, 1% SDS; pH: ~8.25
	Gel buffer (3x)	3 M Tris, 1 M HCL, 0.3% SDS; pH: 8.45
	Reducing sample buffers	12% SDS (w/v), 6% mercaptoethanol (v/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250, 150 mM Tris/HCl (pH 7.0)

4.2 Methods

4.2.1 Tissue Collection and Isolation of Equine Chondrocytes

Articular cartilage tissue was aseptically collected within 72h post-euthanasia from 5 horses that had no history of joint diseases or macroscopically noticeable pathological changes. During the transport cartilage samples were kept intact, covered with a phosphate buffered saline (PBS)-soaked drape and stored on ice. The further cell isolation process was performed under a laminar flow hood. A previously described protocol [398] was slightly modified as described briefly in the following.

The cartilage surface area of the joint was treated with 70% (v/v) ethanol and cartilage was carefully dissected with a scalpel from the bone. Cartilage samples were collected in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% (v/v) heat inactivated fetal calf serum (FCS) and 1% (v/v) penicillin/ streptomycin (Pen/Strep). After washing the cartilage slices three times with Hanks' Balanced Salt Solution (HANKS, HBSS) the cartilage was sliced into 2 mm-pieces in a Petri dish containing fresh medium, and suspended in DMEM, 10% (v/v) FCS and (v/v) 1% Pen/Strep. For digestion of cartilage, an enzyme cocktail, including 30 units/ml of collagenase P and collagenase CLS II, respectively, and 1,000 units/ml hyaluronidase, was added to the suspension. The cartilage enzyme mix was incubated for 16-18 h in a spinner flask on an orbital shaker at 37°C and 5% CO_2. Afterwards, the homogenous cell suspension was filtered through a standard cell filter (pore size 100 µm), centrifuged at *500 g* for 10 min and the sediments were washed with HANKS solution. Final sediments were suspended in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) Pen/Strep. Cell count and cell viability were assessed using a Neubauer chamber and trypan blue exclusion staining.

4.2.2 Chondrocyte Monolayer Cultures

Primary chondrocytes were seeded in tissue culture flasks at a density of $2x10^5$ cells/ml followed by cultivation in a humidified CO_2 incubator (95% air and 5% CO_2) at 37°C. The composition of the growth medium for all cultures was DMEM with 10% (v/v) FCS and 1% (v/v) Pen/Strep unless indicated otherwise. In addition, the growth medium was changed two times per week depending on growth rate and medium consumption, thereby cells were washed twice with 1x calcium-free and magnesium-free PBS solution. When cells reached a confluence of 70-80%, they were passaged by detachment from the dishes with Accutase. To visualize phenotypic changes, photographs were taken at selected passages (P0, P2, P3, P6).

4.2.3 Cryopreservation of Chondrocytes

Freshly isolated as well as first passaged chondrocytes were cryopreserved to stock up for further use. Therefore, 1x10^6 cells/ml were suspended in DMEM, 10% (v/v) FCS, and 10% (v/v) dimethylsulfoxide (DMSO). The cell suspension was put into cryopreservation containers stored at -80°C for 2 days and subsequently transferred to liquid nitrogen until further use.

4.2.4 Alignment Study Comparing the Canine and Equine Cox-2 Promoter

In previous studies the functionality of an inflammation-responsive canine plasmid including the Cox-2 promoter was shown [332, 399]. In order to evaluate the transfer potential of the amplified canine Cox-2 promoter, an alignment study, using Vector NTI AlignX, was performed with the following nucleotide sequences from the National Centre for Biotechnology Information (NCBI): AF027335 (equine Cox-2 promoter base 1-1360) and EU249362 (canine Cox-2 promoter [399]). Owing to the species specificity of IL-4, we were not able to transfer the IL-4 DNA to the equine system. Subsequently, IL-4 and the Cox-2 promoter primer were designed and tested as described in 4.2.5 and 4.2.6.

4.2.5 Primer Design

Sequences for the genes of interest were obtained from the National Centre for Biotechnology Information, and were cross-referenced to the *Ensembl* equine genome data base (www. ensembl.org). Primer sequences were designed using “A plasmid editor”. Thereafter, a Basic Local Alignment Search Tool (BLAST) search was completed using OligoCalc for all primer sequences. The synthesis of primers was performed by TIB-Molbiol. *Table 7* and *Table 8* list the equine primer sequences used for cloning and qRT-PCR.

Table 7: Oligonucleotide primer sequences used for cloning.

Gene	Accession no.	f/r	Sequence 5’ – 3’	Position	Amplicon size (bp)
pCox-2	AF027335	f	TGATTAATGAGCATCTCTGCAGATCCGGGG	1418 - 1439	1199
		r	GCAGATCTCTGGGAGGCAGTGCTGGAGGAG	241 - 262	
IL-4	AF305617	f	GTCTCGAGATGGGTCTCACCTACCAACTG	394 - 414	414
		r	CTGGATCCTCAACACTTGGAGTATTTCTCTTTC	1 - 25	

Table modified and reproduced from Lang et al. 2014 [8]; Abbreviation: p= promoter sequence; Restrictions sides are underlined

Table 8: Oligonucleotide primer sequences used for qRT-PCR.

Gene	Accession no.	f/r	Sequence 5' – 3'	Position	Amplicon size (bp)
IL-1β	ECU92481	f	GGCCTCAAGGAAAAGAACCT	413 - 432	191
		r	TGGGGTACATTGCAGACTCA	242 - 261	
Cox-2	AF027335	f	CAGATCCTAAGCGAGGTCCA	6208 - 6227	547
		r	TGAGGCGGGTAGATCATTTC	5681 - 5700	
IL-4	AF305617	f	CAAAACGCTGAACAACCTCA	189 - 208	161
		r	TTGAGGTTCCTGTCCAGTCC	48 - 67	
IL-6*	AF005227.1	f	CAAGCACCGTCACTCCAGTTGC	413 - 432	191
		r	CATCTTCTCCCAGGGGTAGTGGG	242 - 261	
IL-8*	AY184956.1	f	TGCTTTCTGCAGCTCTGTGT	6208 - 6227	547
		r	GCTCCGTTGACGAGCTTTAC	5681 - 5700	
MMP1*	AY246754.1	f	CCGAAGGGAACCCTCGGTGG	189 - 208	161
		r	TGGCCTGGTCCACATCTGCTC	48 - 67	
MMP3*	U62529	f	GGCAACGTAGAGCTGAGTAAAGCC	413 - 432	191
		r	CAACGGATAGGCTGAGCACGC	242 - 261	
TNFα*	M64087	f	TTCTCGAACCCCAAGTGACAAG	189 - 208	161
		r	GCTGCCCCTCGGCTT	48 - 67	
HPRT*	AY372182	f	AATTATGGACAGGACTGAACGG	413 - 432	191
		r	ATAATCCAGCAGGTCAGCAAAG	242 - 261	
SDHA*	DQ402987	f	GAGGAATGGTCTGGAATACTG	6208 - 6227	547
		r	GCCTCTGCTCCATAAATCG	5681 - 5700	
GAPDH	NM_001163856.1	f	AACAGTGACACCCACTCTTC	352 - 371	245
		r	CTCAATATGTTAGGGGGTCA	127 - 147	

*Table modified and reproduced from Lang et al. 2014 [8]; * These primers were established and designed by Johannes Neuhaus (PhD student, Department of Rheumatology, Charité-Universitätsmedizin Berlin)*

4.2.6 Amplification of the Equine Cox-2 Promoter and IL-4

The equine Cox-2 gene was sequenced by Boerboom et al. [346]. They defined the promoter sequence from nucleotide 1 to nucleotide 1360. Using Vector NTI, several previously described regulatory elements were identified. To ensure functionality, primers were designed to amplify the promoter sequence between nucleotide 241 to 1439.

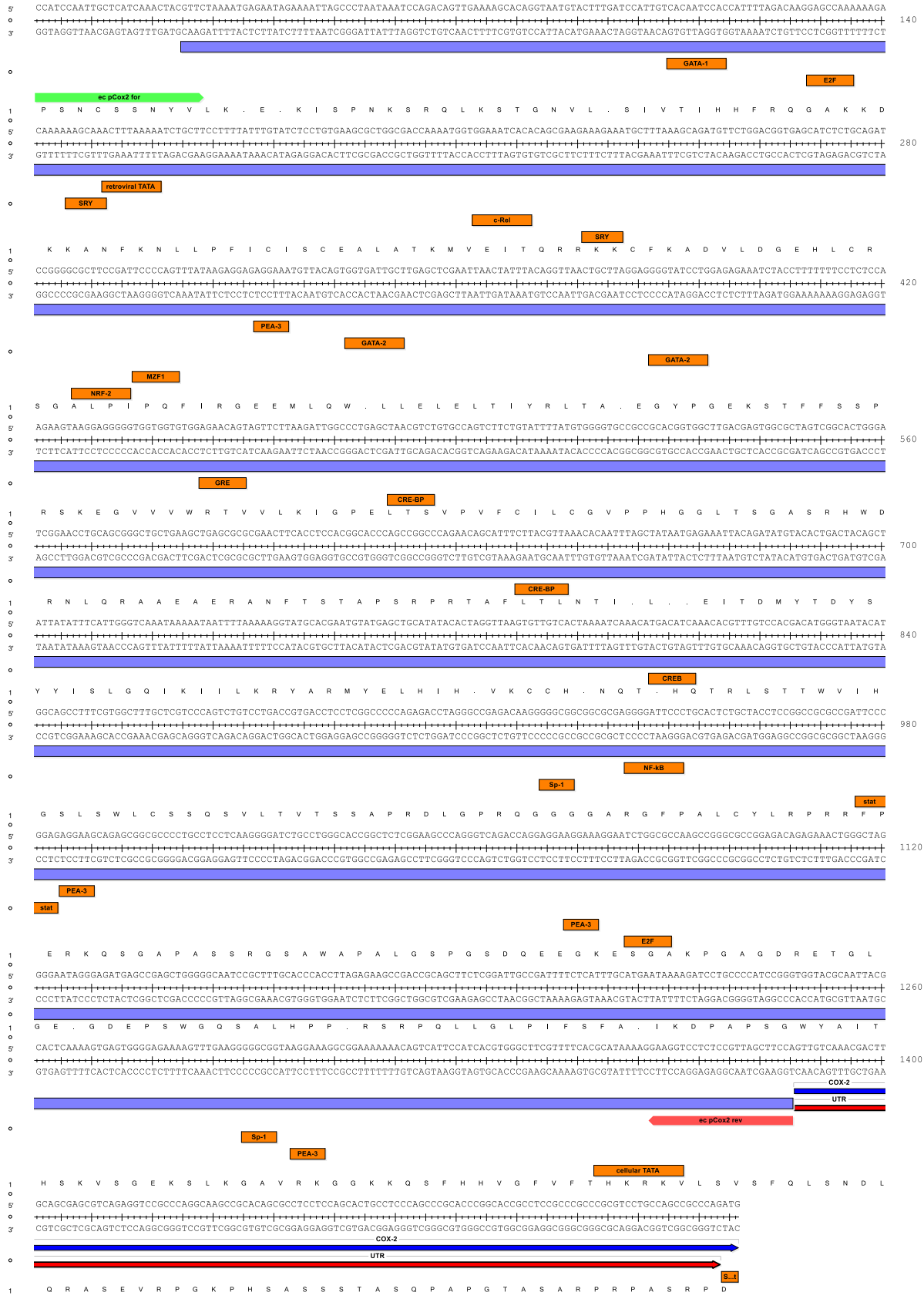

Figure 9: Position of the forward and reverse primer on the Cox-2 promoter nucleotide sequence.

Forward primer are marked green and reverse primer are marked red.

Whole genomic DNA was isolated from equine whole blood containing Ethylenediaminetetraacetic acid (EDTA), using a Nucleo Spin Blood Kit. The DNA concentration was measured via NanoDrop spectrophotometer.

The equine Cox-2 promoter was amplified by a polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase. Therefore, 200 µM dNTPs, 1.0 µM forward primer, 1.0 µM reverse primer, 5x GC buffer (10µl), 1.5 mM Mg^{2+}, 1.0 unit/50 µl Phusion High-Fidelity DNA Polymerase and up to 250 ng DNA template were mixed and filled up with nuclease-free water to a final volume of 50 µl per reaction. Optimized PCR conditions were as follows: 3 minutes of initial denaturation at 98°C (1 cycle), 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 40 seconds (35 cycles); the terminal extension was achieved with 72°C for 10 minutes. The equine IL-4 was also amplified by PCR with the Phusion High-Fidelity DNA Polymerase using the same mixture of components as described above for the Cox-2 promoter PCR with 0.5 µM forward primer, 0.5 µM reverse primer. Conditions were optimized to 98°C for 30 seconds for initial denaturation (1 cycle), 98°C for 10 seconds, 54°C for 10 seconds, 72°C for 15 seconds (30 cycles) and final extension of 72°C for 10 minutes. The amplicons were checked via agarose gel electrophoresis and visualized with a UV-radiation hand lamp to prevent DNA degradation. Positive bands were cut out with a scalpel and amplicons were isolated using an Invisorb Fragment Cleanup Kit. DNA concentration was measured via NanoDrop spectrophotometer and the DNA was stored at -20°C for further use.

4.2.7 Agarose Gel Electrophoresis

Amplicons as well as restriction controls were verified by agarose gel electrophoresis. The separation was performed on a 1% (w/v) agarose gel with TAE buffer supplemented with 0.5 µg/ml ethidium bromide. As marker the GeneRuler DNA Ladder Mix was used. The gel was run electrophoretically at 110 V for approximately 1 h, depending on the desired separation. Product bands were visualized by means of UV-radiation.

4.2.8 Plasmid Cloning

- **Plasmid design and restriction analysis**

Two different basic plasmids were used: pIRES2-EGFP (below designated as: pI2; 5.2 kb) pEGFP-N3 (below designated as: pN3; 4.7 kb). pI2 includes an internal ribosomal entry site (IRES) which facilitates the expression of a gene together with EGFP (Green fluorescent protein) without fusion. In contrast, pN3 is a plasmid for fusing EGFP to the C-terminus of a partner protein.

In 20 µl reaction volume, 1 µg of pI2 or pN3 plasmid DNA was incubated with 1x NEBuffer, and 1.5−5 units of the restriction enzymes AseI (ATTAAT) and BglII (AGATCT) for replacing the CMV promoter by the Cox-2 promoter and Bam HI (GGATCC) and XhoI (CTCGAG) for IL-4 insertion (*Figure 10*). The Cox-2 promoter and IL-4 PCR amplicons were equally digested using 10 µg PCR product. The reaction was incubated at 37°C for 2h, and Antarctic Phosphatase was added after 1h. Afterwards the digestion mixtures were separated via Agarose gel electrophoresis and the required DNA samples were isolated using an Invisorb Fragment Cleanup Kit. DNA concentration was measured via NanoDrop spectrophotometer and transferred to the ligation procedure.

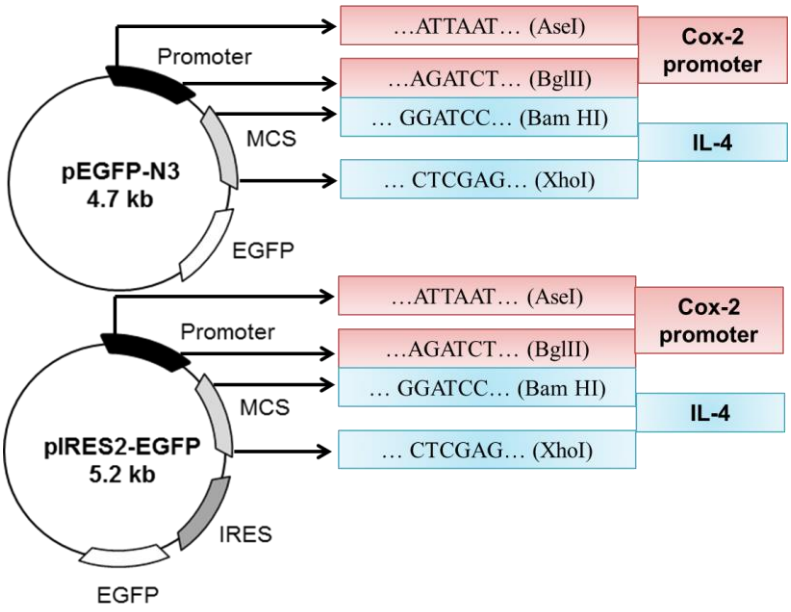

Figure 10: Overview on the basic plasmids and restriction sites for the inserts.

- **Ligation**

The ligation of the Cox-2 promoter and IL-4 was performed in succession. First, the Cox-2 promoter was ligated in the plasmid using 1x T4 ligase buffer, 50-100 ng digested PCR product (Cox-2 promoter or IL-4), 50 ng plasmid DNA (pI2 and pN3), and 0.1 units of T4 DNA ligase in a volume of 20 µl incubated at 16°C overnight. The enzymatic reaction was stopped by heating at 65°C for 15 minutes. Afterwards, the ligation product was stored at −20°C until transformation in bacteria. The same procedure was performed to ligate IL-4 in the basic plasmid and in the plasmid including already the Cox-2 promoter. Finally, the successful ligation was validated via restriction enzyme digestions, using AseI, BglII, Bam HI, XhoI. Therefore, the mixture was loaded on an agarose gel (see 4.2.7). Successful ligation was achieved when each required band was identifiable by its size.

- **Preparation of competent cells**

Competent cells were produced using XL1-Blue cells and the $CaCl_2$ method. Bacterial cells were picked up from the frozen stock by a pipette tip and incubated in 10 ml TYM bacteria growth medium at 37°C overnight on constant shaking. Subsequently, the fresh culture was diluted to 1:100 in TYM medium, incubated at 37°C overnight on constant shaking and grown up to a logarithmic growth phase (OD_{600}) of 0.2-0.4. Afterwards the fresh culture was again diluted 1:10 in TYM medium, incubated until reaching an OD_{600} of 0.4 and cooled down with ice water for 10 min. Cells were harvested by centrifugation at *1000 g* for 10 minutes at 4°C. The pellet was resuspended in 45 ml ice cold TFB I solution and incubated on ice for 10 min, followed by centrifugation (*1500 g*, 8 min, 4°C). After resuspension in 10 ml ice cold TFB II solution, the cells were incubated overnight at 4°C. To store the cells, 0.25 ml of the cell suspension was filled in precooled tubes, frozen in liquid nitrogen and stored at -80°C until further use.

- **Transformation and selection with antibiotics**

Highly competent previously prepared *E.coli* XL-1 blue cells were transformed with the produced ligation products, using the heat shock method. Therefore, 100 µl of competent cells were thawed on ice and mixed with approximately 10 µl of ligation product. The mixture was incubated on ice for 5 min followed by heating at 42°C for 45 s. Subsequently, the reaction was cooled on ice for 2 min, added with 500 µl of SOC medium pre-heated to 37°C and incubated for 1h at 37°C under constant shaking. 100 µl of the reaction mixture was plated onto LB/Kanamycin (final concentration 50 µg/ml) agar plates, which were subsequently incubated overnight at 37°C.

- **Colony screening**

The successful insertion of the plasmids was evaluated by colony screening. Macroscopically well grown colonies were picked from the agar plates and propagated in 5 ml LB/Kanamycin overnight at 37°C.

- **Isolation of plasmid DNA—Mini-Prep and Midi-Prep**

To isolate the Plasmid DNA from the overnight cultures (Mini-Prep), the Invisorb Spin Plasmid Mini Kit was utilized following the manufacturer's instructions.

Propagation of plasmids was achieved by inoculating 250 ml TB/Kanamycin (final concentration 50 µg/ml) with 1 ml of the mini-overnight culture and incubated overnight at 37°C under constant shaking (Midi-Prep). The plasmid isolation was performed using NucleoBond® Xtra Midi according to the manufacturer's instructions. The concentration of the purified plasmid DNA was determined using a NanoDrop spectrophotometer. To measure the

the nucleic acid purity, the ratio of absorbance at 260 nm and 280 nm is measured. A generally accepted ratio for DNA is ~1.8. Low ratios indicate the presence of proteins or other contaminants.

- **Restriction enzyme assay and sequencing**

Proper insertion of the genes into the basic plasmids was analyzed by restriction enzyme assay and sequence analysis. Plasmid sequencing was performed by Source Biosciences (Nottingham, United Kingdom) and subsequently analyzed with the Serial Cloner.

4.2.9 Transfection

- **Transfection reagents**

In order to standardize conditions, cells in passage 3 were utilized throughout all transfection studies. Prior to transfection, cells were utilized at a confluence of 70-80%. Subsequently, cells were cultivated in 12-well plates containing $2x10^5$ cells/well with DMEM, 10% (v/v) FCS and 1% (v/v) Pen/Strep overnight (37°C, 5% CO_2). Cells were washed with PBS twice and incubated for 6 h with DMEM and 1% (v/v) FCS and 4 units/ml hyaluronidase were added prior to transfection [170]. If not stated otherwise, incubation time for transfection was 12 hours at 37°C and 5% CO_2. First, the pI2 plasmid was chosen to compare the transfection reagents and to optimize transection rates. Chemical transfection reagents were selected based on published studies and manufacture's information (suitable for primary and hard to transfect cells). The following transfection reagents were tested in the study: FuGENE 6 and HD, X-tremeGENE 9 and HP, NanoJuice, Turbofect and TransitIT-2020. Transfection procedures were performed according to the manufacturer's instructions and are briefly described in the following:

FuGENE 6 and HD: Serum free medium was mixed with the transfection reagent in a sterile polypropylene tube. After a 5-minute incubation the DNA was complexed to the transfection reagent and after another 5 min the mixture was added dropwise to the cells.

X-tremeGENE 9 and HP: DMEM, the transfection reagent and the DNA were complexed with a total volume of 100 µl in a sterile polypropylene tube, incubated for 15 min at room temperature and afterwards added dropwise to the cells.

NanoJuice: DMEM was mixed with transfection and booster reagent after a 5-minute incubation the DNA was admixed. After an additional incubation time of 15 min the mixture was added dropwise to the cells.

Turbofect: DMEM, transfection reagent and DNA were mixed with a total volume of 200 µl in a sterile polypropylene tube, incubated for 20 min at room temperature and afterwards added dropwise to the cells. The incubation time was reduced to 6 hours.

TransitIT-2020: A mixture including DMEM, transfection reagent and DNA with a total volume of 100 µl was incubated for 20 min at room temperature and added dropwise to the cells.
The conditions for each transfection agent and the different ratios between agent and DNA are compiled in *Table 9*.

Table 9: Used Transfection Reagents.

Transfection reagent	Reagent/DNA ratio (DNA amount in µg)
FuGENE 6	3:1 (1) 3:2 (2)
FuGENE HD	3:2 (1) 4:2 (1) 6:2 (1)
X-tremeGENE 9	3:1 (0.5) 6:1 (0.5)
X-tremeGENE HP	2:1 (1) 4:2 (1)
NanoJuice	2:2 (2) 3:2 (1)
Turbofect	4:2 (2) 6:2 (2)
*Transit*IT-2020	2:1 (1) 4:1 (1)

- **Electroporation and Magnetofection**

In comparison to the chemical transfection reagents, electroporation as well as magnetofection apply physical methods to transfer plasmids into the cell. Electroporation operates by applying electrical impulses to the cells, leading to a cell membrane softening and a subsequently facilitated transformation of plasmids. Therefore, the Amaxa® Human Chondrocyte Nucleofector® Kit was used according to the manufacture's instruction and the optimized protocol for human chondrocytes. Both provided Nucleofector programs (U-24, U-28) were tested. Magnetofection was applied utilizing the PolyMAG system. In detail, the plasmids are complexed to iron beads and supplied to monolayer culture in a 96 well format. Afterwards the 96 well plate is placed on a magnetic plate leading to a transfection driven by a magnetic force on the iron beads.

4.2.10 Fluorescence Activated Cell Sorting (FACS)

For the evaluation of different transfection reagents, cells were analyzed for expression of GFP-fluorescence after 24 h or 72 h. Articular chondrocytes were removed using Accutase, washed once with PBS and determined via flow cytometry using a Coulter Epics XL MCL. Data was analyzed using the Expo 32 ADC software provided by Beckman Coulter and the FlowJo software from Tree Star.

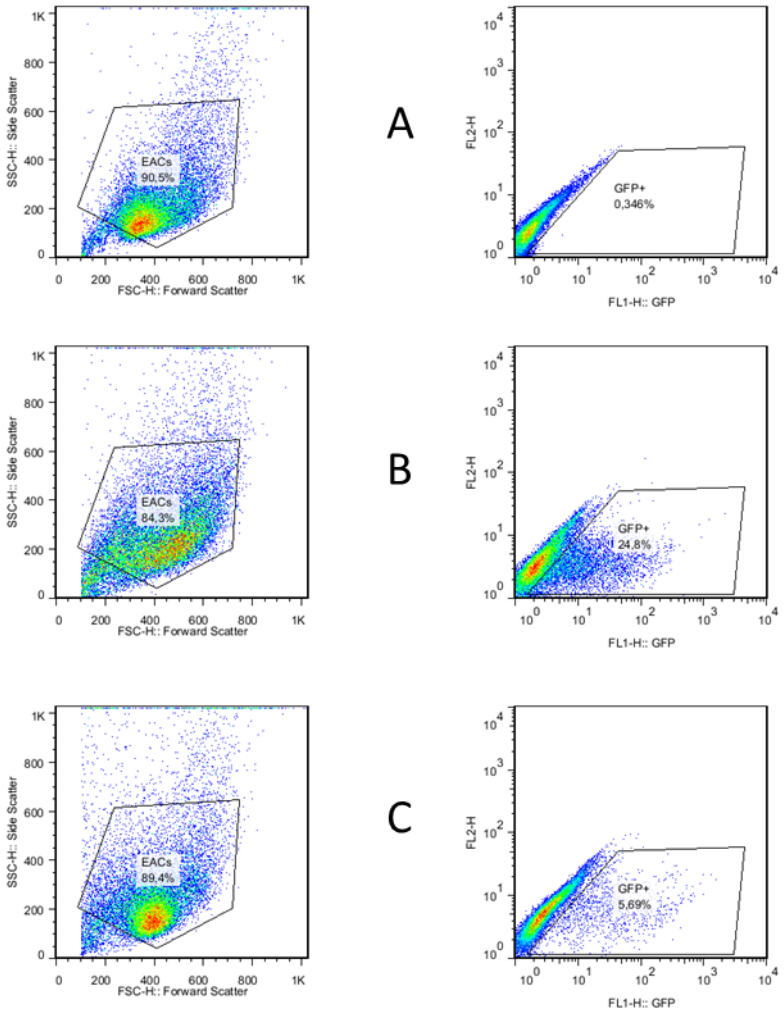

Figure 11: Measurement of transfection efficiency using flow cytometry (FACs).
Settings and analysis procedure are shown by three examples. In the first step the cell population was gathered by putting the side scatter against the forward scatter. GFP+ cells were then calculated by comparison of FL2-H and FL1-H: GFP. The FL2-H was chosen to better determining the auto-fluroscent (dead) cells in the control sample (A). Via PI staining the dead cells could be excluded in each sample before gating on the living cells. (B) and (C) represent the difference between a good transfecting reagent (Turbofect, 4:2) and a not so effective transfecting reagent (X-tremeGENE 9, 6:1). Abbreviations: EACs = equine articular chondrocytes

Non-transfected cells served as negative control. The percentage of fluorescence-positive cells was evaluated. The percentage of GFP-positive cells is defined as transfection efficiency and can rank between 0% and 100%. *Figure 11* exemplifies the gating strategy for the evaluation. To quantify cell viability, propidium iodide (PI) (0.5 µg/mL) was added to each sample prior to measurement.

4.2.11 Stimulation with eqIL-1β or LPS

Cells in passage 3, prepared as described for transfection purposes, were seeded at a density of $2x10^6$ cells/ well in a 12 well plate and incubated for 24 h at 37°C and 5% CO_2 to achieve a confluence of 70-80%. For stimulation, recombinant eqIL-1β (100 ng/ml) or LPS (100 ng/ml) in DMEM containing 1% FCS were added to the culture medium and cells were analyzed for mRNA expression of corresponding genes 72 h later.

4.2.12 RNA Isolation and cDNA Synthesis

At least $5x10^5$ cells were released and centrifuged at *200 g* for 10 min. The cell pellet was washed, resuspended in PBS and stored in RNA*later* overnight at 4°C and afterwards at -20°C until RNA isolation procedure. Total mRNA was isolated using the Universal RNA Purification Kit or RNeasy Mini Kit according to the manufacturer's instructions and stored at -20°C until further use. RNA concentration and purification were determined by NanoDrop spectrophotometer, measuring optical density at 260 nm. Beside the concentration, the 260/280 ratio is of great importance and facilitate to draw conclusions on the purity and quality of the isolated RNA. Low ratios indicate the presence of proteins or other contaminants. A 260/280 ratio of approximately 2.0 is generally accepted for RNA. Subsequently, the RNA quality was also validated via Bioanalyzer (electrophoresis method, performed by a technical assistant). At least 5 µg of RNA were subjected to first-strand cDNA synthesis using Revert Aid First Strand cDNA Synthesis Kit or Sensiscript Reverse Transcription Kit. In accordance to the manufacturer's instructions, RNA and random hexamer primer were incubated 5 minutes at 25°C followed by 60 minutes at 42°C. cDNA was stored at -20°C until further use.

4.2.13 Quantitative Real-time PCR (qRT-PCR)

Quantification of the cytokines was performed using quantitative real- time RT-PCR (qRT-PCR) on an iCycler iQ-5 or Stratagene Mx3000P qPCR cycler. The designed oligonucleotide primers (see 4.2.5) were tested and the mixture conditions were optimized using RNA

samples of plasminogen activator inhibitory (PAI) stimulated equine peripheral blood mononuclear cells (PBMCs). Therefore, the concentrations of the IL-4, Cox-2, MMP-3, HPRT and SDHA primers were 500 nM, whereas the other primers were applied with 250 nM. LightCycler Fast Start DNA Master SYBR Green I Kit was employed and for each reaction 4 µl H_2O, 1 µl SYBRmix, 2 µl $MgCl_2$, 1 µl template, forward and reverse primer, respectively, were mixed. The following program was used: initial denaturation at 95°C for 10 min (1 cycle); denaturation at 95°C for 30 sec, annealing at 55°C at 1 min and extension at 72°C for 1 min (400 cycles); 95°C for 1 min, 55°C for 30 sec, 95°C for 30 sec (1 cycle). Samples were tested in triplicates. As an endogenous reference, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes as well as Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and Succinate dehydrogenase complex (*SDHA*) were used for normalization of cycle threshold (Ct) values of target genes. Thereafter melting curves were examined for specificity of the amplification. A Ct value was obtained from each amplification curve automatically by the optical system software version 2.0 (Bio-Rad). The data evaluation was performed by applying relative quantification (delta-delta-Ct method) without efficiency correction and by using the following calculation formula:

ΔCt = Ct Target gene – Ct Housekeeper gene	→ Normalization
$\Delta\Delta$Ct = ΔCt Transfected cells – ΔCt Controls	→ Standardization
$2^{-\Delta\Delta Ct}$	→ Expression fold change

All values were normalized to either GAPDH or HPRT and SDHA.

4.2.14 IL-4 Enzyme Linked Immunosorbent Assay (ELISA)

Cell supernatants were harvested at different time-points (24 h, 48 h and 72 h) after transfection and eqIL-1β stimulation (100 ng/ml) and stored at -80°C until further use. Sandwich ELISA was performed using Equine IL-4 DuoSet and slightly modified manufacture instructions as published before [400]. A seven point standard dilution row with 2-fold serial dilutions were assayed in parallel with high concentration of 2,000 pg/mL which was adopted when higher concentrations were shown. Briefly, 96-well flat-bottomed plates were prepared with 100 µl capture antibody diluted in PBS overnight at room temperature. After washing 3 times with washing buffer (0.05% (v/v) Tween20 in PBS), plates were blocked with 300 µl reagent diluent (1% (w/v) BSA in PBS, pH 7.2-7.4) for 1 h at room temperature, followed by a washing step. Standards were dispensed in 2-fold steps starting with 2 ng/ml that were incubated on the plate together with the samples for 2 h. After washing, 100 µl of the detection antibody together with normal goat serum in PBS was applied for 1h followed by a 20 min incubation with 100 µl Streptavidin-HRP and a 20 min incubation with 100 µl

substrate solution addition. The reaction was stopped after 10 minutes with 50 µl 2N H_2SO_4 and plates were read at 450 nm and 540 nm (wavelength correction) at a Spectra Max Plus 384 Microplate Reader.

4.2.15 Protein sample preparation

Samples used for western blot were collected 48 h or 72 h post-transfection. The cells were washed with PBS, treated with Accutase and centrifuged as mentioned previously. Whole cell protein was isolated using a protein isolation kit (whole cell protein) and following the manufacturer's instructions.

4.2.16 Western Blot

Western blot analysis was performed via Tricine-SDS-Page as published previously investigating protein from cell lysates [401]. Briefly, a 4% polymerized stacking gel was laid on top of a 16% polymerized separating gel. The previously collected protein was mixed with SDS-containing sample buffer (5 µl of reducing sample buffer for a 15 µl sample) and incubated for 15 min at 37°C. Gels were brought into the vertical electrophoresis apparatus and loaded with anode and cathode buffer before 10 µl of each sample was loaded to the stacking gel. Electrophoresis conditions were as following (5-6 h): initial voltage of 30 V (~80 mA) until the samples have completely entered the stacking gel, next voltage step 200 V and voltage at the end of run was 300 V (~10 W). Subsequently, a semidry blot was performed transferring the protein bands to a nitrocellulose membrane and blocking with BSA (in PBS/Tween) overnight at 4°C. As marker the Page Ruler Prestained Protein Ladder was used facilitating the transfer to the blot and thereby working as control. Equine anti-IL-4 and anti-β-Actin antibodies were applied at concentrations of 0.1 µg/ml and 1 µg/ml, respectively, as previously established by Johanned Neuhaus (PhD student, Department of Rheumatology, Charité-Universitätsmedizin Berlin). Secondary antibodies included anti-goat-HRP and anti-mouse-HRP. Viszualisation was performed by using Luminol (chemiluminescence method) and an ImageQuant LAS 4000.

4.2.17 Statistical Analysis

Results are based on experiments that were performed three to six times in duplicate or triplicate. Statistical analysis was carried out with GraphPad Prism V.5 software. All values are expressed as the Mean ± SD and Student's t-test or one-way analysis of variance (ANOVA) were applied. In the case of a lack of Gaussian distribution, the following statistical parameters were determined: median (75%, 25%) and p-values by nonparametric t-test

(Mann–Whitney test) or the Wilcoxon signed-rank test. $P < 0.05$ was considered as statistically significant.

CHAPTER 5: Results

5.1 Development of an Equine Inflammatory *in vitro* 2D Chondrocyte Model

5.1.1 Phenotypic Changes of Equine Chondrocytes

Chondrocytes were isolated from macroscopically normal articular cartilage of euthanized horses within 72 h post-mortem. Due to the size of equine joints, a high number of cells could be gained ($2x10^7$ cells). Viability after isolations was examined by trypan blue staining and always ranked between 95% and 99%. No correlation between donor age and viability could be found. Cells were seeded in cell culture flasks at an approximate density of $2x10^5$ cells/ml and cultivated in DMEM with 10% FCS and 1% penicillin and streptomycin in a CO_2 incubator (95% air and 5% CO_2) at 37°C and cultivated up to passage 6 (P6). Within 24 h up to 72 h the primary chondrocytes became adherent, showing a polygonal shape and special distribution pattern referred as islet of growth (P0-P2; *Figure 12*).

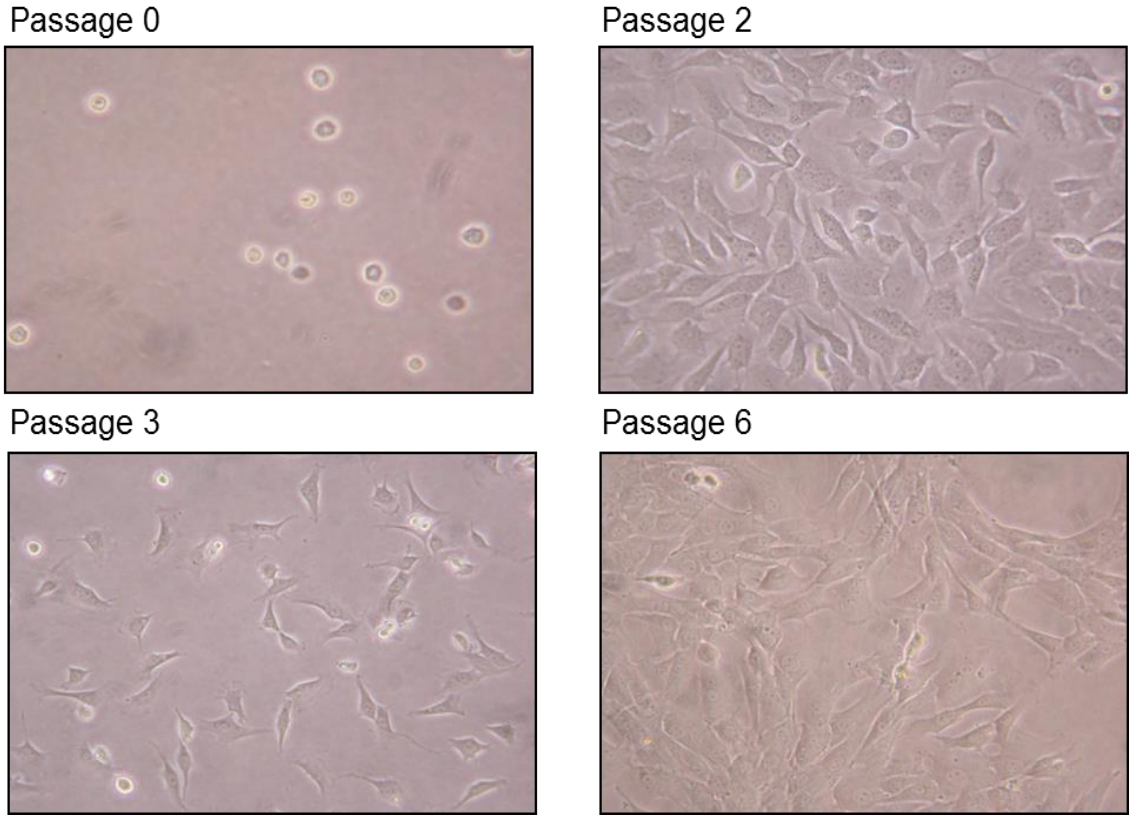

Figure 12: Microscopic morphology of equine chondrocytes in monolayer culture. *Chondrocytes were harvested from articular cartilage by enzymatic digestion and cultivated in monolayer until passage 6 (P6). The pictures show the microscopic phenotype of chondrocytes at selected passages (magnification 200x). The characteristic polygonal morphology of chondrocytes at the first passages alternates to spindle-like shape which is comparable to fibroblasts. In accordance, the growth rate decreases and confluence of 70-80% is hard to be reached. The viability was constant > 95%. These pictures were previously published in the Bachelor Thesis of Moritz Pfeiffenberger [402].*

The polygonal shape can be observed up to P3 where the chondrocytes start to become an elongated or fibroblast-like shape characterized by a spindle-like phenotype (P6 see *Figure 12*). These results confirm that chondrocytes in monolayer cultures assume the characteristic fibroblast-like, flattened and elongated morphology that is consistently reported in the literature for other types of chondrocytes [403].

Based on the observations only chondrocytes in P3 or P4 were utilized for the experiments described below.

5.1.2 Stimulation of Equine Chondrocytes

To simulate the pathogenesis of OA *in vitro*, articular chondrocytes were exogenously stimulated with either recombinant equine (req) IL-1β (100 ng/ml) or LPS (100 ng/ml). After 24 h, endogenous mRNA expression of IL-1β was determined by qRT-PCR. The stimulation with IL-1β enhanced the endogenous IL-1β mRNA significantly compared to the control (*Figure 13*). In addition, the results show that LPS is also able to induce the equine chondrocytes to express high mRNA levels of the pro-inflammatory IL-1β. In further experiments, reqIL-1β was taken to create an inflammatory environment.

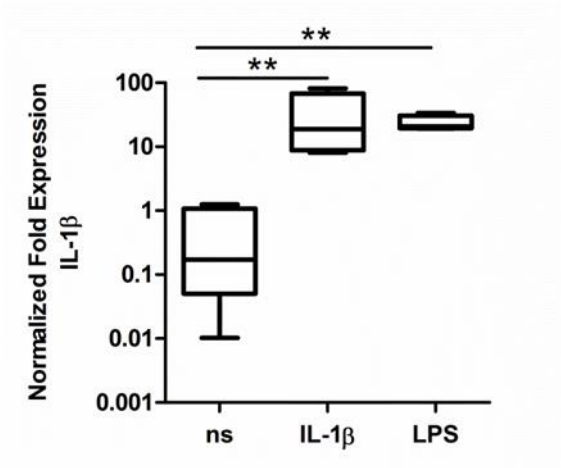

Figure 13: mRNA expression of endogenous IL-1β in equine chondrocytes after stimulation.

*Stimulation was achieved with the supplementation of exogenous reqIL-1β (100 ng/ml) or LPS (100 ng/ml) for 24 h compared to non-stimulated cells (ns). The data were normalized to GAPDH. Graph bars show Whiskers plot (5-95th percentile). Asterisks (*) indicated statistical significance (**p<0.01) as calculated via the Mann–Whitney test.*

5.2 Generation of an Inflammatory-sensitive Plasmid System Comprising the Cox-2 Promoter and IL-4

5.2.1 Alignment Study Comparing the Canine and Equine Cox-2 Promoter

In previous studies, the functionality of an inflammation-responsive canine plasmid including the Cox-2 promoter could be shown [332, 399]. In order to determine the transfer potential of the previously cloned and tested canine Cox-2 promoter to the equine system, an alignment study with Plasmid NTI AlginX was performed.

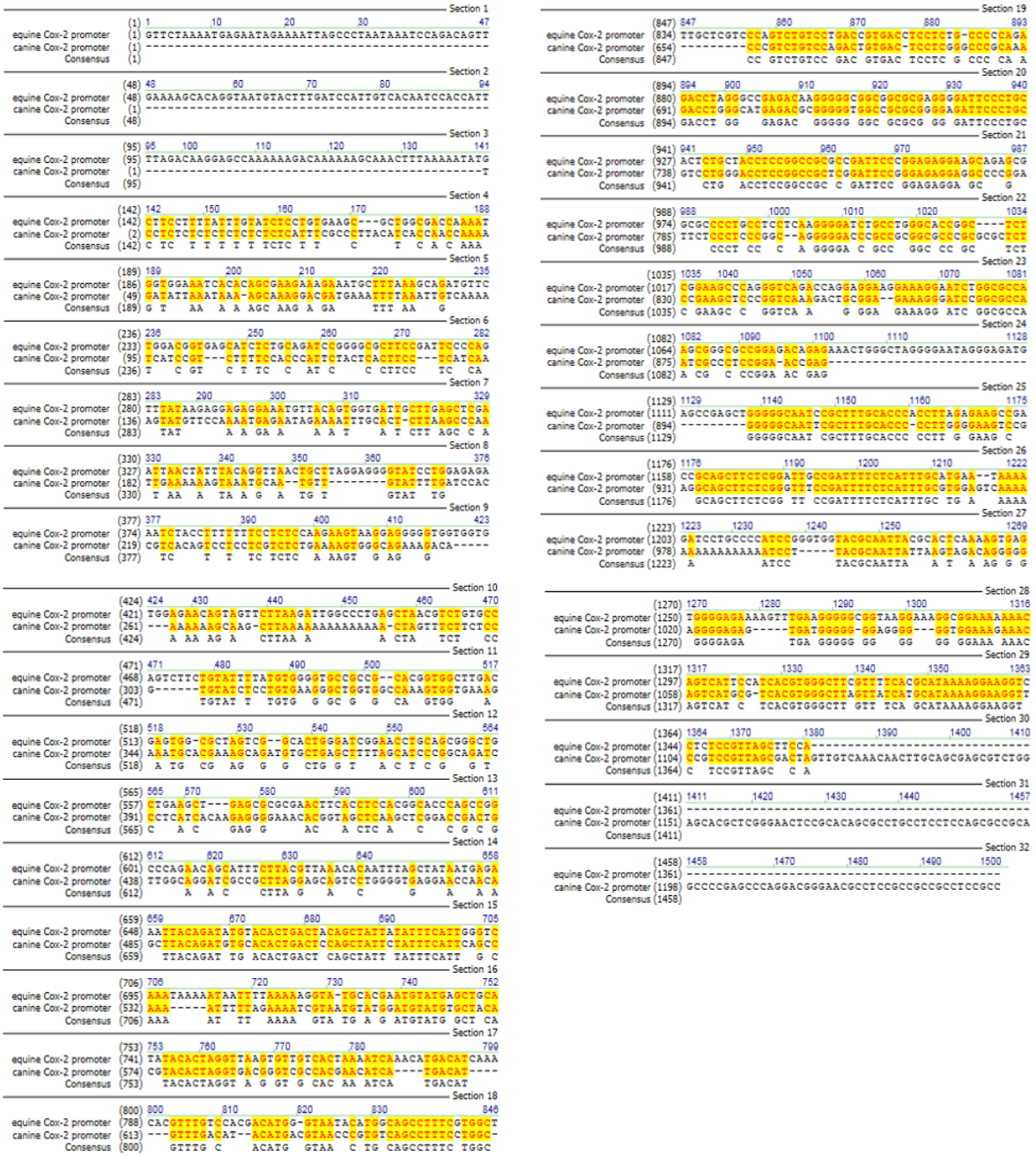

Figure 14: Alignment of equine and canine Cox-2 promoter sequences.

Yellow marked nucleotides = consensus positions = 46.2%; Accession numbers: equine Cox-2 promoter_ AF027335 and canine Cox-2 promoter_EU249362

The practicability of having one promoter sequence that is working in different mammalian systems is obvious in order to elude the cloning procedure. Sequences for the alignment study were obtained from GenBank and were 1360 bp (equine) and 1240 bp (canine) in length. The comparison showed that both nucleotide sequences had 46.4% consensus positions (compare yellow marked sequences in *Figure 14*) which was considered too low to justify application of the canine Cox-2 promoter in equine cells. Consequently, the amplification and cloning of the equine-specific Cox-2 promoter was performed in order to achieve functionality in the equine system. Since IL-4 is described as a strict species-specific cytokine and can only be partly transferred to other mammalians with unknown and possible restricted activity, I resigned to perform an alignment study for IL-4.

5.2.2 Cloning the Cox-2 Promoter Regulated Plasmids Expressing IL-4

Firstly, the pI2 was used as basic plasmid due to the presence of an IRES which facilitates expression of two genes in parallel without fusing the proteins. During extensive investigations, low transfection efficiencies of pI2 in equine chondrocytes were observed, compared to normal cell lines. Therefore, I decided to exclude the IRES from the plasmids and produced comparable plasmids with the pN3.

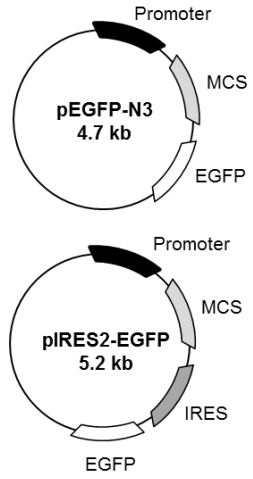

Table 10: Detailed plasmid design.

Plasmid	Designation	Promoter	MCS	Size (kb)
pEGFP-N3	pN3*	CMV	-	4.7
	pN3.IL-4*#	CMV	IL-4	5.1
	pN3.Cox-2#	Cox-2	-	5.5
	pN3.Cox-2.IL-4#	Cox-2	IL-4	5.9
pIRES2-EGFP	pI2*	CMV	-	5.2
	pI2.IL-4*	CMV	IL-4	5.6
	pI2.Cox-2†	Cox-2	-	6.0
	pI2.Cox-2.IL-4†	Cox-2	IL-4	6.4

**used for transfection efficiency studies; †not used for further experiments;*
#used for all further studies;
Table modified and reproduced from Lang et al. 2014 [8].

Figure 15: Plasmid design.
Different plasmids were cloned and applied in the described experiments. As basic plasmids pIRES2-EGFP (pI2) and pEGFP-N3 (pN3) (both BD Biosciences Clontech, USA) were used. Figure reproduced from Lang et al. 2014 [8]. Abbreviations: MCS= Multi cloning site; EGFP= enhanced green fluorescent protein

After amplification from whole genomic DNA, the Cox-2 promoter was cloned at the position of the CMV promoter. The IL-4 gene was placed in the multiple cloning site (MCS) (*Figure 15* and *Table 10*). Different plasmids were cloned including either the Cox-2 promoter or IL-4 as well as both to perform comparative studies with suitable controls. Promoter studies supported the primer design process.

Cloning results were validated via control restriction digestions. *Figure 16* shows an example for a successful cloning procedure verifying the DNA bands to be expected for pI2.IL-4, pI2.Cox-2 and pI2.Cox-2.IL-4.

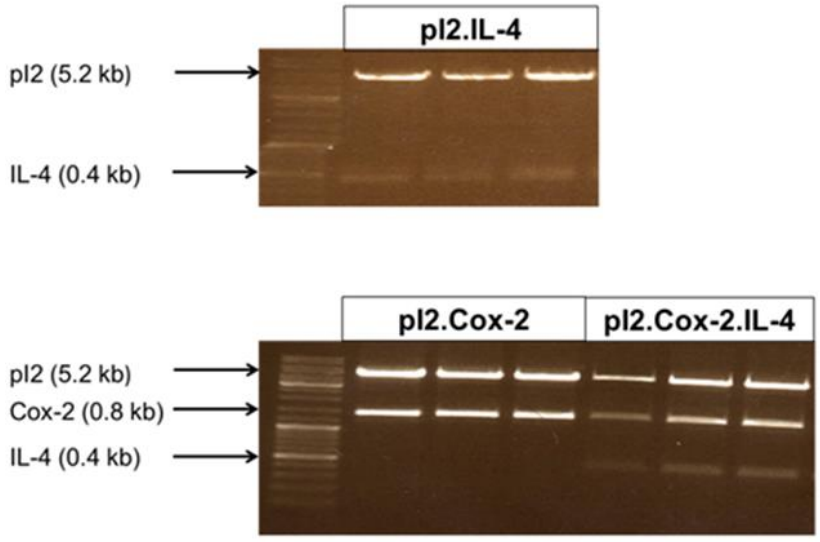

Figure 16: Agarose gel electrophoresis after control digestion of positive clones.
To verify the cloning process, positive clones (pI2.IL-4, pI2.Cox-2, pI2.Cox-2.IL-4) were digested with the previously used digestion enzymes (AseI, BglII, Bam HI, XhoI). With the help of defined markers (left side, DNA Gene Ruler), bands can be assigned to sizes followed by the classification as specific gene.

Positive clones were further expanded and sequenced before they were tested for their functionality. Respective, genes could be demonstrated for their presence in the correct position and orientation as it was further verified by sequencing (compare *Figure 17* and *Figure 18*). Serial Cloner was used for the analysis.

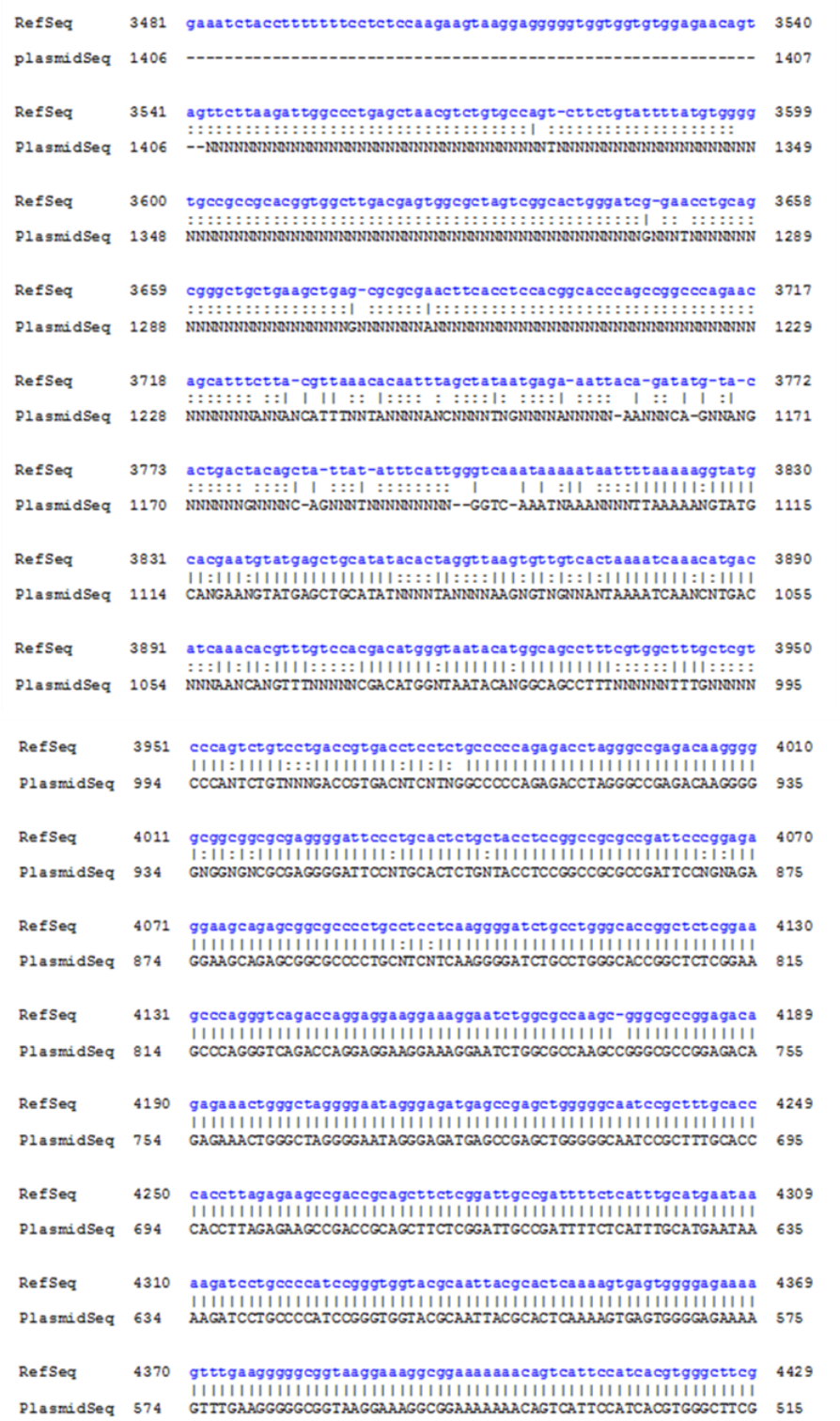

Figure 17: First part of the sequencing analysis starting in the middle of the Cox-2 promoter.

After successful control digestion positive clones were sequenced and analyzed via Serial Cloner. Therefore, the gained plasmid sequence was compared to the reference sequences for the equine Cox-2 promoter (Accession no. AF027335). The Cox-2 promoter sequence is marked blue. Vertical lines indicate consensus positions. Double dots mark discrepancies that are mostly caused in the sequence starting areas where the sequencing process is not yet consistent and efficient. N stands for not identified nucleotides. (RefSeq= reference sequence; PlasmidSeq= sequence of the cloned plasmid)

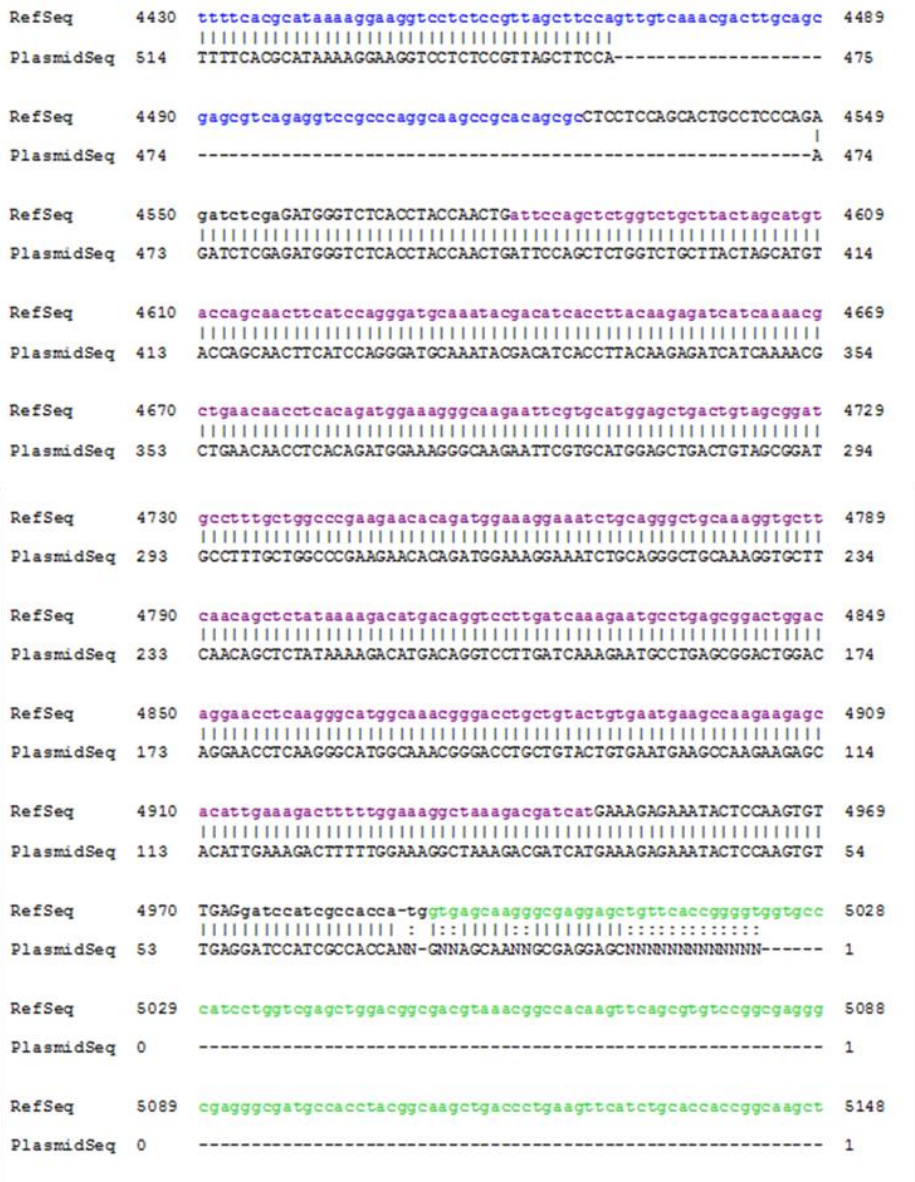

Figure 18: Second part of the sequencing analysis from the end of the Cox-2 promoter to the beginning of EGFP involving the complete IL-4 site.

After successful control digestion, positive clones were sequenced and analyzed via Serial Cloner. Therefore, the gained plasmid sequence was compared to the reference sequences for the Cox-2 promoter (Accession no. AF027335) and IL-4 (Accession no. AF305617). The Cox-2 promoter sequence is marked blue. IL-4 is marked in purple and EGFP is marked in green. Vertical lines indicate consensus positions. Double dots mark discrepancies that are mostly caused in the sequence starting areas, where the sequencing process is not yet consistent and efficient. N stands for not identified nucleotides. RefSeq= reference sequence; PlasmidSeq= sequence of the cloned plasmid

Taken together, since the sequence analysis revealed no relevant discrepancies (indicated by horizontal lines) the cloning of the equine genes was rated successful. The equine-specific inflammatory-sensitive plasmid systems comprising the equine Cox-2 promoter and/ or IL-4 that were used in the following investigations.

5.2.3 Comparing Different Transfection Systems

Several chemical-based transfection reagents are commercially available which have not yet been tested in equine chondrocytes. Consequently, in this study 7 different transfection reagents were compared for their efficiency to transfect non-virally equine chondrocytes in cell culture with the basic GFP-containing plasmid pI2. In addition, two physically based transfection methods were examined. Cells were pretreated with hyaluronidase as described previously [170]. Evaluations were performed via FACS 24 h after transfection. The percentage of GFP-positive cells of all counted cells signifies the transfection efficiency.

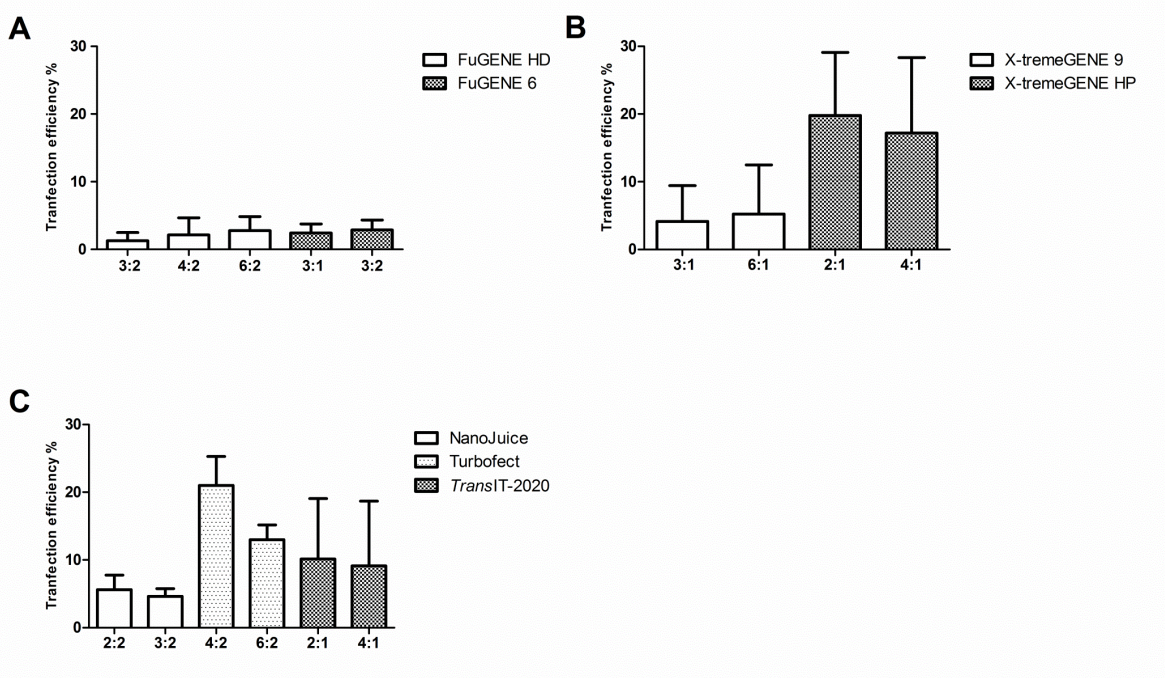

Figure 19: Comparison of the efficiency of different non-viral transfection reagents.
Bar graphs show the varying ability of commercially available reagents to successfully transfect equine chondrocytes using pI2. FACS analysis was performed to obtain the reported results.Turbofect (Fermentas) shows the highest potential with the lowest standard deviation (C). (A) The bar graphs show no comparable transfection efficiency of FuGENE 6 and HD (Promega) as it was tested for dog and human chondrocytes [170, 404]. In horse chondrocytes the transfection efficiency is lower compared to the the successor X-tremeGENE 9 and HP (B). Different lipid/ RNA ratios were tested for each reagent. As control non-transfected cells were consulted showing an autofluorescence of 0.72%. 100% are all counted cells. Transfection efficiency means the percentage of GFP-positive cells of all counted cells. All data are expressed as the Mean ± SD. A summary of the results is published in Lang et al. 2014 [8].

Turbofect (21.02% ± 4.26%) and X-tremeGENE HP (19.78% ± 9.31%) mediated the highest GFP expression as depicted in *Figure 19*. The best lipid/ DNA ratios were for Turbofect 4:2 and for X-tremeGENE HP 2:1. Increasing the reagent/ DNA ratio led to a decreased efficiency (Turbofect 6:2 13% ± 2.16%, X-tremeGENE HP 4:1 17.19% ± 11.14%). In contrast,

FuGENE HD (1.28% ± 1.218%) and FuGENE 6 (2.40% ± 1.36%) promoted the lowest reporter gene expression (*Figure 19*). The transfection efficiency could not be increased by a higher reagent/DNA ratio (3:2, 4:2, 6:2). Also, the results of NanoJuice (5.60% ± 2.15% for 2:2 ratio and 4.64% ± 1.12 % for 3:2 ratio) and *Transit*IT-2020 (10,15 ± 8,91 % for 2:1 ratio and 9.14 ± 9.55 % for 4:1 ratio) showed that the increase of the reagent/DNA ratio does not enhance the efficiency. Using X-tremeGENE 9 at a ratio of 6:1 (5.24% ± 7.26%) instead of 3:1 (4.17% ± 5.26%) only showed a slight increase (*Figure 19*). Electroporation as well as magnetofection constantly showed very low transfection efficiencies (< 5%). In all cases, cell viability was measured by Trypan blue staining microscopically, or by flow cytometry after adding PI to the transfected cells (see *Table 11*). Reagents that promoted high transfection efficiency often mediated a decrease in cell viability. The increase of the reagent/DNA ratio of Turbofect and X-tremeGene HP resulted in a further depression of cell viability without enhancing transfection efficiency. In order to minimize the side effects on the viability the exposure time of Turbofect from recommended 12 h was shortened to 6 h.

Table 11: Cell viability (CV) after transfection with different reagents.

Transfection reagent	Reagent/ DNA ratio	CV in % Mean ± SD
FuGENE HD	3:2	89.8 (± 11.0)
	4:2	83.2 (± 14.6)
	6:2	87.8 (± 9.0)
FuGENE 6	3:1	82.3 (± 16.4)
	3:2	82.0 (± 20.7)
X-tremeGENE HP	2:1	77.5 (± 4.9)
	4:1	74.8 (± 8.7)
X-tremeGENE 9	3:1	84.3 (± 14.4)
	6:1	85.6 (± 11.6)
NanoJuice	2:2	83.2 (± 3.9)
	3:2	85.0 (± 10.2)
Turbofect	4:2	71.8 (± 7.0)
	6:2	63.6 (± 14.5)
*Transit*IT-2020	2:1	84.6 (± 12.3)
	4:1	83.4 (± 12.3)

Electroporation as well as magnetofection lead to enhanced cell death. Survived cells needed more than 48 h to recover and become adherent. Therefore, I decided to not follow up optimization steps for these two methods.

In conclusion, the choice of adequate reagents as well as optimization steps are crucial for achieving acceptable transfection efficiency with equine chondrocytes. According to the described findings, Turbofect was used for further investigations at a lipid/ DNA ratio of 4:2.

5.2.4 Optimizing the Plasmid Design

Compared to other cell lines or other mammalian chondrocytes, the transfection efficiency yielded in equine chondrocytes is comparatively low with ~20%. To investigate whether the IRES or/and the size of a plasmid have an influence on the transfection efficiency, cells were transfected using Turbofect with the following plasmids: pI2 (5.2 kb), pI2.IL-4 (5.602 kb), pN3 (4.7 kb) or pN3.IL-4 (5.102 kb) (see *Figure 15*). Again GFP-expression was measured by FACS and the transfection efficiency was calculated at 24 h and 72 h - after initiating the transfer.

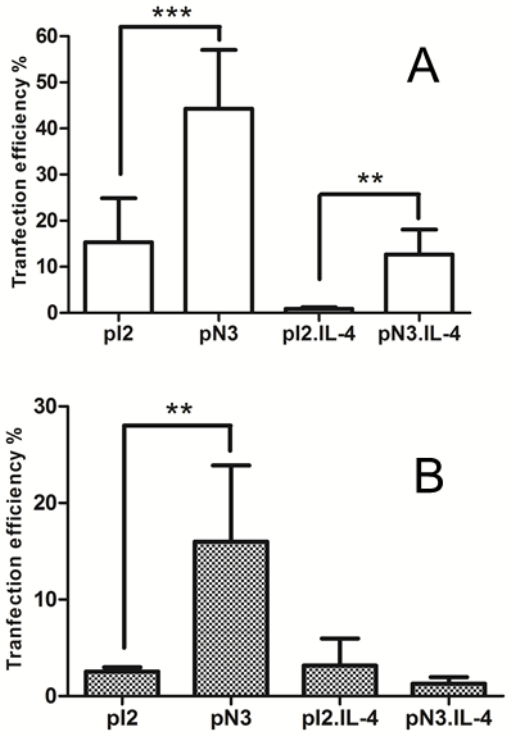

Figure 20: Effect of plasmid design on transfection efficiencies.

*To optimize the transfection rate of Turbofect, plasmids with or without IRES where compared, indicating an influence of the IRES. Transfection values were measured after 24 h (A) and 72 h (B). FACS analysis was performed to acquire the presented results. 100% are all counted cells. Transfection efficiency means the percentage of GFP-positive cells of all counted cells. All data are expressed as Mean ± SD. Asterisks (*) indicate high statistical significance (**p<0.01, ***p<0.001) as calculated via Student's t-test. Figure 20A is reproduced from Lang et al. 2014 [8].*

As shown in *Figure 20A*, the highest significant frequency of GFP expressing cells was obtained after 24 h with the small pN3 plasmid. In contrast, the somewhat larger pI2 plasmid mediated only one third of this yield. Whenever the IL-4 gene was present in the plasmid (pN3.IL-4, pI2.IL-4) the frequency of GFP expressing cells was reduced. For instance, the plasmid pN3.IL-4 yielded less than one third of the expression yields reached with the same plasmid devoid of IL-4.

After 72 h of transfection, the efficiency was reduced collectively (*Figure 20B*). But pN3 promoted a higher expression level than pI2. The GFP expression of pI2.IL-4 did not surpass the results of pN3.IL-4 significantly. Additionally, the cell viability showed no significant variation between the different plasmids (see *Table 12*).

Table 12: Comparison of the cell viability after transfection using different plasmids.

Plasmid	CV after 24 h in % Mean ± SD	CV after 72 h in % Mean ± SD
pN3	87.7 (± 6.4)	80. 9 (± 8.3)
pI2	82.0 (± 2.3)	67.4 (± 17.3)
pN3.IL-4	86.3 (± 11.6)	93.7 (± 1.6)
pI2.IL-4	74.1 (± 16.3)	75.7 (± 18.5)

These results indicate that the plasmid design can have a significant influence on transfection efficiencies and should be considered carefully in further studies to avoid preventable transfection rate reductions.

5.3 Test of the Functionality of a "Genetic Switch" (eqCox-2 Promoter)

Four different plasmids, pN3, pN3.IL-4, pN3.Cox-2, and pN3.Cox-2.IL-4 were compared in an inflammatory equine chondrocytes cell culture model for their ability to induce the expression of mRNA coding for IL-4 in the transfected cells. Supernatants and cell lysate were examined for the presence of IL-4 on the protein level via ELISA and Western Blot. Control cells transfected with the pN3.Cox-2 plasmid which is devoid of the IL-4 gene, produced only basal levels of IL-4 mRNA even after an exogenous inflammatory stimulus by addition of recombinant equine IL-1β (100 ng/ml) to the growth medium. As depicted in *Figure 21A*, cells transfected with pN3.IL-4 and pN3.Cox-2.IL-4 yielded a significant stimulation of IL-4 expression over time when compared with the control cells transfected with the pN3 or pN3.Cox-2 plasmid. Interestingly, the IL-4 mRNA expression decreased after 72 h whereas the protein concentration in the cell culture supernatant remained constant. IL-4 protein production was verified in the cell culture supernatant (ELISA *Figure 21B*) and in the cell lysate (Western blot *Figure 21C*).

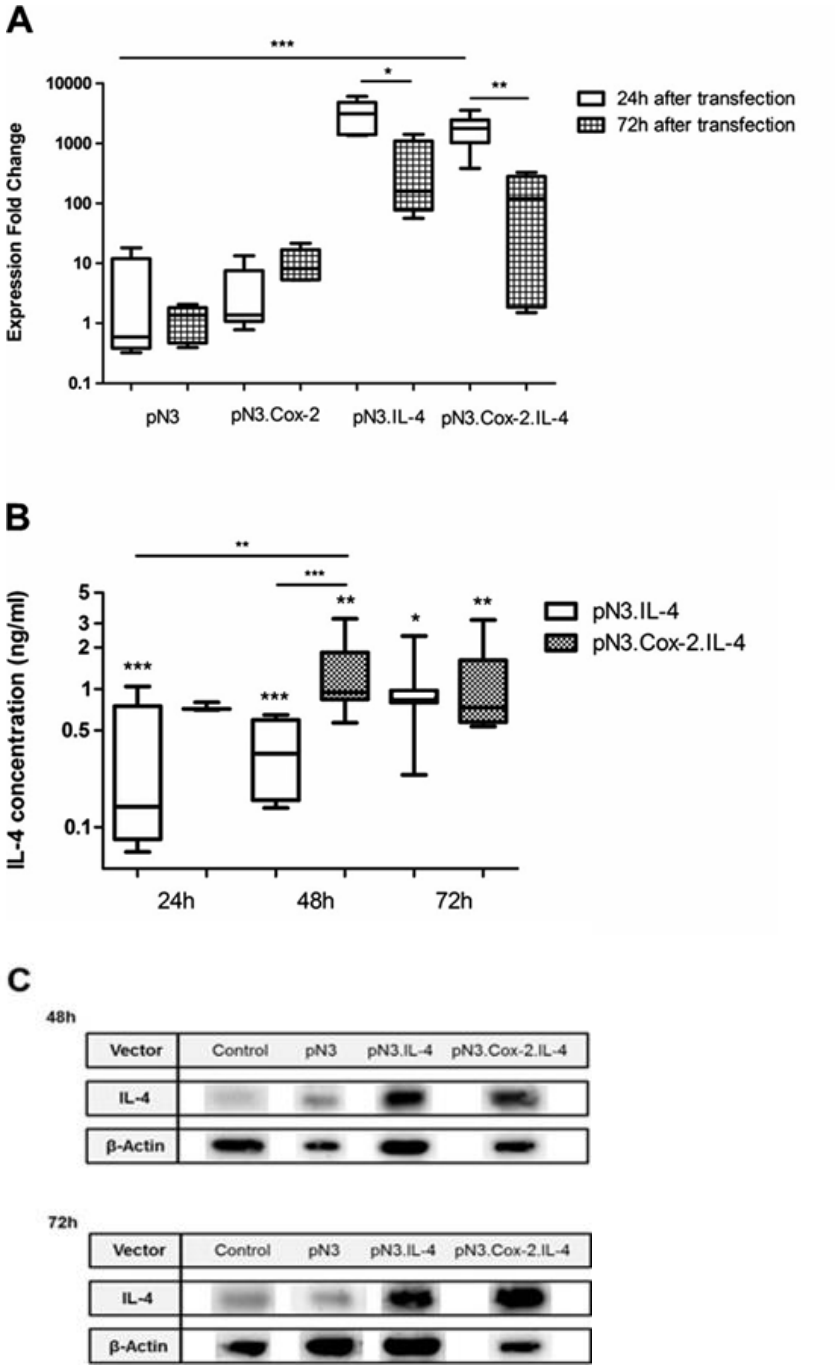

Figure 21: IL-4 expression and translation after transfection and IL-1β-stimulation of chondrocytes.

*(A) The bar graphs show a higher expression of IL-4 mRNA in cells that were transfected for 24 h or 72 h with the pN3.IL4 or pN3.Cox2.IL4 plasmid, compared to the cells containing the pN3 and pN3.Cox2 plasmid. The data were normalized to HPRT and SDHA. IL-4 mRNA expression decreased over time, whereas the protein concentration in the supernatant was almost constant and highly significant as measured via ELISA (B). The lower limit of quantification of the original assay was 31.20 pg/ml and the upper limit was 2 ng/ml, further controls were used until 3 ng/ml. Asterisks (*) indicate high statistical significance (*p <0.05, **p <0.01, ***p <0.001) as calculated via the (A) Mann-Whitney-test or (B) one way analysis of variance (ANOVA) and Wilcoxon signed rank test. Graph bars show a whisker plot (5–95th percentile). (C) Figure shows the results of the IL-4 western blot 48 h and 72 h after transfection using extracted protein from cell lysate. Figures are reproduced from Lang et al. 2014 [8].*

To assess the potential involvement of Cox-2 on the control of inflammation *in vitro*, the mRNA synthesis for Cox-2 was determined and found to be mainly increased after the exogenous stimulus of cells with reqIL-1β. The results in *Figure 22* demonstrate that non-transfected cells which were stimulated with this cytokine produced a pronounced elevation of Cox-2 mRNA levels. Under these conditions, no IL-4 mRNA is expressed. When cells were transfected with pN3.Cox-2.IL-4, high levels of IL-4 mRNA were detected side by side with Cox-2 mRNA in stimulated cells. Control cells that were not transfected and not stimulated showed neither Cox-2 nor IL-4 transcription (*Figure 22*, left side).

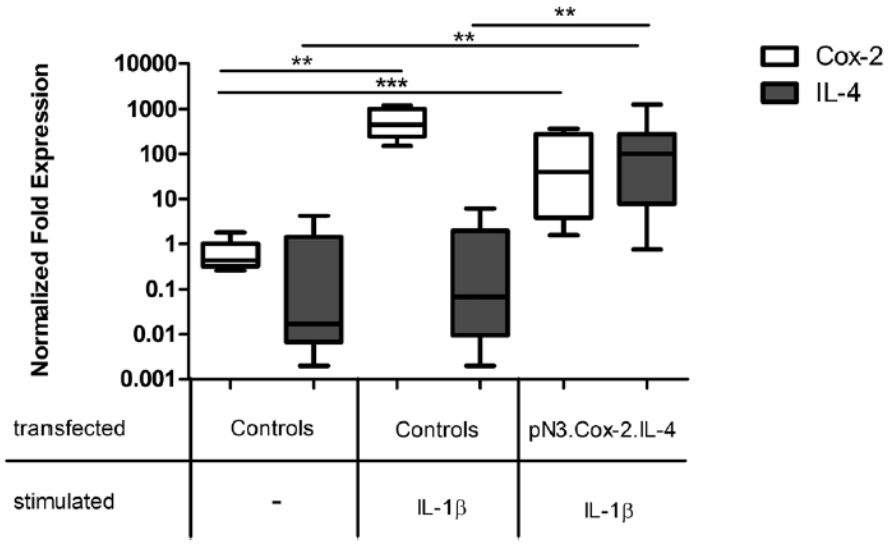

Figure 22: Expression of Cox-2 and IL-4 after transfection with pN3.Cox-2.IL-4 and subsequent IL-1β stimulation.

*Bar graphs present values for Cox-2 and IL-4 mRNA expression in pooled non-stimulated controls, pooled IL-1β stimulated controls and pN3.Cox2.IL4 transfected/IL-1β stimulated cells. Non-transfected and with pN3 transfected cells were pooled as low controls. The data were normalized to GAPDH. Graph bars show a whisker plot (5–95th percentile). Asterisks (*) indicate high statistical significance (**p <0.01; ***p <0.001) calculated via the Mann–Whitney test. Figure is reproduced from Lang et al. [8].*

To summarize, the obtained data revealed the functionality of the Cox-2 promoter to be activated by exogenous reqIL-1β stimulation and to facilitate the IL-4 mRNA expression and protein formation. The hypothesis was confirmed in different experiments to provide more evidence (see below).

5.4 Evaluation of the Anti-inflammatory Potential of eqIL-4

Since it is known from the literature that IL-4 has a regulatory role for the expression of pro-inflammatory cytokines, it was of interest to assess the mRNA expression of inflammatory cytokines (IL-1β, IL-6, IL-8, TNFα) and matrix-degrading enzymes (MMP-1 and MMP-3) under the conditions described above. Equine chondrocytes were therefore transfected with

the pN3.IL-4 plasmid or the same plasmid which, in addition, carries the Cox-2 gene (pN3.Cox-2.IL-4). As a control one set of cultures was transfected either with pN3 or with pN3.Cox-2. After completing transfection, all cultures were stimulated as described above to induce a state of "inflammation" by adding reqIL-1β (100 ng/ml) to the medium. Transcription of cytokines and enzymes was measured 24 h and 72 h later. During the analysis, results of control plasmids (pN3, pN3.Cox-2) and results of IL-4 containing plasmids (pN3.IL-4, pN3.Cox-2.IL-4) were pooled, respectively, to show the efficiency of IL-4. As seen from the results shown in *Figure 23* the control cells express high levels of IL-1β, IL-6, IL-8, MMP-1 and MMP-3 transcription as expected. However, when IL-4 is produced as a result of transfection of the cells with the IL-4-bearing plasmids (pN3.IL-4, pN3.Cox-2.IL-4) (compare *Figure 23*), the expression of these inflammatory mediators was significantly reduced (*Figure 23*) at 24 h and 72 h. This reducing potential was slightly limited after the 72 h indicating a fast degradation of IL-4 or a beginning of the Cox-2 promoter feedback inhibition. The TNFα mRNA expression showed no detectable difference.

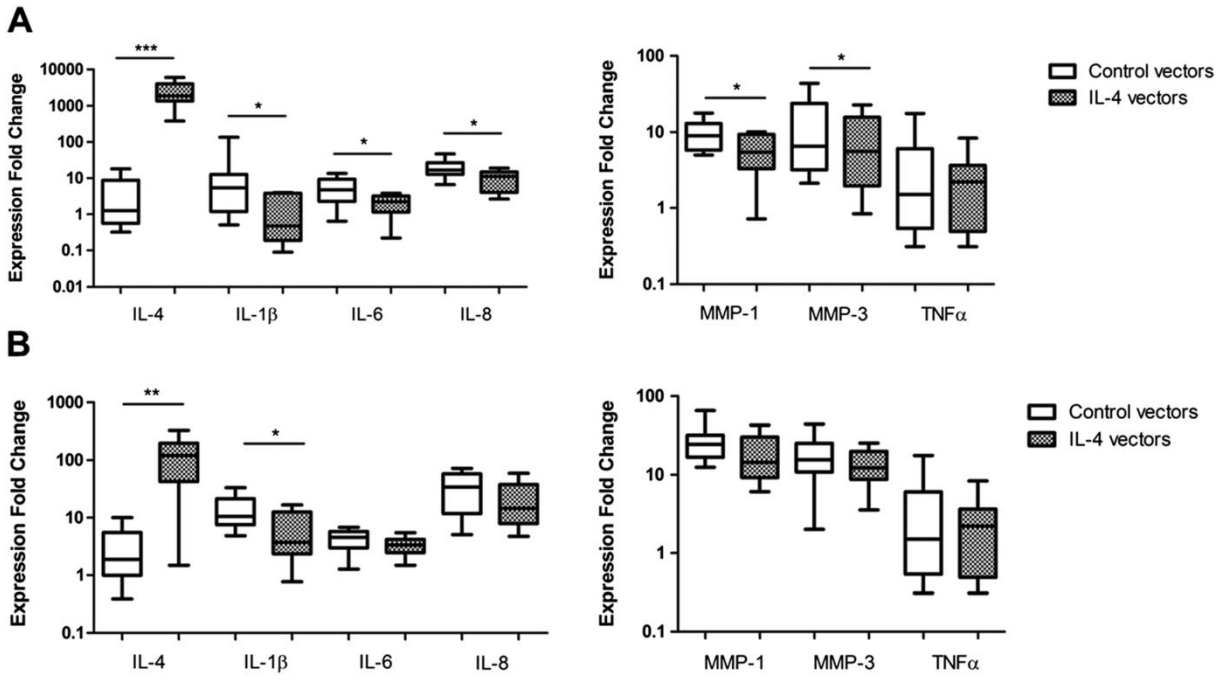

Figure 23: Application of IL-4 as a therapeutic gene in the equine model system.

*The graphs present the quantified endogenous transcription as fold change expression of inflammatory cytokines and matrix-degrading enzymes after 24h (A) and 72h (B). Equine chondrocytes transfected with control plasmids (pN3, pN3.Cox2) or IL-4 bearing plasmids (pN3.IL4, pN3.Cox2.IL4) have been stimulated exogenously with equine IL-1β (100ng/ml). The data were normalized to HPRT and SDHA. Graph bars show a whiskers plot (5–95th percentile). Asterisks (*) indicate statistical significance (*p <0.05, **p <0.01, ***p <0.001) calculated via the Mann–Whitney test. Figure is reproduced from Lang et al.[8].*

The results demonstrate the inhibitory effect of the IL-4-bearing plasmids on different inflammatory mediators in inflammation-stimulated cells, implying a time-dependence. To

summarize, upon exogenous stimulation the Cox-2 promoter leads to increased IL-4 production and subsequent down-regulation of inflammatory cytokines and matrix-degrading enzyme expression.

CHAPTER 6: Discussion

This thesis comprises two parts. Part 1 of this thesis presents my own experimental work (Chapter 4-6). Part 2 includes a complete update for the state of the art in research on OA gene therapy, animal models for OA and the potential and risks of IL-4 as transgene (Chapter 2.2, 2.3, 2.5 and 6.5). In the following chapter Part 1 as well as the potential risk of IL-4 are discussed comprehensively. Parts of the thesis were published in Lang et al. 2014 [8].

Osteoarthritis (OA), a degenerative joint disease, ranks among the most common musculoskeletal disorders around the globe accounting for considerable disabilities and a financial burden on society. Even in veterinary medicine, OA is increasing as the number of patients with severe joint disorders (dogs and horses) is steadily rising. Unfortunately, there is still no therapy available which would offer a selective and lasting treatment of severe joint diseases such as OA [8]. Therefore, daily administration of anti-inflammatory drugs is unavoidable and often related to adverse reactions. At the beginning of the 1990s, gene therapy was noted to be a convenient approach to treat inflammatory disorders locally in the affected joint. Promising approaches link gene therapeutic techniques to tissue engineering methods including the development of next generation cartilage transplants. To overcome critical challenges such as repeated local gene deliveries and undesirable overexpression of the transgene, several approaches focus on the effective control of expression. IL-4 provides great advantages compared to other gene candidates, by inhibiting the expression of the pro-inflammatory cytokines, especially IL-1β.

Hence, to achieve a safe and applicable gene therapy for OA, research at the Institute for Immunology (FU-Berlin) has been aiming at the expression of therapeutic cytokines in a disease responsive manner by using a newly developed “genetic switch”, the cyclooxygenase-2 promoter (pCox-2). To this end, the pCox-2 is cloned upstream of IL-4 and can be inactivated via the absence of inflammatory mediators, leading to disease controlled expression.

The experimental section of this thesis focuses on the establishment of an equine inflammatory cell culture model. This included the evaluation of suitable inflammatory mediators and the optimization of different transfection protocols for equine chondrocytes. The ultimate goal was to provide experimental proof, that the plasmid including the Cox-2 promoter is functional and the therapeutic gene chosen has indeed the potential to silence the inflammatory state of cells in an equine model system.

6.1 Development of an Equine Inflammatory *in vitro* Chondrocyte Model

6.1.1 Phenotypic Changes of Equine Chondrocytes

The dedifferentiation phenomenon of chondrocytes in monolayer cultures is sufficiently known and a problem especially for tissue engineering applications of matrix-associated cartilage transplants. Therefore, an adequate amount of chondrocytes has to be expanded in monolayer cultures within three to four passages. In this thesis, it could be shown, that primary equine chondrocytes show microscopic changes. The cells changed their shape from the initial round/polygonal (passage 1-3) towards elongated fibroblast like cells (passage > 3) (*Figure 12*). This process is defined as dedifferentiation [405]. Dedifferentation can mainly be observed in monolayer culture or under IL-1β exposure as well as in case of biomechanical or physical damage [406-408]. The underlying mechanisms are still unclear. Relationships between the shape changes of chondrocytes and e.g. the production of Col II, the expression of Sox-9 as well as the BMP-signaling are described in the literature [70, 409-411]. Chondrocytes are terminally differentiated cells and Sox-9 is an important transcription factor of chondrogenesis and responsible for the maintenance of the phenotype [410]. Col II is a cartilage specific matrix protein and decreases during longer cultivation time, whereas the expression of Col I increased [412]. Col I is found in many different tissues. These alterations are also known for osteoarthritic cartilage where the cells become hypertrophic. Many efforts have been made to preserve the chondrogenic phenotype especially for therapeutic applications. Therefore, gene therapeutic plasmids including Sox-9, Col II or hedgehog genes as well as methods for immortalization were tested [413, 414].

In addition, the phenotype can be maintained by transferring the cells into a 3D state by embedding them in suitable scaffolds or by 3D self-assembly [415, 416].

To circumvent the problem of phenotypic changes, chondrocytes only in passage 3 or 4 where equine chondrocytes are fully differentiated were utilized. Moreover, experimental examination times were kept short to prevent dedifferentiation. In summary, in this study also equine chondrocytes showed phenotypic changes in monolayer cultures. Therefore, studies in 3D systems should be enrolled in further steps as well as the assessment of Sox-9 and Col II expression changes.

6.1.2 Stimulation of Equine Chondrocytes

For developing new therapeutic approaches to treat OA, suitable models in cell culture as well as in animal models are needed [417]. Instead of using small rodents, large animal models, which represent both naturally occurring models and patients at the same time,

should be preferred (see also Chapter 2.5). Thus, in a first step a standardized and reliable equine cell culture model that allows to mimic molecular aspects of inflammation and to assess therapeutic intervention strategies *in vitro* was established [8]. This cell culture model is based on primary equine chondrocytes which can be stimulated with recombinant equine IL-1β or with LPS which are known to be potential triggers of endogenous production of IL-1β [418]. In this thesis the equine model system is applied to assess the therapeutic potential of regulatory and anti-inflammatory cytokines by recording the production of pro-inflammatory cytokines under various conditions. The results presented here revealed that equine, recombinant IL-1β as well as LPS, respectively, can efficiently stimulate equine chondrocytes to a 20-fold increase of IL-1β transcription (*Figure 13*). Dose-response experiments showed that a final concentration of 100 ng/ml of IL-1β or LPS in the growth medium represent a most effective dose to stimulate equine chondrocytes towards an inflammatory state [8]. In contrast, other studies examined the effect of human IL-1β on equine chondrocytes at much lower concentrations [419, 420]. Additionally, Takafuji et al. (2005) performed a transcription study of various cytokines and other mediators in equine chondrocytes after a short stimulation with equine IL-1β (6 h, 5 ng/ml) [421]. As a result, they found an at least 2-fold increase of markers such as extracellular-matrix proteins, cytokines and growth factors [421, 422]. The cause for this variance in effective concentrations of mediators for the induction of an inflammatory state is not known at present and needs further investigations. However, the concentration of effective IL-1β or LPS may differ in reagents from different suppliers. Another possibility may be the variety in the receptor status in different cell culture systems usually used by researchers.

LPS was tested in parallel to provide a positive control as well as to test an opportunity that is more cost-sparing than reqIL-1β. Additionally, LPS can be used to simulate a septic, bacterial-caused arthritis *in vitro*. Moreover, the Cox-2 promoter is also activated by LPS as depicted in *Figure 6* [8]. Therefore, the potential risk of an unwanted expression of the therapeutic transgene after activation of the Cox-2 promoter in case of a septic arthritis should be considered and carefully assessed in further investigations. One advantage could be the different receptor activation pathways of LPS and IL-1β.

To conclude, in the described experiments high concentrations (100 ng/ml) of reqIL-1β were applied to achieve significant effects that can be shown to be reversed by our approach.

6.2 Generation of an Inflammatory-sensitive Plasmid System Comprising the Cox-2 Promoter and IL-4

6.2.1 Comparing Different Transfection Systems

For unknown reasons, equine chondrocytes appear to be more difficult to transfect than the same cell type from other species [421]. Since the transfection of chondrocytes is a central issue for testing any novel approach for gene-therapeutic intervention, the optimization of the transfection protocols was an essential part of this study. Non-viral gene transfer delivers plasmid DNA into the cells with selected chemical and physical methods and only achieves transient transfection. Therefore, a series of commercially available non-viral transfection reagents were tested [8]. Guided by the high biosecurity requirements, non-viral gene delivery and transient transfection methods were preferred for the initial study presented here. The transfection reagents were selected either based on their previous application in canine or human chondrocytes, or their potential to transfect hard-to-transfect cells [170, 332, 399]. It appeared that equine chondrocytes do indeed behave differently compared to human, canine or even bovine chondrocytes which could be transfected at ease by using FuGENE 6 [170, 224] or others [423-426]. Additionally, higher transfection efficiencies were observed by pretreating the cells with hyaluronidase to break up the strong matrix as described previously [170]. Turbofect was determined as the best transfection reagent for high gene delivery yields in equine chondrocytes, despite the decline in cell viability, which indicates a lower stress threshold of the equine chondrocytes.

Table 13: Transfection reagents and their mode of action.

Transfection reagent	Ingredients and functional principles
FuGENE 6	▪ blend of lipids and other components ▪ non-liposomal transfection
FuGENE HD	▪ blend of lipids and other components ▪ non-liposomal transfection
X-tremeGENE 9	▪ blend of lipids and other components
X-tremeGENE HP	▪ blend of lipids and other components
NanoJuice	▪ nanotechnology Priostardendrimers ▪ polycationic liposomal transfection
Turbofect	▪ cationic polymer ▪ liposomal transfection
*Transit*IT-2020	▪ lipid-polymer ▪ liposomal transfection

Table reproduced from Lang et al. 2014 [8].

The significant differences in transfection efficiencies can be caused by the different functional principles of the reagents (*Table 13*). Most common methods base on liposomal or non-liposomal agents (lipofection) to form DNA-lipid complexes which are taken up by the cell via endocytosis and directly translocated to the nucleus by endosomal-liposomal vesicular transport [168]. Liposomal agents build cationic liposome-DNA complexes, whereas non-liposomal agents comprising lipids envelop micelle-like structure around the DNA and can be combined to non-ionic surfactants [142, 169]. Several reagents for transfecting chondrocytes with differing efficiencies are commercially available [170]. Therefore, liposomal transfection with cationic polymers appears to be suited best for equine chondrocyte transfection. Most of the other species can also be transfected with non-liposomal transfection [170, 399]. But it is hard to make tangible conclusions on the transfer potential of a given reagent. This is at least in part due to the often inadequate information of the manufacturers on the action mode of the reagents. *Table 13* summarizes the information that can be found on the webpages or in information brochures [8].

Electroporation is widely used in the laboratory routine to achieve high transfection rates of different cell lines and does not need cytotoxic chemicals. To gain high transfection efficiencies in chondrocytes, specific protocols using radiographic electroporation are needed and therefore, the demand for specialized equipment is higher [172, 173]. In this study, transfection of equine chondrocytes via electroporation was not sufficiently achieved. Low transfection efficiencies and high mortality rates were observed (see Chapter 5.2.3).

Magnetofection includes the binding of plasmid DNA to magnetic microparticles to be dragged into cells by strong directional magnetic fields [427]. This method does not require cytotoxic transfection reagents, but can be combined to raise the transfection rate. In this study, magnetofection was convincing and therefore, too time-consuming for further efforts.

Another observation was that the cells eliminated the plasmids not long after transfection, which may cause the low transfection yields (see Chapter 5.2.4). In order to experimentally overcome this fragility and to achieve a stable, well integrated gene delivery, the use of viral methods for gene transfer is considered in forthcoming studies. Not only the transfection agent but also the choice of the plasmid was found to be crucial for transfection of equine chondrocytes.

Taken together, to my best knowledge, this is the first study in which a wide range of transfection reagents has been tested for their potential to effectively transfect equine chondrocytes with the result that Turbofect a liposomal transfection reagent was identified to be well suited to achieve transfection rates up to 44%.

6.2.2 Optimizing the Plasmid Design

As a control in the transfection studies, the pI2 plasmid was initially chosen which includes an IRES to allow the independent expression of the two upstream genes (IL-4 and GFP) without fusing the proteins (*Figure 15*). This plasmid offers the possibility to insert two therapeutic transgenes under the control of a single promoter. Unexpectedly, the presence of IRES was found to cause a decrease of GFP expression and transfection efficiency, respectively, when compared with the same plasmid without the IRES site (*Figure 20*). Nevertheless, low transfection efficiencies for chondrocytes are not unusual due to the compact matrix that is produced by the chondrocytes [170]. Different studies also showed the moderate transfection efficiency of chondrocytes of other mammalian species that never reached up to 100% as it is normal in e.g. HEK cells [170, 399]. The basic plasmid includes an EMCV (encephalomyocarditis virus) IRES that is widely used in gene therapeutic approaches due to its high activity compared to other IRES. Therefore, one reason of reduced transfection efficiency could be that the EMCV IRES, in this particular plasmid, influences the expression of the downstream gene and consequently diminishes the expression of GFP as previously reported by others [428-432]. Another explanation for different expression yields could be the plasmids size. For instance, pN3 with a size of 4.7 kb showed the highest transfection efficiency, followed by pI2 (5.2 kb) and pN3.IL-4 (5.102 kb) which have nearly the same size. The pI2.IL-4 plasmid (5.602 kb) reached only low transfection efficiencies. Based on the results obtained the pN3 plasmid appeared to be the best choice for optimal transfection and expression yields and was therefore used for the *in vitro* equine cell culture model to be established.

Finally, the focus of the preliminary study was to optimize the non-viral transfection strategy to go on with the main experiments on the inflammation-controlled expression of IL-4. Consequently, all the afterwards performed experiments applied the same plasmid without IRES showed higher transfection efficiencies and the functionality of the approach. That is why, I desisted from further IRES experiment and liked to focus on the translation of our approach where I do not see a need for an IRES due to the sufficient effects of IL-4 expressed by the pN3.

In summary, the obtained results indicate the plasmid design should be considered carefully in further efforts since the integration of an IRES may have negative influence on the transfection efficiency in equine chondrocytes.

6.3 Test of the Functionality of a "Genetic Switch" (eqCox-2 Promoter)

The described and tested gene therapeutic strategy was previously introduced for canine chondrocytes [347, 348]. Compared to other anti-inflammatory or regulatory candidate genes with therapeutic potential, IL-4 provides great advantages because it inhibits the expression of the pro-inflammatory cytokine IL-1β which is regarded as one of the major propagator of inflammation leading to a cartilage breakdown [266, 433-436]. To regulate the expression of this transgene, the CMV-promoter in the basic plasmid was replaced with an endogenous and "inflammation" responsive element, the Cox-2 promoter (*Figure 15*). Consequently, the therapeutic transgene should be expressed in a controlled manner and the expression products released to the defect site as driven by the severity of inflammation in the OA afflicted joint. Only a few investigations have been implemented in the field of regulated gene therapy with various control elements other than the Cox-2 promoter so far [186, 328, 330, 437-440]. In the equine model described here, the Cox-2 promoter was utilized since it is known to be activated by different markers of inflammation such as IL-1β or LPS [337]. To test the system for its functionality, equine chondrocytes were transfected with the pN3.Cox-2.IL-4 plasmid and then stimulated with IL-1β to mimic an inflamed state of the cultured chondrocytes. As apparent from the results shown, exogenous stimulation causes an increase in the expression of both Cox-2 and IL-4 in such transfected cells, whereas IL-4 is not expressed in non-transfected, but stimulated chondrocytes, which is in line with the reported features of osteoarthritic cartilage [106, 419] (*Figure 22*). The observed simultaneous rise of Cox-2 and IL-4 indicates the responsiveness of the Cox-2 promoter as a "genetic switch" for the trigger IL-1β and thus renders the approach as functional [8]. Beside the successful expression of IL-4 on the mRNA-level, the translation and presence of IL-4 on protein level was proven in our experimental setup (*Figure 21*).

The primary aim of the study was to give a first evidence for the functionality of the approach in an equine cell culture model. In detail, the Cox-2 promoter switches on when stimulated with pro-inflammatory cytokines and consequently the Cox-2 promoter will be down-regulated via feedback-loop. In this study, the activation of the Cox-2 promoter was confirmed but not the switch-off. To verify this pathway, a more consistent gene delivery system that enables the investigation of transfected chondrocytes for a longer time needs to be established. Therefore, the establishment of a viral transduction system has already been started that allows the assessment of the down-regulation of the Cox-2 promoter as well as the reactivation. In addition, I do not think that further specific promoter studies on the functionality are necessary such as mutagenesis of the Cox-2 promoter. One big problem of such mutagenesis is the impossibility to be complete sure of the inactivity. The Cox-2 promoter consists of a huge number of regulatory elements such as PPRE as binding site for PPARs, two CRE as well as a serum response element binding to the SRF, two NRE, SP1 sites as

well as C/EBP, AP-2 binding sites and the E-box that overlaps the CRE. Therefore, complex mutagenesis studies have to be performed, optimizing the deletion sites and testing the activity. Consequently, this would be rather more expensive and time consuming as advantageous for the whole project.

Controlled expression of therapeutic genes in a disease-regulated fashion is of great importance in order to avoid adverse reactions. Therefore, the normally used CMV promoter should be treated with caution especially when applying growth factors (e.g. IGF-1). Subsequent uncontrolled cell divisions may lead to neoplasia and an uncontrolled expression of other therapeutic transgenes may cause potential systemic adverse reactions [324]. In order to provide a higher biosecurity level of gene therapy different systems for the controlled expression are under development. The tet-on system was successfully tested *in vitro* in lapine chondrocytes [327]. The disadvantage of this system is the needed regular injection of doxycycline when translating the approach to human patients. More elegant approaches have taken disease-regulated promoters such as elements of IL-1 and IL-6 promoter, promoters of acute phase proteins, the Saa3 and MMP-13 promoter or the coupled C3-tat/HIV promoter [328-331]. All these approaches aim at the expression activation under inflammatory conditions due to several cytokines, the upregulated transcription of transgenes during acute OA and the inhibition of expression rates after eliminating the inflammation [186, 332]. A study on the proteoglycan-4 expression under the control of Col II promoter was published recently [226].

Taken together, the results of this study clearly demonstrated the IL-1β driven activation of the Cox-2 promoter leading to higher levels of IL-4 mRNA and intracellular as well as extracellular protein concentrations.

6.4 Evaluation of the Anti-inflammatory Potential of eqIL-4

IL-4 is a multifunctional cytokine that e.g. promotes the maturation of Th2-cells and the subsequent down-regulation of Th1-cells and their cytokines. The main advantage of IL-4 is to effectively inhibit IL-1β and TNFα production that was shown in many *in vitro* and *in vivo* studies on OA and RA.

To estimate the anti-inflammatory effect of IL-4 the mRNA expression of IL-1β, IL-6, IL-8, MMP-1, MMP-3, and TNFα were measured under the same conditions. The results shown in *Figure 23A* demonstrate that after 24 h IL-4 is able to significantly decrease the expression of IL-1β, IL-6, IL-8, MMP-1, and MMP-3 in the cell cultures which produce high amounts

of IL-4 mRNA. This inhibition is supposed to decrease over time, whereas the mRNA expression of IL-4 is reduced in parallel *Figure 23B [8, 404]*. Unlike the protein concentration in the cell supernatants remained constant. IL-4 is known to have a short half-life between 15 and 20 min in the serum [283, 305]. Consequently, the IL-4 mediated suppression is hypothesized as concentration dependent. Nevertheless, the cell culture model is not yet suitable to perform long-term tests. Since IL-4 controls Th2-cells, it has the capacity to reduce Th1-cell released pro-inflammatory cytokines and to enhance the synthesis of those inhibitors (e.g. IL-10, IL-13) [441, 442]. The Th1-cell inhibition initiates suppression of macrophage activation and subsequently reduction of IL-1β, IL-6, IL-8 and TNFα [443]. But the main advantage of IL-4 is to effectively inhibit IL-1β and TNFα production also in synovial tissue obtained from RA as well as OA patients [263]. Moreover, IL-4 is able to suppress the MMP release of macrophages, monocytes and fibroblasts, and promotes the production of TIMPs by chondrocytes [264]. As described previously, the mechanism behind is that IL-4 represses NF-κB and mediates the anti-inflammatory activity through STAT-6 [107, 270]. Nevertheless, the anti-inflammatory potential of IL-4 in OA or arthritis has not been tested in an equine cell culture model as well as any kind of other large animal model in the literature before. However, there is only one study investigating the effect of IL-4 against OA in a mouse model [283]. In contrast, the efficacy of IL-4 was shown in several RA mouse models mainly CIA using gene therapeutic approaches. But also alternatives are under development as described in the study of Hemmerle et al. In this study the anti-inflammatory activity of IL-4 in a collagen-induced mouse model connecting IL-4 to a fusion antibody that recognizes the alternatively spliced extra domain A of fibronectin which is strongly overexpressed in arthritic synovial membranes [292]. In addition, IL-4 was described to reduce neuropathic pain which is relevant in OA. The study of Üçeyler et al. showed that IL-4 knock-out mice exhibit higher mechanical hypersensitivity after chronic constriction injury of the sciatic nerve paralleled by an elevated expression of IL-10 in this area [444]. They hypothesized that this is one of the other observed mechanisms that protects IL-4 knock-out mice from increased pain behavior. In addition, the group of Eijkelkamp (Utrecht) could show that a synerkine of IL-10 and IL-4 could block the pain in neuropathic pain model of the hinder limb in mice [445]. Neither IL-10 nor IL-4 alone could block the pain effectively. In conclusion, it seems that IL-4 also plays a great role in neuropathic pain and can be used as inhibitor.

It is clear that mRNA expression is only an indirect measure for the production of the corresponding protein which needs to be assessed directly in future experiments. However, since transcription of a gene gives a first evidence for the appearance of a particular expression product, the results based on qRT-PCR-evidence strongly indicate a functional link between

the synthesis of IL-4 and the inhibition of osteoarthritic mediators. Nevertheless, further experiments should also include protein studies on inflammatory markers.

As our results showed, recombinant equine IL-4 is able to inhibit inflammatory markers on the mRNA-level in an equine cell culture chondrocyte model and is therefore a promising transgene for therapeutic approaches towards OA.

6.5 General Discussion on IL-4 – Possible Toxicity

Several clinical studies investigating the toxicity of IL-4 in a dose escalation study or the efficacy against specific cancer or autoimmune disease as psoriasis in a phase II study have been enrolled (see Chapter 2.3.5 *Table 4*, *Table 5*) [251, 253, 255, 303-308, 311-313, 315, 317, 446, 447]. Some important remarks should be considered when assessing these studies. Most of these studies investigated the toxicity or efficacy in cancer patients where the immune system was involved. Furthermore, the applied rhuIL-4 was purified from *E.coli*. Consequently, the conclusions have to be drawn carefully on the possible toxicity of IL-4 applied in a joint. But the observation of these studies can give first hints towards possible risks. In each study a MTD was stated or could be assumed by carefully reading the adverse effects that were described. Doses of rhIL-4 ranked between 20 fg/d (p.os.) and 45 µg/kg/d (i.v.) using several treatment protocols [303, 310]. Based on an average patient with a body size of 1.80 m and a body weight of 75kg, the maximum tolerated (phase I escalation studies) or used (phase II studies) doses ranked for the s.c. application between 37.5 µg/d [255, 308] and 450 µg/d [316] and for the i.v. application between 150 µg/d [448] and 3,375 µg/d [303]. The overall lowest dose was 1.500 fg/d p.os [310]. In addition, the toxicity was mainly Grade 1-2 and Grade 3-4 adverse effects were mostly observed in high doses. However, the incidence and severity of toxicological effects depended besides the dosage also on the treatment duration that differed a lot between the studies. Sosman et al. showed in a 3 cycle study that a treatment with IL-2 after a 7-day application of IL-4 had no influence on adverse effects in a further IL-4 treatment cycle [311]. Compared to the studies on cynomolgus monkeys that were treated subcutaneously or intravenously with human IL-4 where the highest not lethal doses were 2 µg/kg/d i.v. for one month, 25 µg/kg/d s.c. for one month and 5 µg/kg/d s.c. for 6 months, the human studies showed that IL-4 is good tolerated to a certain degree even in cancer patients [299-301]. The observed adverse reactions such as chronic focal hepatitis, hepatic necrosis and chronic end- and periarteritis could not be observed in clinical trials. In some clinical trials the elution of hepatic enzymes was reported as well as a leak of peripherial endothelial cells leading to edema [311, 315]. Hepatic toxicity symptoms only appeared in patients with hepatic disease history [304, 313]. The risk of neurologic and cardiac toxicity should not be underestimated. Therefore, the clinical protocols of

recent studies defined strict criteria even for this kind of toxicities leading to an interruption of IL-4 treatment when achieving Grade 2 toxicity [303, 315, 319]. For the application in the joint, we do not expect to achieve such doses systemically. Therefore, the two studies on psoriasis with doses of 20 fg/d (p.os.) and 0.5 µg/kg/d (s.c.) that showed no adverse reaction are of importance and should be considered.

Beside the possible toxicity the question on the possible amount of IL-4 that can diffuse in the vasculature of the joint capsule and therefore enters the systemic blood stream should be addressed. To date there has not been enrolled any clinical study investigating the toxicological effect of intra-articular application of IL-4. Preclinical studies in rats and mice focused on the beneficial effect of IL-4 in the treated joint. A few studies can be used to draw conclusions on the systemic dissemination of IL-4 after direct joint application. Tarner et al. transferred T-cell hybridomas that were retrovirally transducted to express IL-4 and that verifiably migrated into the joint with the result that no significant difference could be found on day 8 and 15 between IL-4 levels in lymphnodes from treated and untreated site [289]. Another study applying adenoviral plasmids in a mouse model investigated the IL-4 serum levels and IL-4 concentration in the patella washout via ELISA. The patella washout showed IL-4 concentrations between 650 and 750 pg/ml whereas in the serum no IL-4 could be detected (limit of detection was 10 pg/ml) [449]. Controversially, a study performed in a rat model using retroviral plasmids found similar IL-4 concentrations on day 8 in the treated and untreated joint [450]. Simultaneously, the serum levels of IL-4 were increased to 2810 ± 180 pg/ml compared to the controls (272 +/- 17 pg/ml). The untreated joints were examined for mRNA expression of IL-4 with the result that no expression could be found leading to the hypothesis that the high serum levels lead to the increase in the untreated joint. However, as described previously the interpolation of data from rodent models to the human is challenging even in the case of RA and OA gene therapy. According clinical data using IL-4 as gene therapeutic target in OA and RA patients, one's conclusions can only be drawn from other clinical studies that have been conducted using IL-1Ra or TNFR–Fc (etanercept) as transgenes. Two *ex vivo* studies transducing retrovirally autologous synovial fibroblasts with IL-1Ra and applying them in the metacarpophaleal joints II and VI in 9 and two patients reported high amounts of IL-1Ra in the treated joints after 7 and 14 days, respectively [177, 451]. No severe adverse reactions could be observed. Mease et al. performed a clinical study in 2009 (15 patients) and 2010 (127 patients) using AAV plasmids to transfer TNFR-Fc in different joints (e.g. knee, ankle) [452]. In a dose-dependent manner, different titers of rAAV2-TNFR:Fc were observed in the blood of treated patients whereas no associated adverse effects could be observed. Most of the reported adverse effects were caused by the

intra-articular injection whereas one patient died due to an infection with *Histoplasma capsulatum* but no relation could be found to the treatment procedure.

Furthermore, as recorded in the literature review (Chapter 2.3.6) IL-4 is known to have a short half-life leading to the assumption that IL-4 is quite fast degraded after being expressed and translated by genetically modified cells in the joint when using gene therapeutic approaches. In implication the gene therapeutic application of IL-4 seems to be promising to provide a constant concentration of effective IL-4 in the joint, especially, when the expression is inflammation-controlled and self-limiting as in our approach.

CHAPTER 7: Conclusion and Future Perspectives

In conclusion, this study provides the experimental framework for the establishment of an *in vitro* inflammatory equine cell culture model of OA. Presently, the experimental system consists of an optimized non-viral transfection protocol for equine chondrocytes which is combined with the generation of an inflammatory environment by use of exogenous IL-1β or LPS. Based on the results presented here, it is now possible to generate a new species-specific, self-limiting and disease-controlled gene therapy for the treatment of OA or other joint defects with inflammatory causes in horses as well as humans. The key element of this new strategy is the integration of a “genetic switch”, the Cox-2 promoter which allows an inflammation triggered expression of therapeutic genes such as IL-4. Since such a controlled production of therapeutic proteins will be limited to the site of disease, such gene therapy to treat OA is regarded as both safe and effective. It thus has significant potential for public acceptance despite of the general skepticism which often comes up when the term “gene therapy” is in play.

In addition, in ongoing studies we focus on the fast application of IL-4 in the equine model in different ways such as chemical stabilization of IL-4 by protein modifications or its application in combination with newly developed nanocarriers that are available from biomedical companies. Several clinical studies had shown the risk of adverse effects of systemic rhuIL-4 (purified from *E.coli*) application in high doses and strong treatment protocols, which should be carefully considered in further efforts. Nevertheless, the risk of achieving these high doses when applying IL-4 via gene therapy in the joint is minimal. From personal communication, we know that the new approach of Hemmerle et al. who developed a fusion protein with IL-4 is now in the recruiting phase of patients for a clinical phase I trial [292].

Efforts towards the development of a 3D equine *in vitro* cartilage model are presently under way [453]. Autologous transplants suitable for testing in the equine animal model and/or in equine OA-patients will be available in the near future [386]. The limited transfection efficiency observed with non-viral transfection strategies (*ex vivo*) of equine cells especially with large plasmids may necessitate the additional or alternative application of viral plasmids in the future. Since in the projected therapy approach viral plasmids will only be present in the enclosed cartilage environment of a diseased joint, their eventual acceptance by both, the public opinion as well as the biosafety legislation is more likely than with systemic application. Furthermore, positive progress in patient education as well as several clinical studies on gene therapeutic approaches for cancer, against HIV or Eppstein-Barr virus will pave the way for the introduction of gene therapy to treat OA in the veterinary as well as

the human health system. Moreover, EU projects such as GAMBA or the company Gene-Quine Biotherapeutics (Germany) support the introduction of OA gene therapy in Germany.

CHAPTER 8: Summary/Zusammenfassung

Summary

Annemarie Lang: **Reviewing Challenges in Osteoarthritis Gene Therapy and Introduction of a Molecular Therapeutic Approach in an Equine Model System**

Osteoarthritis (OA) is one of the most common musculoskeletal disorders around the globe. Affected patients suffer from a huge loss in quality of life, and the treatment of OA has a strong financial impact on society. The underlying mechanism is based on cartilage destruction accompanied by an inflammatory response that leads to chondrocyte apoptosis and matrix degradation. However, an effective causal therapy which provides long-term remedy has not been found yet.

One new promising strategy is the application of local gene therapy. In order to avoid continuous overexpression, a controlled expression of therapeutic transgenes which should be self-limiting and operate only in case of inflammation is of main interest. Therefore, a “genetically switchable” plasmid was developed by placing the Cox-2 promoter in front of the chosen transgene IL-4. Previous studies by others proved the feasibility of the new gene therapeutic approach in a canine *in vitro* model [332, 399]. The aim of this thesis was to transfer and test the inflammation-controlled and self-limiting gene therapeutic approach in an *in vitro* equine cell culture model. Additionally, different questions on the feasibility and state of the art in OA gene therapeutic research, the possible risk and toxicity of IL-4 as effective transgene, and the suitable animal model for OA research were addressed in a copious literature search.

The first step in the experimental work included the establishment of an inflammatory *in vitro* 2D equine cell culture model to create an OA-like system. Therefore, equine chondrocytes were used in passage 3 to 4 and stimulated with 100 ng/ml recombinant equine IL-1β and LPS. The successful treatment was shown by proper inflammatory increased IL-1β mRNA expression. In a further step, alignment studies on the canine and equine Cox-2 promoter were performed with the result that only 46.4% consensus positions were determined. For the cloning of equine Cox-2 promoter regulated plasmids expressing equine IL-4, pIRES2-EGFP and pN3-EGFP were used as basis plasmids. During the optimization of the transfection protocols and adjustment to equine chondrocytes, different non-viral transfection reagents were examined, that yielded transfection rates up to 21% by combining Turbofect and pIRES2-EGFP. The transfection efficiency could be raised up to 44% by delivering a plasmid without IRES (pN3-EGFP). Further investigations focused on the functionality of the equine plasmid pN3.Cox-2.IL-4. Equine chondrocytes were transfected with

pN3.Cox-2.IL-4 and exogenously stimulated, which leads to a significant increase in IL-4 mRNA expression and protein translation. Simultaneously, endogenous IL-1β expression as well as expressions of specific OA markers decreased conceivably due to the presence of newly synthesized IL-4.

The literature part covers the potential risk and toxicity of an intra-articular injection and expression of IL-4. Several clinical studies were summarized, which showed the risk of adverse effects of systemic rhuIL-4 application in high doses (up to 45 µg/kg/d i.v.) and strong treatment protocols in late stage cancer patients. Nevertheless, the risk of achieving these comparable doses when applying IL-4 via gene therapy in the joint was rated as low.

In addition, the choice of a suitable animal model is of great importance for the translational process as well as the interpolation possibility to the human patient. Because of the size, joint anatomy and cartilage thickness, the horse provides a number of advantages as animal model for OA research. As natural occurring model, OA pathogenesis parallels the human disease in many facets and the translation to patient horse and afterwards to the human is accelerated. Furthermore, during the last years, many efforts have been made towards the safety and acceptation of OA gene therapy. First clinical trials have been successfully conducted and further studies are in preparation.

Nevertheless, very few researches focus on the controlled expression of therapeutic transgenes. In this thesis, the transformation of a molecular therapeutic approach to the equine system could be demonstrated. This new approach should be exploited to develop an anti-inflammation therapy initiating itself by an inflammatory microenvironment. Molecular tools such as the described therapeutic plasmid pave the way for a successful controlled, self-limiting and local gene therapy [8].

Zusammenfassung

Annemarie Lang: **Wissenschaftliche Betrachtung von Herausforderungen gentherapeutischer Entwicklungen für die Arthrosebehandlung und Einführung eines molekularen therapeutischen Ansatzes in ein equines *in vitro* Modell**

Osteoarthrose (OA) zählt zu den weltweit am häufigsten auftretenden muskuloskelettalen Erkrankungen, die mit einem deutlichen Verlust an Lebensqualität, erheblichen körperlichen Einschränkungen und finanzieller Belastung der Gesellschaft einhergehen. Die Knorpeldestruktion wird von einem entzündlichen Prozess begleitet, der die Apoptose von Knorpelzellen weiter fördert. Diese führt zu arthrosetypischen Veränderungen im Gelenk, von denen ebenso die Synovialmembran und der subchondrale Knochen betroffen sind. Zum jetzigen Zeitpunkt gibt es keine Therapie, die eine längerfristige Besserung oder Heilung gewährleisten kann.

Einen neuen therapeutischen Ansatz stellt die intra-artikulär angewandte Gentherapie dar. Dabei ist die unkontrollierte, kontinuierliche Überexpression von therapeutischen Transgenen ein mögliches Risiko für unerwünschte, systemische Nebeneffekte. Zur Vermeidung, kommt der Entwicklung von kontrollierten, entzündungs-regulierten und selbst-begrenzenden Expressionen eine große Bedeutung zu. Um dieses Ziels zu erreichen, wurde der Cox-2 Promoter als „genetischer Schalter“ vor das therapeutische Transgen IL-4 kloniert. Die Umsetzbarkeit dieses Ansatzes konnte erfolgreich in vorherigen Studien im *in vitro* Hunde-Knorpelzell-Modell gezeigt werden.

Das Ziel dieser Doktorarbeit war die Übertragung dieses neuen Ansatzes auf das equine System und somit die Testung in einem zuvor etablierten equinen *in vitro* Knorpelzell-Modell. Des Weiteren sollten verschiedene Fragestellungen zum Stand der Forschung im Bereich der Gentherapie bei Arthrose, der möglichen Risiken und Toxizität von IL-4 und der Wahl des geeigneten Tiermodells für die Arthrose-Forschung mittels Literaturrecherche untersucht und diskutiert werden.

Der erste Teil der experimentellen Arbeit bestand in der Etablierung des inflammatorischen equinen 2D Zellkultur-Modells, um ein arthrose-ähnliches Testsystem herzustellen. Dafür wurden equine Knorpelzellen in Passage 3 und 4 verwendet und mit 100ng/ml IL-1β oder LPS stimuliert bis eine typische Zunahme der IL-1β mRNA-Expression beobachtet werden konnte. Zudem wurden die Sequenzen des caninen und equinen Promoters verglichen, um eine etwaige Übertragbarkeit und Verwendung des caninen Promoters zu validieren. Nur in 46.4% der Positionen konnte eine Übereinstimmung gefunden werden. Im anschließenden Klonierungsprozess wurden die Sequenzen des equinen Cox-2 Promoters und des equinen IL-4 Gens in ein pIRES2-EGFP oder pN3-EFGP Basisplasmid eingebracht. Während der

Transfektions- und Protokolloptimierung an equinen Knorpelzellen, wurden verschiedene nicht-virale Transfektionsreagenzien getestet. Als besonders gut stellte sich Turbofect heraus, welches eine Transfektionseffizienz von 21% mit dem pIRES2-EGFP erreichte. Durch die Nutzung eines Plasmids ohne IRES (pN3-EGFP) konnte die Transfektionseffizienz signifikant auf 44% gesteigert werden. pN3.Cox-2.IL-4 exprimierende Knorpelzellen zeigten nach Stimulation mit IL-1β eine signifikante Zunahme der IL-4 mRNA- und Proteinlevel sowie die Abnahme spezifischer Arthrose-Marker.

Im Literaturteil wurden Risikopotentiale der intra-artikuläre Injektion oder Expression von IL-4 sowie die mögliche Toxizität genauer beleuchtet und diskutiert. Verschiedene klinische Studien zu Risiken für unerwünschte Nebenwirkungen von einer systemischen Applikation von rhuIL-4 in hohen Dosen (bis zu 45 µg/kg/d i.v.) und nach strengen Behandlungsprotokollen in Spätstadium-Krebspatienten wurden zusammengefasst. Die Wahrscheinlichkeit, vergleichbar hohe systemische Dosen aufgrund einer intra-artikulären Injektion oder Expression zu erreichen, konnte gering eingestuft werden. Weiterhin spielt die Wahl des geeigneten Tiermodells eine wichtige Rolle im translationalen Prozess und der Möglichkeit der Erkenntnisübertragung auf den humanen Patienten. Aufgrund der Größe, der Gelenksanatomie und der Knorpeldicke, bietet das Pferd viele Vorteile als Tiermodell in der Arthroseforschung. Da das Pferd selbst einen Patienten darstellt, ist die Pathogenese vergleichbar und ermöglicht dadurch eine Beschleunigung der Translation auf den Menschen. Zusätzlich wurde in den letzten Jahren viel unternommen, um die Gentherapie sicherer zu gestalten.

In dieser Arbeit konnte die Übertragung eines molekularen therapeutischen Ansatzes in ein equines *in vitro* Knorpelzell-Modell gezeigt werden. Dieser neue gentherapeutische Ansatz soll genutzt werden, um eine anti-entzündliche Therapie zu entwickeln, die selbst-aktivierend und regulieren in einem entzündlichen Milieu agiert. Innovative molekulare Ansätze, wie die beschriebenen Plasmide ebnen den Weg zu einer kontrollierten, selbst-regulierenden und lokalen Gentherapie [8].

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Anteilserklärung von Annemarie Lang

(1) Anteilserklärung von Annemarie Lang an der vorliegenden Monografie gemäß § 8 Abschnitt 3 Promotionsordnung des Fachbereichs Veterinärmedizin der Freien Universität Berlin (Stand 14.09.2011)

Annemarie Lang hat die Experimente, Ergebnisse und Abbildungen/Tabellen, die in dieser Monografie dargestellt sind, selbständig durchgeführt, analysiert und erstellt. Abbildungen/Tabellen und Ergebnisse die in Zusammenarbeit mit anderen Wissenschaftlern entstanden und in anderen Arbeiten/Veröffentlichungen publiziert sind, wurden kenntlich gemacht und werden im Folgenden detailliert aufgelistet:

Figure 1: Der histologische Schnitt (Acanblau-Färbung, Gelenk, Pferd) wurde Annemarie Lang von Gabriela Korus (Julius- Wolff-Institut, Charité- Universitätsmedizin Berlin) zur Verfügung gestellt. Die Bilder wurden von Annemarie Lang aufgenommen und dürfen mit freundlicher Genehmigung von Gabriela Korus in dieser Monografie veröffentlicht werden.

Table 1: Annemarie Lang hat diese Tabelle eigenständig auf Grundlage der angegebenen Publikationen erstellt.

Figure 2: Die Abbildung sowie die Legende wurden aus der Publikation [63] entnommen. Die Genehmigung zur Verwendung in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Figure 3: Die Abbildung sowie die Legende wurden aus der Publikation [77] entnommen. Die Genehmigung zur Verwendung in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Table 2 und ***3***: Die Tabellen wurden von Annemarie Lang eigenständig für die Veröffentlichung in der folgenden Publikation erstellt:

Rai, M.F., Lang, A., Sieber, M. & M.F.G. Schmidt (2011). Conditioning and Scaffolding of Chondrocytes: Smart Steps towards Osteoarthritis Gene Therapy. In: Chunsheng Kang (ed.), Gene Therapy Application. In: Tech Open Access Publisher. Rijeka, Croatia. ISBN 978-953-307-541-9

Die Quellenangabe wurde entsprechend den Forderungen in der Copyright Policy von InTechOpen angegeben: ® 2011 M.F. Rai, A. Lang, M. Sieber, M.F.G. Schmidt. Adapted from [213]; originally published under CC BY-NC-SA 3.0 license. Available from: 10.5772/1013

Table 4-6: Die Tabellen wurden eigenständig von Annemarie Lang auf Basis der angegebenen Publikationen erstellt.

Figure 5: Die Abbildung sowie die Legende wurden aus der Publikation [337] entnommen. Die Genehmigung zur Verwendung in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Figure 6: Die Abbildung sowie die Legende wurden aus der Publikation [339] entnommen. Die Genehmigung zur Verwendung in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Figure 7: Diese Abbildung wurde von Annemarie Lang und Matthias Sieber für die Veröffentlichung im folgenden Magazin erstellt:

Schmidt MFG, Lang, A., Sieber, M., Rai, M.F. Smarte Transplantate - Ein neuer Therapieansatz zur Heilung der Osteoarthrose. hundkatzepferd, succidia AG: Darmstadt, 2010; 6-12.

Die Genehmigung zur Verwendung der modifizierten Abbildung in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Table 7 und 8: Die Tabellen wurden eigenständig von Annemarie Lang erstellt und publiziert [8]. Die von Johannes Neuhaus (Klinik für Rheumatologie, Charité-Universitätsmedizin Berlin) designten und etablierten Primer wurden kenntlich gemacht. Alle anderen Primer wurden von Annemarie Lang designt und etabliert. Die Genehmigung zur Verwendung der modifizierten Tabellen in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Figure 12: Die Bilder wurden gemeinsam mit Moritz Pfeiffenberger (Bachelor-Student) aufgenommen und in der Bachelorarbeit von Moritz Pfeiffenberger veröffentlicht [402]. Zusätzlich Optimierung der Kultivierung von equinen Chondrozyten sowie Untersuchungen zu phäntotypischen Änderung auch auf mRNA-Ebene wurden von Moritz Pfeiffenberger unter Anleitung von Annemarie Lang durchgeführt und in seiner Bachelorarbeit veröffentlicht [402]. Diese Untersuchungen sind nicht in dieser Monografie enthalten.

Figure 13: Annemarie Lang hat die Vorgehensweise bei den Versuchen geplant und etabliert. Moritz Pfeiffenberger hat unter Anleitung von Annemarie Lang erste Untersuchungen zur exogenen Stimulation von equinen Chondrozyten mit IL-1β oder LPS durchgeführt. Erste Vorergebnisse der Untersuchungen wurden in der Bachelorarbeit von Moritz Pfeiffenberger veröffentlicht [402]. Die Vorergebnisse wurden anschließend eigenständig von Annemarie Lang weiter validiert, ausgewertet und in entsprechenden Abbildungen dargestellt.

Table 10, Figure 15: Diese Abbildung wurden eigenständig von Annemarie Lang erstellt und publiziert [8]. Die Genehmigung zur Verwendung der Tabelle in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

Table 11, Figure 19: Annemarie Lang hat die Vorgehensweise bei den Versuchen geplant und etabliert. Moritz Pfeiffenberger hat unter Anleitung von Annemarie Lang erste Untersuchungen zur Transfektionseffizienz verschiedener Reagenzien in equinen Chondrozyten sowie die anschließende Beurteilung der Vitalität der Zellen durchgeführt. Erste Vorergebnisse der Untersuchungen wurden in der Bachelorarbeit von Moritz Pfeiffenberger veröffentlicht. Die Vorergebnisse wurden anschließend eigenständig von Annemarie Lang weiter validiert, ausgewertet und in entsprechenden Abbildungen dargestellt.

Figure 20A, 21-23 und Table 10, 13: Diese Abbildungen und Tabellen wurden eigenständig von Annemarie Lang erstellt und publiziert [8]. Die Genehmigung zur Verwendung der Tabelle in dieser Monografie liegt Annemarie Lang vor und kann auf Rückfrage vorgewiesen werden.

(2) Anteilserklärung von Annemarie Lang an der erfolgten Publikation gemäß § 8 Abschnitt 3 Promotionsordnung des Fachbereichs Veterinärmedizin der Freien Universität Berlin (Stand 14.09.2011)

Lang, A., Neuhaus, J., Pfeiffenberger, M., Schröder, E., Weber,Y., Ponomarev, I., Gaber, T. & M.F.G. Schmidt (2014) Optimization of a non-viral transfection system to evaluate Cox-2 controlled IL-4 expression for osteoarthritis gene therapy *in vitro*. Journal of Gene Medicine. Nov-Dec; 16(11-12): 352-63. doi: 10.1002/jgm.2812

Beitrag von Annemarie Lang im Einzelnen:

- Beteiligung an der Versuchsidee
- Planung aller Versuche
- Zellisolierung und Etablierung der Zellkultur von equinen Chondrozyten
- Durchführung der Klonierungen (inkl. Primer- und Plasmiddesign)
- Etablierung und teilweise Durchführung der Transfektionsstudien zum Vergleich unterschiedlicher Transfektionsreagenzien
- Durchführung der Plasmid- und Transfektionsoptimierungsstudie
- Etablierung und Durchführung der exogenen Stimulation von equinen Chondrozyten mit IL-1β oder LPS
- Etablierung und teilweise Durchführung der Durchflusszytometrie sowie Einweisung und Anleitung von Moritz Pfeiffenberger in die Methodik

- Teilweise Etablierung der Primer für die qRT-PCR (Hilfe von Johannes Neuhaus) und Durchführung der qRT-PCR
- Etablierung und Durchführung des IL-4 ELISAs
- Durchführung des Western Blots (Etablierung und Anleitung bei der Durchführung durch Johannes Neuhaus und Erik Schröder)
- Statistische Auswertung aller Ergebnisse
- Erstellung aller Abbildungen
- Erstellung des Manuskripts und Einarbeitung der Korrekturen von Prof. Schmidt, Timo Gaber, Igor Ponomarev und Johannes Neuhaus

Publications

From the present work

Publications (Peer reviewed)

Lang, A., Neuhaus, J., Pfeiffenberger, M., Schröder, E., Weber,Y., Ponomarev, I., Gaber, T. & M.F.G. Schmidt (2014) Optimization of a non-viral transfection system to evaluate Cox-2 controlled IL-4 expression for osteoarthritis gene therapy *in vitro*. *Journal of Gene Medicine*. Nov-Dec; 16(11-12): 352-63. doi: 10.1002/jgm.2812

Book Chapter

Rai, M.F., **Lang, A.**, Sieber, M. & M.F.G. Schmidt (2011). Conditioning and Scaffolding of Chondrocytes: Smart Steps towards Osteoarthritis Gene Therapy. In: Chunsheng Kang (ed.), Gene Therapy Application. In: Tech Open Access Publisher. Rijeka, Croatia. ISBN 978-953-307-541-9

Abstracts & Participation in Conferences

Neuhaus, J., **Lang, A.**, Gaber,T., Schmidt, M.F.G. (2015) Kontrollierte Überexpression von IL-4 als gentherapeutische Strategie zur Behandlung von Osteoarthrose. Osteologiekongress, Berlin

Lang, A., Neuhaus, J., Pfeiffenberger, M., Schröder, E., Buttgereit, F., Schmidt, M.F.G. & T. Gaber (2014) Interleukin-4 as Promising, Anti-inflammatory Transgene for Gene Therapeutic Application in Rheumatic Disorders. ACR/ARHP Annual Meeting, Boston - Poster Presentation

Lang, A., Neuhaus, J., Pfeiffenberger, M., Gaber, T., Ponomarev, I., Buttgereit, F., Barnewitz, D. & M. F. G. Schmidt (2014) Controlled Expression of Endogenous Interleukin-4 for Gene Therapeutic Approaches towards Rheumatic Disorders. Annual European Congress of Rheumatology EULAR, Paris - Proceeding publication

Lang, A., Pfeiffenberger, M. & M.F.G. Schmidt (2013) Controlled Expression of Endogenous Genes for Osteoarthritis Gene Therapy. Regenerative Medicine, Vol. 8, No. 6s: 44. World Congress on Regenerative Medicine, Leipzig - Oral Presentation

Lang, A., Pfeiffenberger, M., Barnewitz, D. & M.F.G. Schmidt (2011): Controlled Expression of Endogenous Genes: Smart Steps towards Osteoarthritis Gene Therapy; PhD Symposium, Berlin-Brandenburg School of Regenerative Therapies (BSRT), Berlin - Poster Presentation

Pfeiffenberger, M., **Lang, A.**, Barnewitz, D. & M.F.G. Schmidt (2011): Smart steps towards osteoarthritis therapy using an equine cell culture model; bone-tec, Hannover

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die hier vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 04.09.2015

Annemarie Lang