TECHNISCHE UNIVERSITÄT MÜNCHEN

Abteilung für Sportorthopädie

Klinikum rechts der Isar

Direktor: Univ.-Prof. Dr. A. Imhoff

The Behavior of Bovine Articular Chondrocytes of distinct topographical Knee Joint Regions in Monolayer - and mechanically stimulated 3-D Culture

Anna Maria Stefanie Buchberger

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. E. J. Rummeny

Prüfer der Dissertation:

1. **Univ.-Prof. Dr. A. Imhoff**
2. **Priv.-Doz. Dr. K. Beitzel**
3. **Univ.-Prof. Dr. R. von Eisenhart-Rothe**

Die Dissertation wurde am 09.02.2015 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 03.02.2016 angenommen.

Meiner Familie

In Liebe und Dankbarkeit

Content

Content

1. Index of Abbreviations

ACI /ACT	Autologous Chondrocyte Implantation / Transplantation
%	Percentage
~	About
°	Degree
°C	Degree Celsius
3-D	Three Dimensional
AD	Anno Domini
Agg	Aggrecan
atm	Atmospheric Pressure
BC	Before Christ
BMP	Bone Morphogenic Protein
ca.	Circa
cm	Centimeter
cm^2	Square Centimeter
CO_2	carbon dioxide
Col I, II, X	Collagen I, II, X
COMP	Cartilage Oligomeric Matrix Protein
dest.	distilled
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribinuclear Acid
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular Matrix
EDTA	Ethylen-Diamino-Tetraacetat
et al.	et altera
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycan
h	hour

H_2O	Water
HE	Haematoxylin-Eosin
HMG	High Mobility Group
ICD-10	International Statistical Classification of Diseases and Related Health Problems
ICRS	International Cartilage Research Society
IGF	Insulin-like Growth Factor
Ihh	Indian Hedgehog
Img	Image
LF	Lateral Femur Condyle
LT	Lateral Tibia Plateau
Macht	Matrix Associated Chondrocyte Transplantation
MF	Medial Femur Condyle
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mmol	Millimol
MMP	Matrix Metallo Proteinase
MPa	Mega Pascal
mRNA	Messanger Ribonuclear Acid
MT	Medial Tibia Plateau
µg	Microgram
µl	Microliter
µm	Micrometer
μm^2 / μm^3	Square/Cubic Micrometer
µmol	Micromol
N	Newton
NaCl	Sodium Chloride
ng	Nanogram
NWB	Non Weight-Bearing

O_2	Dioxide
OA	Osteoarthritis
OATS	Osteochondrales Autologes Transfer-System
P	Patella
p	Page
P [number]	Passage [number of passages in culture]
PBE	Phosphate Buffer
PBS	Phosphate Buffered Saline
PD	Population Doubling
PG	Proteoglycan
PRG-4	Proteoglycan 4
PTHrp	Parathyroid Hormone related peptide
rpm / g	Rotation per Minute
rtPCR	reverse transcription Polymerase Chain Reaction
sec	Second
SOX 9	Sex Determining Region Y Box 9
SZP	Superficial Zone Protein
TBSS	Tyrode's Balanced Salt Solution
TGF-beta	Transforming Growth Factor
TRI	Trizol
Vol	volume
WB	Weight-Bearing
X	Femoral Notch
Y	Proximo-Medial Femur Condyle

2. Introduction

"The narrowest hinge in my hand puts to scorn all machinery." [Whitman 1982] This observation by Walt Whitman in 1892 still stands unchallenged, despite all the remarkable advances in joint replacement and repair. No current prostheses or other surgical repair method comes close to duplicating the function and durability of synovial joints. These complex structures, crucial for movement and thus for a normal life, as we define it, are formed by an arrangement of multiple distinct tissues including the joint´s capsule, synovial tissue, ligaments, menisci, subchondral bone and hyaline articular cartilage.

Intact hyaline cartilage in joints permits movement with low friction and reduced pressure and shear. About 80% of its mass is made of water while the solid components are composed of mainly collagen type II and proteoglycans like Aggrecan. When load is applied during joint movement, cartilage tissue responses with an elastic deformation due to the structure of its extracellular matrix (ECM). Water is released into the joint space during load and can, during decompression reintegrate into the ECM. Thus, the structural integrity of the ECM with its distinct biochemical composition is crucial for the function of hyaline cartilage.

Already small injuries of the articular cartilage of joints can, progressing over time, lead to the manifestation of arthrosis [Hayes 2001 p. 35-53; Mankin 1982 p. 460-466]. This is not only a therapeutic but also a socioeconomic problem, since the demographic development of society is one of aging with a progression in the prevalence of age-dependent osteoarthritis. In 2006 the direct costs for the German health system were 236 billion Euros, 17.2 billion Euros more than in 2002. The category of ICD-10 XIII diseases of the musculo-skeletal-system shared the third place together with ICD -10 V psychological and behavioral disorders and was responsible for 11.3% of the expenses (26.6 billion Euros). In the age group of 30-44 years, which accounted for 30 billion Euros of all expenses, diseases of the musculo-skeletal system were already placed on third position being responsible for 10.5% of all expenditures (9.6% female/11.8%male). Osteoarthritis was accountable for 3.2% of the direct spending. A survey from 2003/04 conducted by the Robert Koch Institute revealed that 23% of all female and 15% of all male citizens asked suffered from Osteoarthritis. In the age group of > 69 years of age, this percentage was even higher (50% female/25% male) [Nöthen 2009 Vol. 48: p. 13].

Especially the knee joint is one of the main sites of chondral and osteochondral lesions, leading to osteoarthritis (OA). Van Saase et al. performed a cross-sectional population study in a Dutch village radiologically assessing 6585 inhabitants. In the age group of 45-54 years, OA of the knee joint already accounted for 9.3% (female) /14.2% (male) of the findings, with numbers increasing with age [Van Saase 1989 p. 275-276]. In a clinical trial of arthroscopic reevaluation after traumatic knee injury

in 31516 cases, in 62.9 % (19827 cases) lesions of the articular cartilage were found. The majority of these lesions were lesions of the patella as well as lesions of the medial femur condyle [Curl 1997 p. 458]. Another study reported similar findings. Chondral lesions were found in 60% of the 25124 knee arthroscopies with 36% located on the patella and 34% on the medial femur condyle [Widuchowski 2007 p. 179]. An arthroscopic study providing data of 1000 knee arthroscopies also published very similar results. Chondral and osteochondral lesions were found in 61% of the patients, with 58% of them localized at the medial femur condyle. In 19% of the cases this defect was a focal one with a mean defect area of 2.1 cm^2 and an associated trauma in 61% of the patients [Hjelle 2002 p. 730-34].

2.1. Cartilage Defects

The famous London anatomist and surgeon William Hunter (1718-1783) published the first scientific study about articular cartilage up to that time point. He described very detailed structure, components and function of articular cartilage. But he also wrote about the nature of cartilage defects and stated in 1743, "From Hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and that, once destroyed, is not repaired" [Hunter 1743 42: p. 521].

Approximately 250 years later we have investigated the structure, microstructure and biochemical components as well as biomechanical properties of articular cartilage in detail but the main problem of articular cartilage "**when destroyed, it is never recovered**" [Hunter 1743 42: p. 521] still remains. Joint degeneration involves all the tissues that form the synovial joint however the primary changes consist of the loss of articular cartilage, remodeling of the subchondral bone and formation of osteophyts. Compared to many other tissues, the cell to matrix ratio, mitotic activity and turnover rate are very low in articular cartilage. Although individual chondrocytes are surprisingly metabolically active, showing similar glycolytic rates like other cells of vascularized tissue, the tissue in sum shows only a low metabolic activity since there are only few cells in the tissue of the articular cartilage [Buckwalter 1998 p. 478; Stockwell 1978 31: p. 9]. The higher glycolytic rates are probably explained by the fact that chondrocyte metabolism has to operate at low oxygen tension within the cartilage matrix, ranging from 10% at the superficial zone down to less than 1% in the deep zone [Goldring 2006 p. 1006].

Unfortunately, articular cartilage has only a limited ability to maintain and repair itself and with age, these capacities decline. While young cartilage has a high concentration of transforming growth factor beta (TGF-β) which accelerates synthesis of extracellular matrix, aged cartilage shows a decrease in chondrocyte activity and number of marrow mesenchymal stem cells. Once the cartilage

is matured in the adult, chondrocytes maintain a low turnover rate of replacement of ECM with a collagen half-life of more than 100 years and an aggrecan core protein half-life between 3 and 24 years [Goldring 2006 p. 1006]. Thus, in mature tissue the potential for spontaneous, quality repair is comprised, normally resulting in primarily fibrous tissue at the superficial layers and fibrocartilage in lower zones. These repair tissues are unable to withstand the high compressive loads in joints and eventually deteriorate [Buckwalter 2000 p. 487].

Cartilage defects can be divided into three main types. Matrix disruption, partial thickness defects, and full thickness defects which do not penetrate the subchondral bone. These are distinct from osteochondral defects, involving the subchondral bone. Clinically, there have been several score systems introduced to grade arthroscopic findings, the ICRS Grading is today considered to be the gold standard [Borne, van den 2007].

Table 1: Clinical Grading of Arthroscopic Findings

ICRS Grading	
Grade 0	Normal
1 A/B	Nearly normal; soft indentation (A) and/ or superficial fissures and cracks (B)
2	Abnormal; lesions extending down to < 50% of cartilage depth
3 A/B/C/D	Severely abnormal; lesions extending to > 50% of cartilage depth (A), down to the calcified layer (B), until but not through the subchondral bone (C), including blisters (D)
4 A/B	Severely abnormal; through the sub-chondral bone, not across the entire diameter of the defect (A), penetration across the entire diameter of the defect (B).

Matrix disruption occurs from blunt traumas, such as patella luxation or rupture of the anterior cruciate ligament. The ECM is damaged but the injury is not extreme and the remaining viable chondrocytes will increase their synthetic activity to repair the tissue. Already small, partial thickness defects that resemble clefts and fissures caused for example by mechanical damage and thus interrupt the surface will fail to heal and degenerate over time. Articular cartilage degeneration, the progressive loss of the normal structure and function of cartilage, leads to the clinical syndrome of (osteo)-arthritis [Buckwalter 2000 p. 481]. In mature tissue, a limited repair process can take place in response to the trauma within the tissue surrounding and adjacent to the site of defect. It has been observed that the cells at the wound margins first undergo cell death. However after 24 hours there is an increase in cell proliferation or cluster formation. Simultaneous with this proliferation starts an increased matrix synthesis and catabolism. Since this response is short lived, it will fail to repair the defect [Mankin 1982 p. 465]. It has also been described that cells can be induced to migrate from the

synovium across the articular surface to the lesion and fill the defect under the influence of growth factors. But in the absence of a fibrin matrix and mitogenic factors, these synovial cells will fail to fill the void due to the anti-adhesive properties of proteoglycans [Hunziker 2001 p. 25; Hunziker 1996 p. 723].

The full thickness defects are more consuming injuries of the articular cartilage that can perforate into the underlying bone exposing the defect to a supply of blood that opens up the possibility to fill the defect with a formation of a fibrin clot. Spontaneous repair is more likely in these osteochondral defects because a response from the mesenchymal stem cells, from which chondrocytes originate can be triggered [Athanasiou 2001 p. 226]. This intrinsic repair response is utilized in many arthroscopic repair strategies, like microfracturing, abrasion arthroplasty or Pridie drilling to alleviate joint pain. Mostly, the repair tissue is fibrocartilage, with inferior mechanical and biochemical characteristics, a poor organization in structure and significant amounts of collagen type I. Even though in some cases, the formation of hyaline-like cartilage can be found this is still a poor substitute. With time, there is marked degeneration of the repair tissue and continuous degeneration of the native articular cartilage eventually leading to a premature osteoarthritis [LaPrade 2008; Henderson 2007; Kreuz 2006; Hunziker 2002 p. 570; Buckwalter 1998 p. 199]. Shapiro et al. showed that during this process, the tissue adjacent to the wound margins becomes necrotic and little to no remodeling occurs. Empty lacunae observed in the native tissue at the wound margins were not filled by either active migrating chondrocytes nor by mesenchymal cells from the defect void [Shapiro 1993 p. 549].

The goal in cartilage defect repair is not only any repair tissue of inferior quality within the defect, but to implement a repair tissue that has the same functional and mechanical properties as the native hyaline articular cartilage.

To achieve this goal, techniques were developed involving the excision of healthy cartilage to fill the defect. In the MosaicPlasty/OATS (osteochondral autolog transfer system) osteochondral plugs from non load-bearing regions of the joint are biopsied for the implantation into the defect. A cell-based approach is the autologous chondrocyte implantation. It's advantage is a smaller degree of donor site morbidity due to reduced amounts of healthy cartilage needed.

2.2. Autologous Chondrocyte Implantation

The autologous chondrocyte implantation (ACI) or transplantation (ACT) in humans was first published by Lars Peterson and his group in 1994 [Brittberg 1994]. In this clinical approach, patients

presenting with symptomatic small and medium size articular cartilage defects are treated with autologous chondrocytes biopsied from a non weight-bearing region of the joint and re-implanted into the defect after monolayer expansion ex vivo. ACI has been investigated since in many comparative studies with other therapeutic approaches and has been evaluated in several mid- and long-term studies as discussed below.

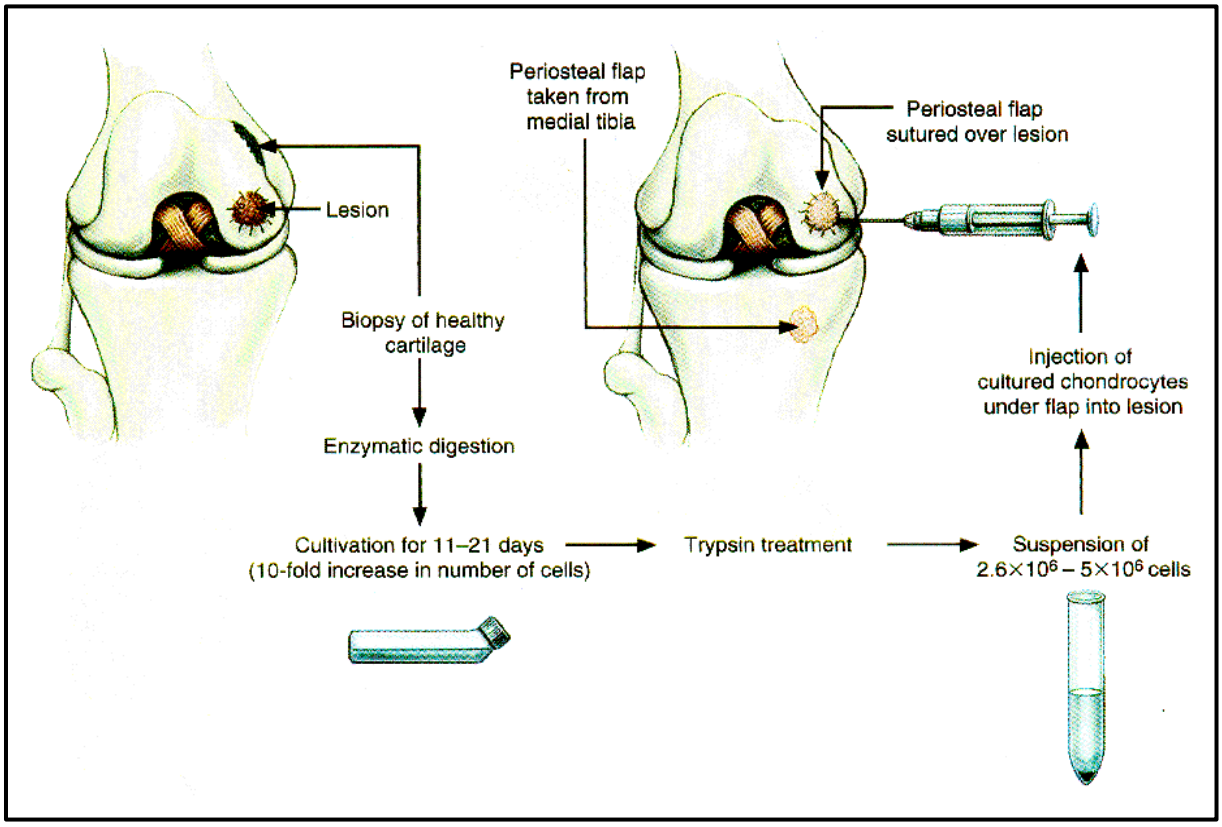
The indication for ACI as a therapeutic approach is evaluated by arthroscopic means, including the above mentioned ICRS score. Location, depth and size of the lesion, the quality of the surrounding tissue, the degree of undermining cartilage as well as the status of the opposing chondral surface are evaluated by this procedure as gold standard. Patients who are possible candidates for ACI present ideally with symptomatic full thickness chondral or osteochondral defects, surrounded by healthy, normal cartilage and no other signs of osteoarthritis or degeneration in the knee joint. ACI should be regarded as a second line treatment for defects less than 2 cm^2 after other simpler bone marrow stimulating techniques have failed. In defects larger than 2-3 cm^2 ACI is considered to be a first line treatment [Brittberg 2008 p. 40-41]. Very recently the working group of "Tissue Regeneration" of the German Society of Orthopaedic Surgery and Traumatology published a review concerning the indications and limitations of ACI. As an indication for ACI is thus advocated a symptomatic cartilage defect with an ICRS score of III or IV with a defect size > 2,5 cm^2 in young and active patients, of > 3-4 cm^2 or with a subchondral defect and large diameter (including bone augmentation) [Niemeyer 2013 p. 42].
Contraindications for ACI include severe osteoarthritis with bipolar lesions, an active rheumatoid arthritis, active autoimmune connective tissue diseases, uncorrected meniscal deficiency, uncorrected mechanical leg axis alignment, severe ligament laxity leading to pathologic stress pattern within the joint and patients with malignancies.

The following classification of first, second and third generation of ACI is based on the review of Marlovits et al. "Cartilage Repair: Generation of autologous chondrocyte transplantation" [Marlovits 2006 p. 24-31].

First Generation ACI

The first generation ACI is a two-stage procedure. After a cartilage biopsy is obtained from a non weight-bearing region within the injured knee joint [Niemeyer 2010], articular chondrocytes are enzymatically isolated and cultured ex vivo in a monolayer expansion culture to gain a sufficient cell number. These expanded cells are then re-implanted into the defect site with approximately $2*10^6$ cells/cm^2 which is sealed with a periosteal flap. This flap is sutured over the defect and water-tightly sealed with fibrin glue [Brittberg 2008 p.41-43; Brittberg 1994].

Image 1: Original Protocol by Brittberg et al. for ACI



Original protocol for the procedure of autologous chondrocyte implantation. Published 1994 by Brittberg et al. [New England Journal of Medicine, Volume 331:889-895, October 6, 1994, Number 14; Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation]

This technique is characterized by a combination of two chondrogenic factors: the implanted suspension of chondrocytes and the cambium cells of the periosteum which might contribute to the formation of cartilaginous tissue [Brittberg 2005].

But there are also certain disadvantages including potential leakage of chondrocytes from the defect site, the dedifferentiation of the chondrogenic phenotype during monolayer culture, an uneven distribution of cells at implantation, periosteal hypertrophy, delamination of the transplant, arthrofibrosis and transplant failure [Marlovits 2006 p. 26; Marlovits 2004; Micheli 2001; Driesang 2000; Brittberg 1999]. It was reported that these disadvantages resulted in revision of surgery in up to 25% to 36% of the patients [Minas 2001].

Although Brittberg et al. as well as other researchers were able to demonstrate good long term results and durable benefits for patients in terms of diminishing pain and improved function [Peterson 2002; Peterson 2000; Brittberg 1994], complications can occur and other authors presented results, challenging this therapeutic approach. Knutsen et al. for example compared ACI to

microfracture in a short-term study and concluded that both methods had acceptable clinical results with no significant difference in macroscopic or histologic results between the two treatment groups [Knutsen 2004]. A large meta-analysis of Vasiliadis et al. comparing 9 trials with a total of 626 patients found no superiority of ACI compared to other techniques, revealing comparable complication rates except for periosteal hypertrophy in ACI of the first generation [Vasiliadis 2010].

In a prospective study of Bentley et al. from 2003, ACI outcome was compared with mosaicplasties in the knee joint. They followed up a 100 patients with symptomatic chondral and osteochondral lesions of the knee after treatment for 19 months with either ACI or mosaicplasty. Postsurgical outcome revealed a good or excellent clinical result in 88% of ACI patients versus 69% of mosaicplasty treated patients. Although this clinical difference was statistically not significant, arthroscopy revealed excellent to good results in 82% of the ACI procedures compared to 34% of the mosaicplasties. Interestingly, all five patellar mosaicplasties failed [Bentley 2003 p. 228-229].

Saris et al. published a large multicenter randomized controlled trial in 2008 comparing ACI with microfracturing in 118 patients, but only for defects of the femoral condyles. Patients were age 18-50, with symptomatic grade III-IV cartilage defects of the femoral condyles and evaluated one year after therapy, both histologically and clinically. ACI was performed with selected autologous chondrocytes, using ChondroCelect following the original protocoll of Brittberg et al. While the clinical assessment scores were similar for both groups, histological differences were significant, favoring ACI as repair method [Saris 2008]. In 2009 they published follow up data of this same randomized controlled multicenter trial up to 36 months, clearly favoring ACI now also in all clinical scores [Saris 2009]. However in 2011 the same group then showed in their 5-year follow up that the greatest impact on the benefit for ACI was due to the early onset of treatment. ACI and microfracturing did not differ anymore in their outcomes but patients treated within the first three years of symptom onset showed the best overall outcome, regardless of their age [Vanlauwe 2011].

Such studies led to further generations of ACI, combining the original technique with other tissue engineering methods to further improve clinical and histological outcome compared to other methods of repair.

Second Generation ACI

The second generation of ACI includes the use of bi-layer collagen membrane rather than a periosteal flap. Comparable to the first generation ACI, these purpose-designed biomaterials are sutured over the prepared cartilage defect and the cell suspension is injected underneath, simplifying the surgical procedure by reducing length and the number of incisions. Furthermore, the complication rates for

periosteal hypertrophy could be reduced [Haddo 2004]. Materials used include Chondro-Gide™, a bi-layer type I/III collagen membrane (Geistlich Biomaterials, Wolhausen, Switzerland) and others.

Third Generation ACI

The third generation ACI combines cultured chondrocytes with three-dimensional biocompatible scaffolds for an "all-in-one" tissue-engineered approach. It has been shown in numerous publications that chondrocytes, which dedifferentiate in monolayer expansion culture [Cheng 2012; Lin 2009; Chacko 1969], may regain their chondrogenic phenotype in 3-D culture [Grad 2005 / 2006; Benya 1982]. These findings led to the combination of ACI with the use of 3-D culture methods by the use of scaffolds. Various work has been published already in the 1990's showing the redifferentiating potential of 3-D culture and it's possible use for ACI [van Susante 1995; Hendrickson 1994; Freed 1993]. As an important advantage in this technique, implants do not have to be covered with a periosteal or other membrane cover and are not fixed with stitches but mostly by using fibrin glue, there is no requirement to do an open arthrotomy but mini-arthrotomy techniques can be used to perform this procedure [Marlovits 2006 p. 27].

The MACI™ technique (Matrix-induced autologous chondrocyte implantation; Sanofi) uses for instance resorbable collagen scaffolds such as Chondro-Gide™ (Geistlich Biomaterials, Wolhausen, Switzerland) or Maix™ (Matricel, Herzoenratz, Germany) [Behrens 2006]. Clinical data for matrix-associated cartilage cell transplantations have mostly been collected only on short term studies. The rate of complete attachment by only using fibrin glue when performing the MACI™ technique was shown to be successful in 14 out of 16 patients [Marlovits 2004], indicating that stitching and suturing will not be necessary in the future. Cherubino et al. showed in 13 patients with a mean defect size of 3.5 cm^2 and treatment with MACI™ improved clinical outcomes in all patients between 2-15 months after surgery [Cherubino 2003 p. 14].

Other materials in clinical use are the hyaluronan-based polymer scaffold Hyalograft C™ [Pavesio 2003; Marcacci 2003] and Bioseed-C™ scaffolds (BioTissue, Freiburg Germany) from polymers of polylactin and polyglactin [Erggelet 2003]. A cohort study of 67 patients treated with Hyalograft-C™ showed subjective improvement in 97% after 17.5 months. Objective evaluation revealed an improvement in 87% with an increase in quality of life in 94%. In the majority of control biopsies analyzed, hyaline-like regenerated tissue was found [Pavesio 2003 p. 240]. Marcacci presented excellent results of a clinical study for 141 patients with a treatment with Hyalograft C™ in 2005. Mean follow up time was 38 months. At this time 91.5 % had improved clinical evaluation and arthroscopic evaluation of the cartilage repair site revealed normal or nearly normal appearance in 96.4% [Marcacci 2005]. Ossendorf and coworkers evaluated the cell-seeded two-component, gel-

polymer composite of BioSeed-C in the arthrotomic and arthroscopic treatment of post-traumatic and degenerative cartilage defects of the knee. The clinical outcome was assessed in 40 patients within a 2-year follow up before and after implantation. Results showed a significant improvement of clinical scores and histology revealed an integration of the graft with the host tissue. Seeded chondrocytes were retained more efficiently at the site of implantation and integration was promoted. Also, the second look biopsies showed mostly hyaline cartilage in the presence of viable chondrocytes and an absence of calcification, necrosis or fibrous repair. Fibrocartilage was observed only in some cases. The failure rate was very low with only 2.5% (2/79) [Ossendorf 2007 p. 4-7].Thus, a transplant failure was considerably less than in standard ACI (5%-13%) [Minas 2001; Peterson 2000].

In an Food and Drug Administration approved phase-II study in a prospective-randomized comparison between microfracturing and scaffold based ACI, the clinical outcome of ACI was significantly better for all time points after 6, 12 and 24 months [Crawford 2012].

Crawford et al. even combined a collagen type I scaffold (NeoCart, Histogenic Corporation, Waltham, Massachusetts) with a bioreactor setting, mechanically training the constructs ex vivo before implantation into the patient [Crawford 2009 p. 1335].

Note: A commentary of Rocky S Tuan was published 2007 where he defined the 3-D scaffold approach as second generation ACI and the combination of a 3-D environment with the storage and release of growth or redifferentiating factors as third generation ACI. These third-generation scaffolds would be capable of delivering biofactors, such as cytokines in a programmed manner and in sufficient quantities to induce a favorable chondrogenic response [Tuan 2007 p. 111]. However, such ideas have not been implemented into clinical application until today.

Defect Site Dependency and Biopsy Site

But already in the original work of Brittberg et al. from 1994, midterm outcomes of the ACI depended strongly on the location of the original defect site. Of 23 young symptomatic patients with a mean age of 27 years, 13 patients presented with traumatic femoral cartilage defects, three with osteochondral lesions and seven patients had patellar cartilage defects. Two years post surgery, 14 out of 16 patients (87,5%) initially presenting with femoral defects showed good to excellent results. Patients with patellar lesions showed less satisfying results, with good outcomes only in two out of seven cases. 15 biopsies were then taken 12-46 months postoperatively which demonstrated hyaline-like cartilage in 73 % (11 cases) and fibrocartilage in 4 biopsies (27%) [Brittberg 1994].

In 2000, a long term follow up report was published by Peterson et al., presenting the 2-9 year data for 101 patients treated with ACI. Good to excellent clinical scores were found in 93 % of the patients with isolated defects on the femoral condyle (and 75 % if combined with the rupture of the anterior cruciate ligament), 67% in those with multiple lesions and 65% in patients with patella lesions. Transplant failure was only observed in 7 % of the cases [Peterson 2000]. A higher rate of defect repair failure of the patello-femoral joint has been reported in several studies [Peterson 2010; Niemeyer 2008; Brittberg 1994] compared to other regions of the knee joint. In conclusion this data shows that therapy success in ACI seems to depend on the defect site.

In concerns of the biopsy site for the healthy cartilage sample, no gold standard has been installed yet. Initially described by Brittberg in 1994 the native tissue biopsy was originally obtained from a minor load-bearing area on the upper medial femoral condyle. Other groups tried different biopsy sites like the non weight-bearing (NWB) proximo-medial femoral condyle rim, also used in this work, [Saris 2008] and the NWB femoral notch as used in this work [Niemeyer 2010; Simonian 1998].

These studies raise the question whether a site dependency in the clinical and histological outcome of ACI is due to the specific topological characteristics of the defect site or might even be related to the biopsy site chosen. So far, by our knowledge, nobody tested chondrocytes from different biopsy locations in an ex vivo mechanical setting, simulating in vivo conditions, for their possibly different behavior.

2.3. Tissue Engineering and Bioreactors

The rapidly growing field of tissue engineering is aimed at the generation of functional tissue as substitutes for various tissues from head to toe. Since articular cartilage is a tissue free of lymph – and blood vessels as well as nerves and lacks the ability to sufficiently heal itself it is an attractive tissue to engineer ex vivo.

But a matrix formed by mixing appropriate concentrations of water and cartilage macromolecules such as collagens, proteoglycans and non-collagenous proteins will never achieve the properties of articular cartilage. To produce a tissue that could duplicate the normal function of the synovial joint, the chondrocytes must first synthesize the right types and amounts of molecules for the ECM and then assemble, organize and structure them into a highly ordered framework [Buckwalter 1998 p. 478 / 2005 p. 466].

Besides biochemical signals known to regulate chondrogenesis, scientific investigations have shown that chondrocyte activity is stimulated by the mechanical demands made by the environment [Jin 2001; Grodzinsky 2000; Buschmann 1995; Bachrach 1995; Parkkinen 1989].

Hence, stresses and/or strains in and around the cells and interstitial fluid flow, resulting from mechanical loading may be applied using tissue-specific bioreactor systems to facilitate tissue growth.

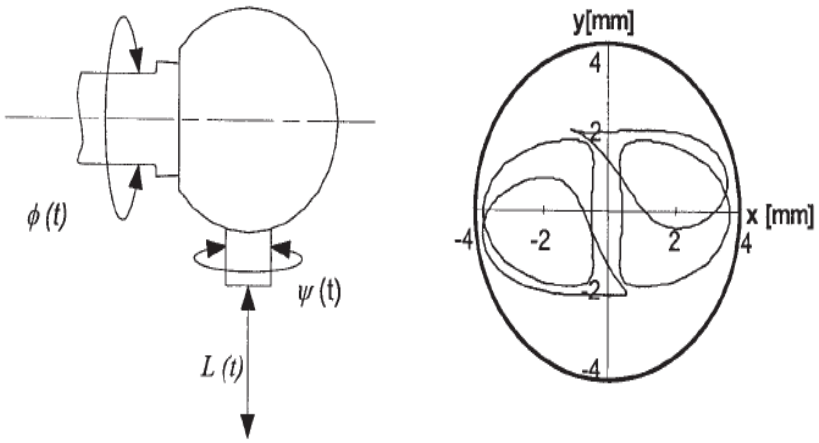
Chondrocytes, directly connected to their microenvironment by focal adhesions, experience these significant mechanical stimuli through direct mechanical effects as well as changes in their microenvironment influencing their synthesis activity. It has been shown in numerous studies, those mechanical parameters such as load duration and magnitude change the metabolic activity of chondrocytes [Correia 2012; Grad 2006; Grad 2006; Wiseman 2004]. Shear stress appears to be the most potent biomechanical stimulator. Shear appears to have an effect on cell metabolism and ECM synthesis, whereas hydrostatic pressure affects the cytoskeletal organization without influencing matrix synthesis [Athanasiou 2001 p. 239].

Bioreactors for tissue engineering have been used in a diversity of experimental settings to promote chondrocyte redifferentiation after monolayer culture, tissue growth and maturation [Lin 2006 p.1977; Nugent 2006 p. 1889-1891; Darling 2003 p. 15-21; Bonassar 2001; Temenoff 2000 p. 431; Quinn 1998 p. 574-575; Buschmann 1995 p.1498-1499] and have even been introduced into clinical settings [Crawford 2009 p. 1335]. Since cartilage is a tissue highly influenced and shaped by a biomechanical setting, it is only a logical conclusion to improve structural, compositional and mechanical characteristics of artificially produced cartilage by applying mechanical stimuli. Several more advantages make tissue engineering with a bioreactor an appealing approach. They provide uniform mixing and precise control over mass transfer rates, facilitating maintenance of nutrient levels and pH. Several types of bioreactors have been used in engineering articular cartilage in the last years. Spinner flasks [Falsafi 2000 p. 760; Freed 1997 p. 383], perfusion cultures [Edlich 2001; Smith 1995] in which e.g. scaffolds, seeded with cells are hooked to a perfusion system delivering a constant flow of media [Pörtner 2005; Davisson 2002; Bujia 1995; Sittinger 1994] that can also be combined with intermittent hydrostatic pressure [Heyland 2006 p. 1642], rotating wall bioreactors [Freed 1997 p.1997; Marlovits 2003], wavy-wall bioreactors [Bueno 2004 p. 769], concentric cylinders [Saini 2003 p. 513], rotating-shaft bioreactors [Chen 2004 p. 1803-1804] and stretching systems [Das 2008 p. 386].

Wimmer et al. designed a bioreactor following considerations of the basic structure of a tribological system [Wimmer 2004 p. 1438]. A tribological system consists of a body, a counter body, interfacial medium and surrounding environment [Deutsches Institut für Normung]. The natural joint can be

defined as such a tribological system with two bodies in contact (facing articular cartilage layers), interfacial medium (synovial fluid) and the surrounding environment defined by the human body milieu. These considerations taken into account a bioreactor was designed with a biaxial “pin-on-ball” concept. The interface of the two bodies is composed of a cell-seeded scaffold or cartilaginous tissue, which is pressed onto a conforming ball. The interfacial medium is nutrition fluid and the surrounding environment is the incubator air with 5.7 % CO_2, 37 °C, 95 % humidity. Either static or cyclic compressive loads may be applied simultaneously along the cylindrical axis of the pin. The latter causes a complex shear force pattern on the surface of the pin and is intended to mimic the characteristics of the natural joint

Image 2: Possible Movements of the Tribological System



Wimmer et al. 2004 p. 1438 Tribology approach to the engineering and study of articular cartilage

2.4. Hypothesis

Up to date, there is no gold standard concerning the site where the biopsy for ACI is taken. Most common non weight-bearing locations biopsied are the proximo-medial as well as the proximo-lateral edge of the femoral condyle and the intercondylar notch [Brittberg 2008 p. 41].

It has also been shown extensively that within the knee joint differences in cartilage depth, cellularity, biochemical composition and structure as well as biomechanical response can be found depending on the compartment, zone and applied compressive stress and shear [Franz 2001; Shepherd 1994; Athanasiou 1991 p. 336; Kiviranta 1987 p. 274]. Already in mosaicplasty the question arose whether plug biopsies taken from non weight-bearing regions were actually capable of enduring the stress and strain of a weight-bearing defect site [Bentley 2003]. Also, ACI outcome has shown to be dependent on the site of reimplantation [Brittberg 1994] possibly due to distinct reactions of the implants to the differing loads applied.

Given all the previous information, the question arises whether topographic differences observed in the native cartilage in the knee joint can be found not only on a compositional level, but on a cellular one.

How durable are these differences?

Can they still be observed after the isolation of chondrocytes, a monolayer expansion phase and maybe even after reentering into a 3-D culture system?

Moreover, do these topographically distinct subpopulations of chondrocytes show differences in their response to a biomechanical stimulation, to simulated in vivo condition of the joint?

Can a gold standard biopsy site be suggested or should cartilage defects be treated with tissue replacement from structural and compositional matching locations?

To our knowledge, this question has not been approached in an experimental setting so far.

Should differences in cellular behavior and chondrocyte specific gene expression profiles occur between topographically distinct populations of chondrocytes in monolayer as well as 3-D culture and under simulated in vivo conditions, one might wonder if cells from a non weight-bearing area are a favorable cell source for ACI after all.

3. Materials and Methods

3.1. Introduction

The following laboratory research work was carried out at the AO Research Institute, Davos, Switzerland, in the laboratories of the Musculoskeletal Regeneration Program, head: Professor Mauro Alini. It was supported by the AO Foundation Research Fund number S-07-61S.

3.2. Experimental Setup

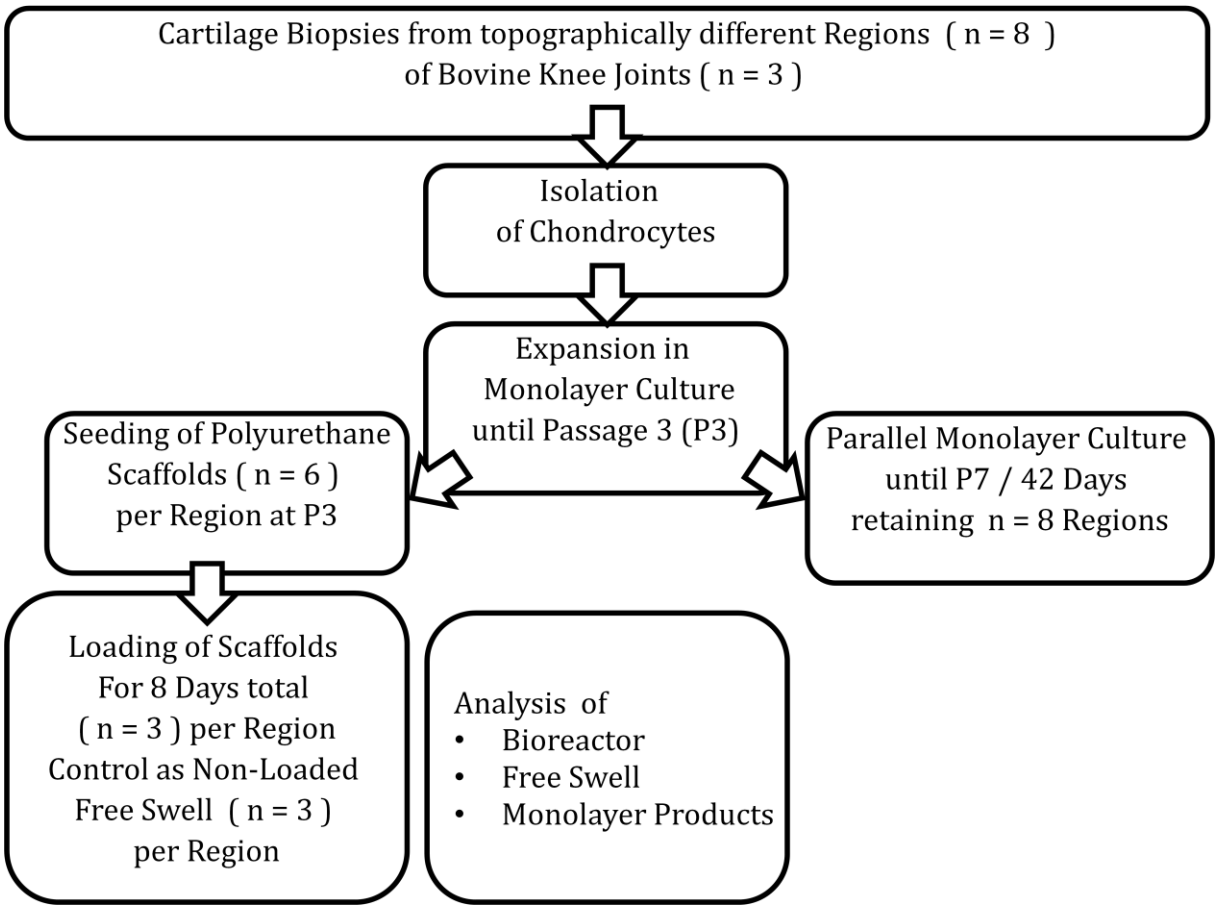
To compare different donor sites for healthy cartilage biopsies in ACI, biopsies where taken at eight topographically distinct locations in three freshly slaughtered bovine stifle joints. After an enzymatic isolation of the chondrocytes from the ECM, cells were expanded in a conventional monolayer expansion culture. In the clinical protocol of ACI, this step is still crucial to gain the required cell number for implantation. During this monolayer culture (42 days until Passage 7) the different subpopulations were analyzed for their proliferation and dedifferentiation behavior.

At passage 3 (P3), after approximately 6.8 population doublings, cells were seeded into biodegradable polyurethane-scaffolds. These scaffolds were either cultured as free-swelling control groups or mechanically loaded to simulate in vivo conditions. Biomechanical stress was induced with a customized bioreactor installed in a standard cell-culture incubator (5% CO_2, 37°C, 95% humidity).

The bioreactor was designed by Wimmer et al. at the AO Research Institute's Musculoskeletal Regeneration Program for the purpose of generating articular cartilage ex vivo [Wimmer 2004]. A special loading protocol was followed that had already been shown to have redifferentiating effects on chondrocytes in earlier works of the group [Grad 2006].

After 21 days of 3-D culture the constructs were finally analyzed biochemically as well as for mRNA expression levels of 11 chondrocyte specific genes and histologically examined.

Image 3: Experimental Design



3.3. Analysis

To evaluate the dedifferentiation extent during monolayer culture as well as redifferentiation ability during 3-D culture and biomechanical loading of the eight topographically distinct chondrocyte populations, the following chondrocyte specific and typical parameters were analyzed.

Biochemically, DNA and GAG content were analyzed in the 3-D constructs. DNA content was evaluated to gain information about proliferation activity during 3-D culture and to put into perspective the GAG content to cell number and thus examine the differences in cellular productivity.

Chondrocyte characterizing gene expression levels were evaluated using rtPCR to gain information about mRNA expression levels. For this purpose the following markers were chosen: Collagen I, II, X, cartilage oligomeric matrix protein (COMP), Aggrecan, PRG-4 (Superficial Zone Protein), Sox9, Matrix Metalloproteinase (MMP) -1,-3,-13 and Parathyroid hormone related peptide (PTHrp).

Articular cartilage contains multiple, genetically distinct types of collagens (Type II, VI, IX, X, XI). Types II, IX and XI form the cross-banded fibrils seen with electron microscopy. 90% up to 95% of the collagen content consists of type II [Buckwalter 1998 p. 480]. The alternatively spliced isoform of collagen II, type IIa, is synthesized by immature chondrocytes and chondroblasts. Collagen type X is primarily present near the cells of the calcified cartilage zone (zone four) and the hypertrophic zone of the growth plate [Buckwalter 2005 p. 467] and is known to be a hypertrophic chondrocyte marker [Goldring 2006 p. 38]. Collagen type I is considered to be a sign for dedifferentiation of chondrocytes. It has been found to show higher expressions in passaged monolayer chondrocytes [Darling 2005 p. 428/429; Mayne 1976 p. 1675] as well as in osteoarthritic cartilage [Lin 2008 p. 1231].

Cartilage oligomeric matrix protein (COMP) is an acidic protein, concentrated primarily within the chondrocyte territorial matrix. It appears to be present abundantly within cartilage and to have the capacity to bind to chondrocytes [DiCesare 1994 p. 934-935; Hedbom 1992 p. 6133-6134]. It might have a value as a marker of cartilage turnover and of progression of cartilage degeneration in patients with osteoarthritis [Lohmander 1994 p. 10].

Aggrecan has large numbers of chondroitin sulfate and keratan sulfate chains attached to a protein core filament. Aggrecan molecules fill most of the interfibrillar space of the cartilage matrix, contributing about 90% of the total cartilage matrix proteoglycan content [Buckwalter 1998 p. 480] and are non-covalently associated with hyaluronic acid and link proteins.

Proteoglycan 4 (PRG-4), is homologous to SZP, the superficial zone protein that is specifically expressed by the superficial zone chondrocytes at the cartilage surface and to lubricin, a major component of the synovial fluid that participates in the lubrication of the synovial joint [Flannery 1999]. PRG-4 has been shown in previous works to be upregulated in ex vivo experiments due to mechanical load with compression and shear [Grad 2005 p. 252, 2006 p. 3175].

The Sry-type, high-mobility group (HMG)-box containing transcription factor SOX9 comes closest to serving the function of a master regulator of the chondrocyte lineage [Shum 2002 p. 99]. In humans, SOX9 haploinsufficiency (Online Mendelian Inheritance in Man (OMIM) number 114290) results in campomelic dysplasia with XY sex reversal, a lethal skeletal malformation syndrome [Wheatley 1996]. Sox9 is involved in mesenchymal cell proliferation and condensation as well as chondroprogenitor proliferation and differentiation. It is one of the earliest marker expressed in cells undergoing condensation and required for the expression of the type II collagen gene (Col 2a1; Sox9 has shown the ability to bind to and activate to a consensus sequence in the Col2a1 enhancer region) and other cartilage-specific matrix proteins prior to matrix deposition in the cartilage anlagen. The expression of SOX proteins is dependent upon BMP (bone morphogenic protein) signaling via

BMPR1A and BMPR1B [Goldring 2006 p. 36]. Sox9 genes are expressed throughout the chondrogenesis pathway but are completely turned off in hypertrophic chondrocytes [Lin 2006 p. 1973].

Enzymes produced by chondrocytes presumably are responsible for degradation of the matrix macromolecules and chondrocytes seem to respond to the presence of fragmented matrix molecules by increasing their synthetic activity to replace the degraded components of the framework [Buckwalter 1998 p. 479]. Interleukin 1 induces expression of matrix metalloproteinases that can degrade the matrix macromolecules and it interferes with the synthesis of matrix proteoglycan at the transcriptional level. Matrix metalloproteinase (MMP-) 13, for example, is a downstream target of Runx2 which is expressed by terminal hypertrophic chondrocytes. It is chiefly expressed in the deep zone [Rannou 2006] and is the collagenase with the highest affinity for Collagen type II [Reboul 1996 p. 2014-2016]. A MMP-13 deficiency results in significant interstitial collagen accumulations leading to the delay of endochondral ossification during bone formation in the growth plate. MMP-1 is mainly found in the superficial layer [Rannou 2006; Ehrlich 1977 p. 232]. Both collagenases are able to degrade the collagen triple helix and can be found in osteoarthritis or other inflammatory states. The same is true for MMP-3 [Goldring 2006 p. 1006] which has additionally been lately shown to decrease during monolayer expansion culture and to be a possible predictory marker for ex vivo cartilage formation [Pelttari 2008 p. 473].

Chondrocyte proliferation and maturation in the growth plate as well as in the lower proliferative and prehypertrophic zones are regulated by a negative feedback loop mediated by the secreted signaling molecules PTHrp (Parathyroid Hormone related peptide) and Ihh (Indian Hedgehog) [Mau 2007 p. 34; Goldring 2006 p. 37]. Vortkamp et al. suggested in 1996 that PTHrp expression is induced in the perichondrium by Ihh [Vortkamp 1996]. PTHrp signaling then stimulates cell proliferation via its receptor expressed in the periarticular chondrocytes [Lanske 1996 p. 665]. PTHrp acts by preventing premature differentiation into prehypertrophic and hypertrophic chondrocytes, thereby suppressing premature expression of Ihh [Kobayashi 2005 p. 1741]. Thus, it plays a key role in maintaining a pool of proliferating cells near the articular ends [Shum 2002 p.101].

3.4. Material, Reagents and Laboratory Equipment

Material	
12-well plates	FALCON
200 µl-micro reaction tubes	Thermowell TM Tubes, 0,2 ml with Dome Cap; DNase/RNase free; Lot:35506001
Caps	Prepared from 1.5 ml Eppendorf tubes; steam sterilized
Cell Strainer	FALCON; 40 µm; REF 352340
Ceramic Hip Ball	used for standard hip joint replacement
96 Well- Plate (DNA)	FALCON, 35, 3296, Opaque Tissue Culture Plate; BD Labware
Tubes	Eppendorf
Filter tips	Aerosol-resistant pipet tips, Eppendorf
96 Well-Plate (GAG)	Costare # 3590
Haemocytometer	Improved Neubauer Chamber
Knee joint	With intact joint capsule; Slaughterhouse Stiffler, Davos
Multipipette	SOCOREX, Calibra 852 with Tips, Lot. 6270402
Multipipette	Eppendorf Multipette ®plus; Vaudaux-Eppendorf
Petridishes	FALCON
Pipettes	FALCON
Pipett-Tips	Eppendrof Combitips, different Sizes
Polyurethane (PU) Scaffolds	Batch numbers 071209 , 080206, GLM-0238-11
Scalpels	10/12 blades
Sterile polypropylene centrifugation tubes	FALCON
Tally counter	Cellcount
Tubes 5ml, 10ml, 25ml, 50ml	FALCON

Solutions / Chemicals /Assays	
18S rRNA	primer/probe mix 20x ; Applied Biosystems cat. # 4310893E, Lot # 0803496
1-Bromo-3-chloropropane	BCP C3H6BrCl; SIGMA Molecular Biology, cat. # B9673 Batch # 075 K 3727
Collagenase II	BioConcept no. 4177, Worthington
Assay-on-Demand TaqMan® Gene Expression Assay	Non-inventoried SMALL-Scale; Applied Biosystems primer/probe mix 20x
	MMP1: Bt 03212822_m1; 360 µl 20x Mix; Lot: 807131 486 H09
	PTHrp: Bt 03224327_m1; 360 µl 20x Mix; Lot:807133 482 H10
Cryocompound	Jung embedding medium; Leica Instruments GmbH, Nussloch, Germany
DEPC treated water	0.1% DEPC in Milli-Qwater ; sterilized;
DNA standard Calf Thymus DNA Invitrogen	LuBioScience, cat. # 15633-019; dissolved in DPBS
DPX Mountant for histology	Fluka,44581; Lot:1329713
Ethanol	Alcosuisse; 75% Ethanol Merck; Lot: 1434543 823
High Salt Precipitation Solution	Molecular Research Center, cat. # PS-161, Lot # 365; for isolation of RNA from samples containing large amounts of polysaccharides and/or proteoglycans
DAB	Peoxidase stubsrate Kit SK-4100;Vector Laboratories Inc

	2.5 ml H_2O
	2 drops buffer
	2 drops DAB
	1 drop H_2O_2
	1 drop Nickel
Fetal Calf Serum FCS	Gibco no.10500-064 (Rd); LOT#: 07F4472K
Fibrinogen Solution	Baxter Biosurgery, Vienna Austria Diluted Fibrinogen stock (100 mg/mL) 1:3 with dilution buffer F
Forward and reverse primers (Microsynth):	Dissolved to 45 µM with Tris-EDTA buffer (prepared from 100x concentrate (SIGMA cat. # T-9285) with DEPC-treated water) by incubation at 65°C for 5 min, shaking, 65°C for 3 min. Store at -20°C for 2 years.
Horse Serum	Provided by the Histology Department AO Institut Davos
Isopropanol	2-Propanol C3 H8 O; FLUKA MicroSelect for molecular biology, cat. # 59304, Lot # 137712442/080/6)
Phosphate buffer (PBE)	in Milli-Q water with adjusted ph (6,5)
	10.68 g/l NaH_2PO_4 $2H_2O$
	8.45 g/l Na_2HPO_4 $7H_2O$ and
	3.36 g/l Disodium-EDTA
Methanol	100 ml + 1 ml 30% H2O2; Alcosuisse
Meyer´s Hematoxylin	Provided by the Histology Department AO Institut Davos
Column-based RNA Isolation Kit	SV total RNA Isolation System; Promega; Lot: 259104; SIGMA GEnElute Mammalian Total RNA Kit, cat. # RTN-70
Polyacryl Carrier™	MRC, cat. # PC-152, Lot # 269
Primary Antibody: 3A4	provided by Dr. Milz, AO Research Institut
DMEM	4-8°C, store for 1month; prepared with Milli-Q water
	13.38 g/L DMEM (LuBio-Science no. 52100-021 (Gibco), LOT#: 105K07232) 3.7 g/L $NaHCO_3$ (Sigma no. 71628; LOT#: 1161595)
	0.11 g/L Na-pyruvate (Sigma no. P5280; LOT#: 375045) (LuBio-Science no.11840-030 (Gibco)) 20 mL/L Hepes 1M (LuBio Science no. 15630-850; (Gibco)LOT#: 8217) DMEM supplemented with 4.5 g/L Glucose
Primocin	InvivoGen San Diego, USA; Cat#: 11K03-SV
Pronase	from Streptomyces griseus, Roche, no. 1 459 643
Proteinase K	recombinant; PCR Grade Roche; cat. # 1 000 144 Lot # 03115801001; 0.5 mg/ml in PBE
Stock Dye Solution	1 mg/ml stored in the dark at 4 °C
	10 mg Hoechst 33258; 10 ml distilled water
Stocksolution of chondroitinsulfate	Polysciences, Inc., cat. # 09460; stored in the dark; chondroitin-4-sulfate sodium salt from bovine trachea, mixture of isomers, Fluka BioChemika, cat # 27042 in distilled H_20
Sucrose 99 %	Sigma; Lot: 112K0997
TaqMan Probe (Microsynth):	Oligonucleotide labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5´end and with the quencher dye TAMRA (6-carboxy-N, N, N´, N´-tetramethylrhodamine) at the

	3´end Dissolved to 12.5 µM with Tris-EDTA buffer (prepared from 100x concentrate (SIGMA cat. # T-9285) with DEPC-treated water) by incubation at 65°C for 5 min, shaking, 65°C for 3 min.
TaqMan Universal Master Mix	Applied Biosystems, cat. # 4324018; 2x concentrate, containing AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference, and optimized buffer components. Stored at 4°C.
TE (Tris-EDTA) Buffer:	prepared from 100x concentrate (SIGMA cat. # T-9285) with DEPC-treated water
TaqMan Reverse Transcription Reagents	Applied Biosystems, Foster City, CA, Lot # B04823:
	10 x PCR Buffer II (500 mM KCl, 100 mM Tris/HCL, pH 8.3) and 25 mM Magnesium chloride, cat. # N8080010 DeoxyNTPs mixture (2.5 mM each dNTP), cat. # N8080260 Random Hexamer (50 µM), cat. # 8080127 RNase Inhibitor (20 U/µl), cat. # N8080119 MultiScribe Reverse Transcriptase (50 U/µl), cat. # 4311235
TE (Tris-EDTA) Buffer:	prepared from 100x concentrate (SIGMA cat. # T-9285) with DEPC-treated water
Thrombin	Baxter Biosurgery, Vienna Austria; Diluted Thrombin stock (500 u/ml) 1:500 with dilution buffer T
DMMB Color reagent	16 mg DMMB ; 1,9-Dimethyl-methylene blue, Aldrich cat. # 34,108-8 dissolved in 1 l water containing 3.04 g glycine and 2.37 g NaCl After stirring over night at room temperature aluminium covered, pH to 3.0 is adjusted with 1 M HCl (approx. 9.5 ml) to give an absorbance of 0.31 at A525. The reagent is stable in a brown bottle at room temperature for at least 3 months
TRI REAGENT	Molecular Research Center MRC, cat. # TR-118, Lot # 3691
PBS Phosphate buffered saline;	pH 7.4 ; adjust pH with 5N NaOH, ca. 4.5 ml 800ml distilled water 2M NaCl 25mM KH2PO4 3.402 g 25mM Na_2HPO_4 3.55 g 2.0M NaCl 116.88 g add distilled water to 1000 ml
Trypan Blue	Fluka, cat. # 500287, 0.4% solution in PBS, filter through 0.45 µm filter
Trypsin-EDTA	10x concentrate, GIBCO, cat. # 35400-019.Diluted in TBSS
Vectastain ABC Kit	(Vector Laboratories Inc
1 % Toluidin Blue solution	per 100ml: 1 g Toluidine Blue (Fluka, 89640; Cl # 52040) 1 g Sodium Tetraborate, anhydrous (Fluka, 71998) 100 ml dd H_2O Stirred over night and filtered
Xylene C8H10	(Schweizerhall Chemie AG, 82510-550)
TBSS	(4-8°C, 1 month) prepared with Milli-Q water

	0.056 g/L $NaH_2PO_4 \cdot 2\ H_2O$ (Sigma no. 71500)
	0.2 g/L KCl (Sigma no. 60130)
	1.0 g/L $NaHCO_3$ (Sigma no. 71628)
	1.0 g/L $C_6H_{12}O_6 \cdot H_2O$ (Sigma no. 49159)
	8.0 g/L NaCl (Sigma no. 71380)
	20 mL/L HEPES buffer 1M (LuBio-Science no. 15630-056 (Gibco) Lot: 454983)
	2mL/L Phenolred sodium salt-solution 0.04% in H_2O (Sigma no. 32954)
PBS 10x:	500 ml Mili Q Water
	10.05 g / l Na $H_2PO_4 2H_2O$
	2.7 g/l KH_sHPO_4
	42.5 g/l NaCl
PBS-Tween:	1 ml/lTween (Sigma-Aldrihk;Tween ® 20 SigmaUltra; Lot:096K00995)
	0,32 g / l Na H_2PO_4
	1,42 g/l Na_sHPO_4
	9,0 g/l NaCl

Laboratory Equipement	
7500 System Software	Applied Biosysstem; Sequence Detection Software Version 1.2.3. 7500 System SDS Software
AB 7500 Real Time PCR System	Applied Biosystems, AB
ABI PRISM Optical 96-well Reaction Plates	ABgene® PCR Plates; Thermo Scientific; Lot: FC0N2)ABI PRISM Optical Adhesive Covers (AB-1170;Absolute QPCR Seal; Thermo Scientific; Lot: WD6U6L
Bioreactor	Heraeus Cytoperm 2 Incubator; 5 % CO2, 37 °C, 95 % Humidity; Thermo Electron Cooperation
Centrifuge	Hettich Laborapparate; Hettich Rotantor/RP
CO_2-Incubator	Heraeus Instruments BBD 6220; Thermo Fisher Scientific; 5 % CO2; 95% Humidity; °C 37)
Cryotome	
Filter	Excitation wavelength of approximately 360 nm with a band of 100 nm and to detect the emission at 465 nm; 535 nm
Gene Amp®5700 Sequence Detector	With Thermal Cycler 9600, Applied Biosystem
Incubator for Proteinase K mixture	Heraeus Instruments, Functionline Type B20, Kendio Laboratory Product
Labview Software	Texas Instruments; Cartload V3.95; created by ALEA Solutions GmBH; programmed with Lab View 6.0 and Flex Motion 5.1.1 National Instruments
Magnetic Stirrer	Techne MCS-104 S, biological stirrer; witec-ag; approximately 70 rpm
Nano Drop	ND-1000 Spectometer ; witec. ag
Plate Reader	Perkin Elmer precisely 1420 Multilabel counter Victor 3
Scale	Mettler PE 360, Delta Range
Thermal Cycler 9600	With Gene Amp 5700 Sequence Detector, Applied Biosystems

Thermostat 5320	Eppendorf; for inactivating the Proteinase K
Tissue Lyser	Quiagen, manufactured by Retsch; 25 Hz, 3 minutes
Vertical Laminar Flow Hood	
Vortex-Genie 2	Scientific Industries
Zeiss Microscope	with Camera (Kappa); Axio Vision; Axiovert 25
Centrifuge	Eppendorf; centrifuge 5417R; rcf
Centrifuge	small; Gilson AG, switzerland; Laboratory and Medical Supplies (LMS), Minicentrifuge MCF 2360
Centrifuge	Hettich Centrifuge Rotanta 46, rcf 1200; for 96-well plates
Centrifuge	Table centrifuge; eppendorf; centrifuge 5415D

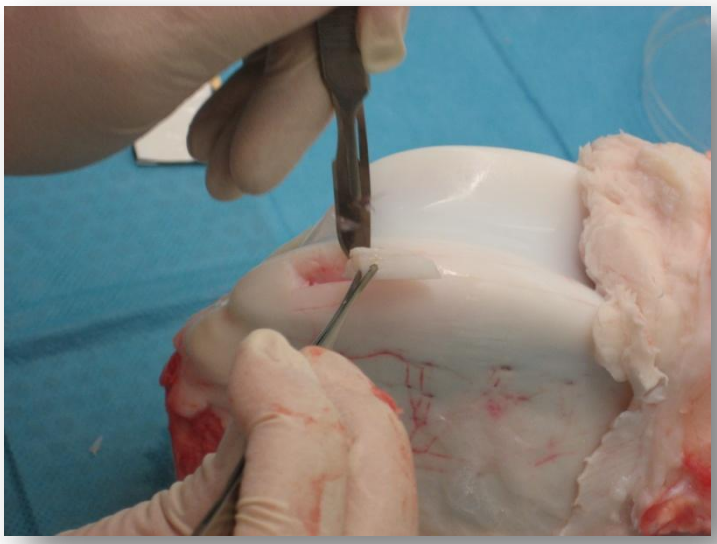
3.5. Cartilage Biopsy

To obtain chondrocytes full-thickness cartilage biopsies were taken across the bovine knee joint. Male juvenile domestic cattle ranging from 3-5 months of age served as donor animals. Biopsies were obtained directly after slaughter in a sterile manner from eight topographically different locations representing the different anatomical weight- and non weight-bearing areas:

Table 2: Biopsy Sites and Abbreviations

Biopsy Regions	Abbreviation
Weight-Bearing Locations	WB
Medial and Lateral Femur Condyle	MF and LF
Trochlea Femoris	T
Patella	P
Medial and Lateral Tibia Plateau	MT and LT
Non Weight-Bearing Locations	NWB
Femoral Notch	X
Proximo-Medial Femoral Condyle	Y

Image 4: Sterile Biopsy Harvest from Bovine Knee Joints

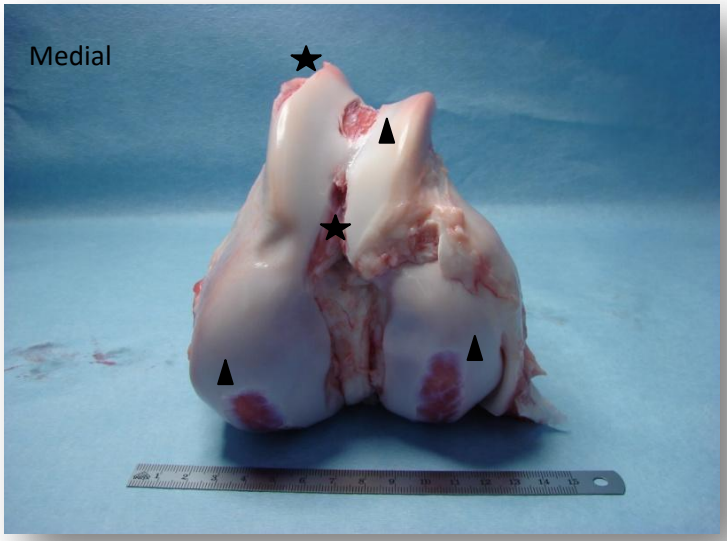


To represent the common weight-bearing areas at the femoral part of the stifle joint, the weight-bearing medial femoral condyle (MF), weight-bearing lateral femoral condyle (LF), weight-bearing trochlea femoris (T) as well as weight-bearing patella (P) was chosen. The weight-bearing medial tibia plateau (MT) and weight-bearing lateral tibia plateau (LT), not distinguishing between meniscus covered or uncovered parts served as weight-bearing biopsy sites of the tibial part of the joint. To simulate the common clinical biopsy sites used for autologous chondrocyte transplantation, the non weight-bearing femoral notch (X) and non weight-bearing proximo-medial femoral condyle (Y) was chosen.

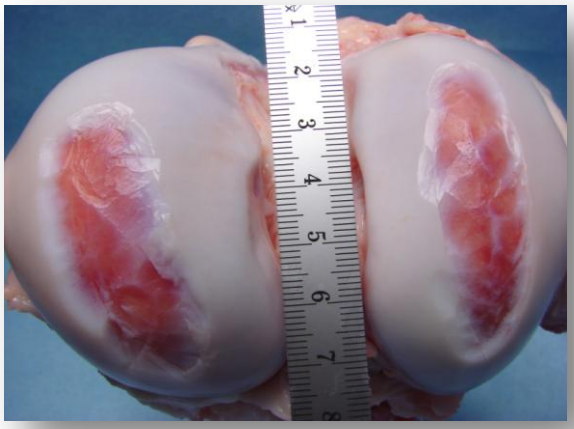
These eight topographically different subpopulations were treated as separate groups throughout the entire experiment.

Image 5: Biopsy Locations

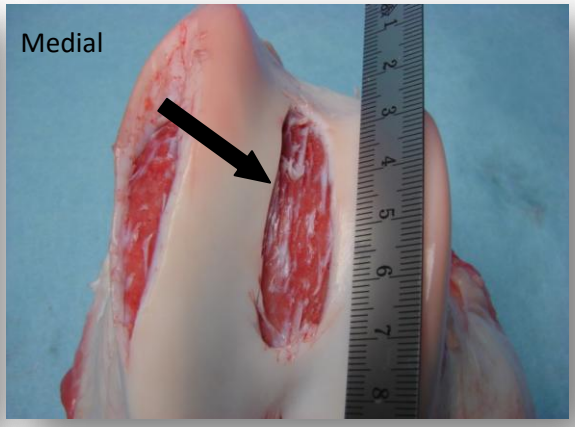
a: Overview of the distal Femur
b - e: Weight-Bearing Locations
f - g: Non Weight-Bearing Locations



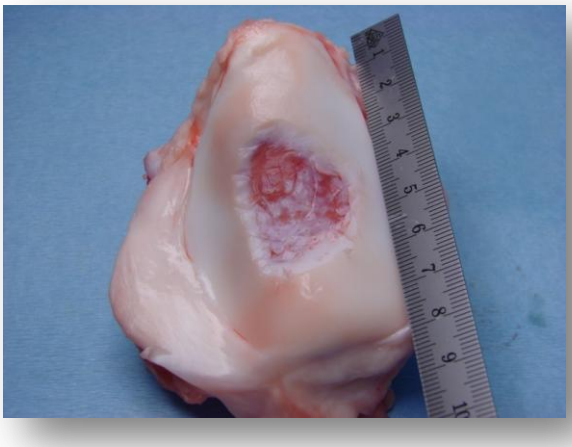
a, Distal Femur with three weight bearing ▲ and two non-weight-bearing biopsy locations ★ ;



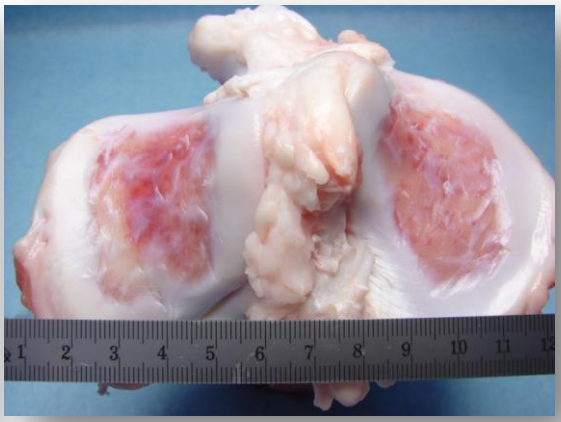
b, Femur Condyles with two major weight-bearing areas, the medial (MF) and lateral (LF) Femur Condyle;



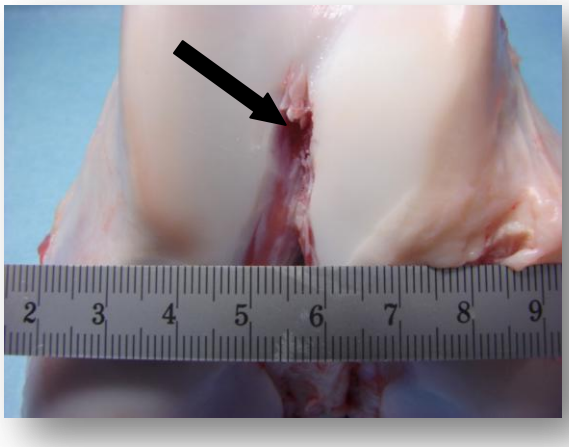
c, Weight-bearing femoral part of the patello-femoral joint; Trochlea (T)



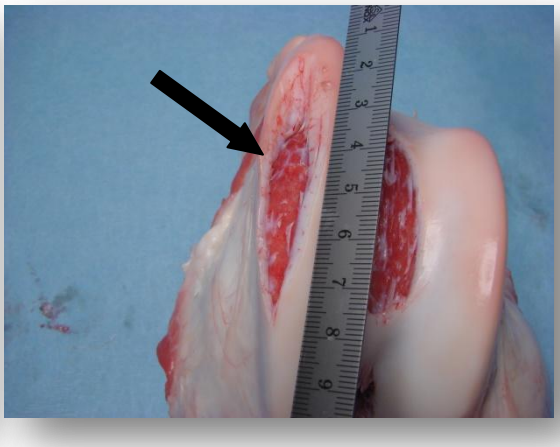
d, Weight-bearing biopsy region Patella (P) of the patello-femoral joint;



e, Tibia Plateau, weight-bearing biopsy locations, meniscus and non-meniscus covered; medial (MT) and lateral (LT) Tibia;



f, Non weight-bearing biopsy location: Femoral Notch (X)



g, Non weight-bearing biopsy location: proximo-medial Femur Condyle (Y)

After the aseptical biopsy was performed, cartilage pieces of each location were transferred into different tubes, containing approximately 30ml TBSS. Total wet weight of each biopsy region was recorded to compare cell density per gram cartilage biopsy of each region, after the enzymatic digestion.

3.6. Cell Isolation

Directly after the articular cartilage biopsies were obtained, samples were enzymatically digested to isolate the chondrocytes out of the extracellular matrix. The following protocol was followed under a laminar flow hood to secure sterility.

The cartilage samples were cut into pieces of approximately 9-25mm² and transferred into spinner flasks containing 60ml TBSS with 50mg/ml Primocin.

Subsequently the cartilage pieces were washed 2x15min with 60ml TBSS (with 50mg/ml Primocin) and pre-digested with 0,1% Pronase in DMEM in the spinner flasks, stirring for 120min at 37°C, 5% CO_2 in the incubator. After another three washing steps of the pre-digested cartilage (with 60ml TBSS and 50mg/ml Primocin), the samples were further digested with 600U/ml (250U/mg) Collagenase Type II in DMEM (serumfree) for 14h under the same conditions as before.

Image 6: Spinner Flask for Chondrocyte Isolation

In the next step, the solution containing the isolated chondrocytes was diluted, filtered through a 40µm cell strainer and centrifuged at 565g for 7min at 4°C. Cells were then resuspended with DMEM containing 25% fetal calf serum (FCS) and 50mg/ml Primocin and counted using a the Neubauer chamber and Trypanblue stain to asses cell viability and cell number achieved by the isolation.

Since the biopsy location X (femoral notch) is anatomically the smallest region, providing the least amount of cartilage, the number of isolated cells of all locations was adapted to this region to subsequently culture them in equal numbers.

3.7. Cell Culture

Cells were cultured in monolayer culture with standard medium containing DMEM, 10% FCS and 50mg/ml Primocin. Glucose in the DMEM serves both as the major energy source as well as an essential precursor for glycosaminoglycan synthesis. Growth medium was changed under the laminar flow every second day in a sterile manner. All eight groups were cultivated under this monolayer standard cell expansion conditions for 42 days with 7 passages during all experiments.

Cell-scaffold constructs were cultured using the same standard medium, containing DMEM, 10% FCS and 50mg/ml Primocin. All constructs, free swelling as well as loaded groups were cultivated in 3ml of growth medium. During this 3-D culture period, medium was preserved for subsequent glycosaminoglycan analysis and frozen at -20°C.

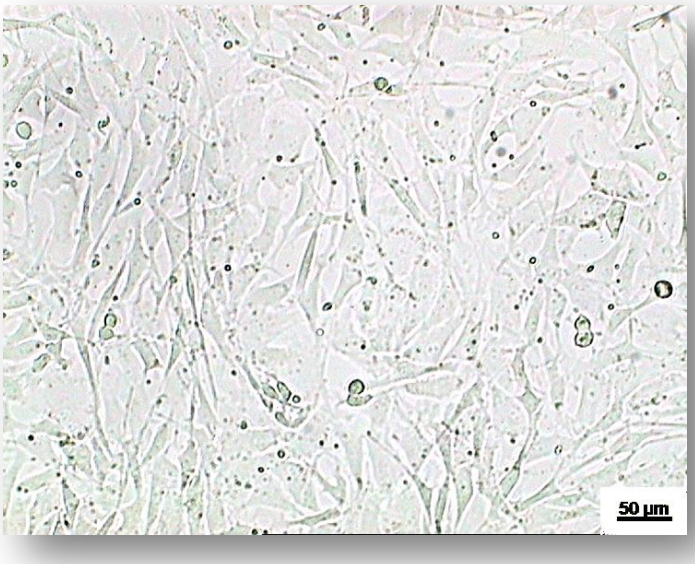
3.7.1. Subculture

Subculture with a standard protocol was performed at subconfluency with identical cell numbers to guarantee the comparability and reproducibility between the groups and experiments. For each group, cell number and cell viability was determined with the Neubauerchamber and Trypan Blue Stain.

First, TBSS, the dissociation agent and DMEM were pre-warmed to 37°C. The flasks containing the cells were brought under the laminar flow hood to secure sterile working conditions. After the medium was removed, using sterile pasteur pipettes, TBSS (0.2ml/cm^2) was added to the cells. The flasks were gently shaken in a circle- like manner to rinse the cells and the washing solution was discarded afterwards. This step is designed to remove traces of serum and calcium, which would inhibit the action of the trypsin, used to detach the cells from the flasks. To guarantee interference free detachment of the cells, this washing step was performed twice. The cells were then incubated for five to seven minutes with 2-10 ml Trypsin, depending on the density of the cell layer and size of culture dish, at at 37°C/5% CO_2. Cells should not be left longer than necessary in the trypsin solution to prevent cell damage or clumping. When cells rounded up and the monolayer started sliding, DMEM with 20%FCS and 50mg/ml Primocin was added to stop the action of trypsin. Cells were dispersed by pipetting them up and down (without producing foam) and transferred into a 50ml Falcon tube. The flask was then rinsed with an equal volume of growthmedium with 20% FCS and 50mg/ml Primocin to collect remaining cells and the rinse was added to the cell suspension. Afterwards the cell suspensions were centrifuged at 500g (or 1830rpm) at 4°C for 7min. The supernatant was removed and cells were resuspended with fresh DMEM containing 10% FCS and 50mg/ml Primocin and centrifuged again. This washing step was performed three times. After these washing steps, cells were resuspended in an appropriate volume of growth medium (with 10% FCS

and 50mg/ml Primocin) and counted with the Neubauer Chamber (Trypan Blue Stain) to check cell viability and document the growth behavior. After cells were counted, the cell suspension was diluted to an appropriate seeding concentration. At this step all the groups were seeded into new container with the same cell number to ensure comparability of cell growth between the subcultures of the different locations. At least 10^6cells were kept for rtPCR gene expression analysis.

Image 7: Subconfluency



Picture: Cells of Medial Condyle, Zeiss Microscope with Camera (Kappa), Axio Vision; Axiovert 25

3.7.2. Population Doubling

The surface of the haemocytometer slide and the coverslip was first cleaned with 70% alcohol. The edges of the coverslip were very slightly wetted and pressed down over the grooves and counting area. The appearance of interference patterns ("Newton's rings", or rainbow colors between the coverslip and the slide) indicates that the coverslip is properly attached, thereby determining the depth of the counting chamber.

The cell samples were mixed very thoroughly to disperse any clumps. 25µl of cell suspension were then gently mixed with an equal volume of 0.4% Trypan blue in a 96 well plate and let stand for 5 min at room temperature. About 20µl were collected into the tip of a pipettor and 10µl of cell-Trypan Blue mix were placed in the Hemocytometer. The suspension was let run out of the pipette and be drawn under the coverslip by capillarity. The number of viable (unstained) cells was counted and the average number of unstained cells in each quadrant was calculated, and multiplied by $2*10^4$ to find

the number of viable cells/ml. The number of population doublings was calculated based on the cell number counted with the Neubauerchamber (with Trypan Blue Stain) at each passage with the formula for exponential growth: $\mathbf{N_{(t)} = N_0 * e^{t*\lambda}}$

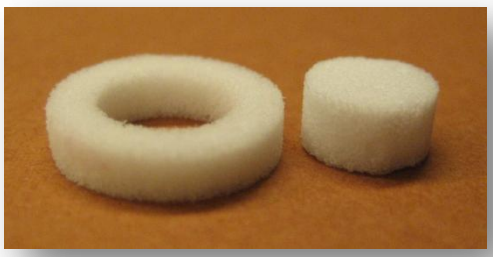
N(t) represents the number of cells counted at a specific time point, **N(0)** the original cell number, **t** the time since the original cell count and **λ** the factor of growth.

The actual population doubling was calculated, using the modified formula **ln (2) / λ =T** with T representing the doubling time.

3.8. Polyurethane Scaffolds

Porous polyurethane (PU) scaffolds were prepared as described elsewhere [Gorna 2002]. The scaffolds with interconnected pores had an average pore size of 90 to 300µm. The polymers used for scaffold preparation were synthesized using hexamethylenediisocyanate, poly(*e* caprolactone)-diol with a molecular mass of 530 daltons and isosorbide diol (1.4:3.6 dianhydro-Dsorbitol) as a chain extender. Cylindrical scaffolds (8mm diameter, 4mm height) surrounded by a ring made of the same PU material were sterilized in a cold-cycle (37°C) ethylene oxide process and subsequently evacuated at 45°C and 150mbar for 3 to 4 days.

Image 8: PU Scaffold and Ring



3.8.1. Scaffold Seeding

At passage 3, after approximately 6.8 population doublings, chondrocytes were seeded onto the polyurethane scaffolds to provide a 3-D environment for the subsequent Bioreactor loading phase.
The PU scaffolds were pre-wetted with medium (DMEM) supplemented with 50mg/ml Primocin and 10% FCS under a vacuum for 1 h. Isolated chondrocytes ($4x10^6$ cells/scaffold) were suspended in 75µl fibrinogen solution and distributed into sterile caps (75µl/cap in 12-well plates). Then this mix was

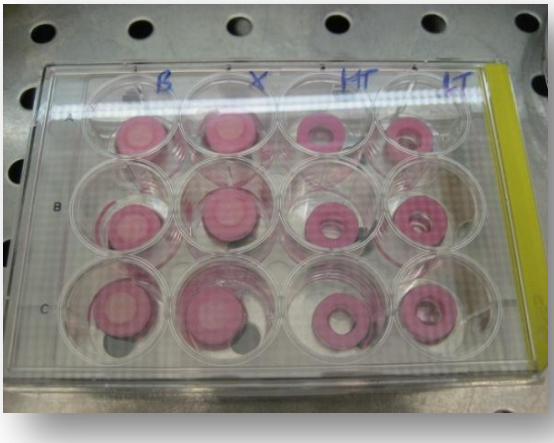
combined with 75µl thrombin solution immediately before seeding. Last, the scaffolds were added to this cell-fibrinogen-thrombin mixture. Constructs were incubated for 60min (37°C, 5% CO_2, 95% humidity) to permit fibrin gelation. PU rings were put around the scaffolds and loaded groups were installed into the special holding device for the bioreactor. The free-swell group was transferred into 12-well plates for subsequent culture. Then, 3 ml medium (DMEM, 10% FCS and 50mg/ml Primocin) was added to which 500kIU/mL of aprotinin was substituted.The final concentrations of the fibrin gel were 17mg/mL of fibrinogen, 0.5U/mL of thrombin, and 665 KIE/mL of aprotinin.

After 5 days in culture the cell–scaffold constructs were either exposed to mechanical loading as described below or remained in static culture for another 2 weeks to define 16 different groups. Each subgroup comprised a total of three scaffolds per experiment.

Image 9: Seeding of PU Scaffolds with monolayer culture expanded articular chondrocytes

Pre-Wetted PU Ring in Petridish

Scaffolds incubated with cell-fibrin



Control Scaffolds (free swell) in 12-well

Loaded Scaffold in special holding device

3.9. Bioreactor

The mechanical conditioning of cell-seeded scaffolds was performed using a bioreactor system. The bioreactor with its four loading stations was installed into a standard Heraeus Cytoperm 2 Incubator running with 5% CO_2, 37°C, 95% humidity. Rotation of the pin and ball is directly applied via a hybrid stepper motor (Zebotronics, Munich, Germany) (900rpm, 1.8° step angle), while the compression of each scaffold is achieved with a stepper motor connected to a translation screw (ROS, Apen, Germany) (5µm/full step; resolution, ~ 0.7µm if sequenced with microstepping). The generated compression force can be controlled with a load sensor. The custom-made software, programmed under LabView (National Instruments, Austin, Texas) allows the specification of load and motion target values over a wide range of 0-250N, ±360° and speeds of20mm/s, 360°/s for all three axes [Wimmer 2004 p. 1437-1438].

A ceramic hip ball (32mm in diameter) was pressed onto the cell-seeded scaffolds. Interface motion was generated by oscillation of the ball about an axis perpendicular to the scaffold axis. Superimposed compressive strain was applied along the cylindrical axis of the construct. Mechanical loading was applied in a displacement controlled manner with the starting point at the ball–scaffold contact; thus, all displacements were related to the center of the scaffold. After application of a preload of 0.2mm (5% of the scaffold height), the ceramic ball sinusoidally oscillated between 0.2mm and 0.6mm (5–15% of the scaffold height) at a frequency of 1 Hz. Simultaneously, the ball oscillated around an axis perpendicular to the scaffold axis over the scaffold surface at ±25° and 1 Hz, as described previously [Salzmann 2009, Grad 2006].

Image 10: Ceramic Hip-Ball used for in vivo simulating Loading

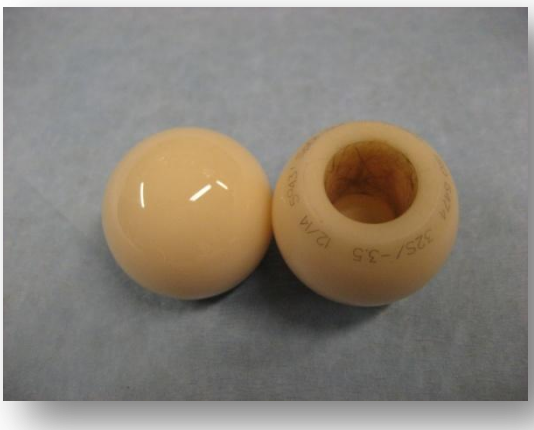
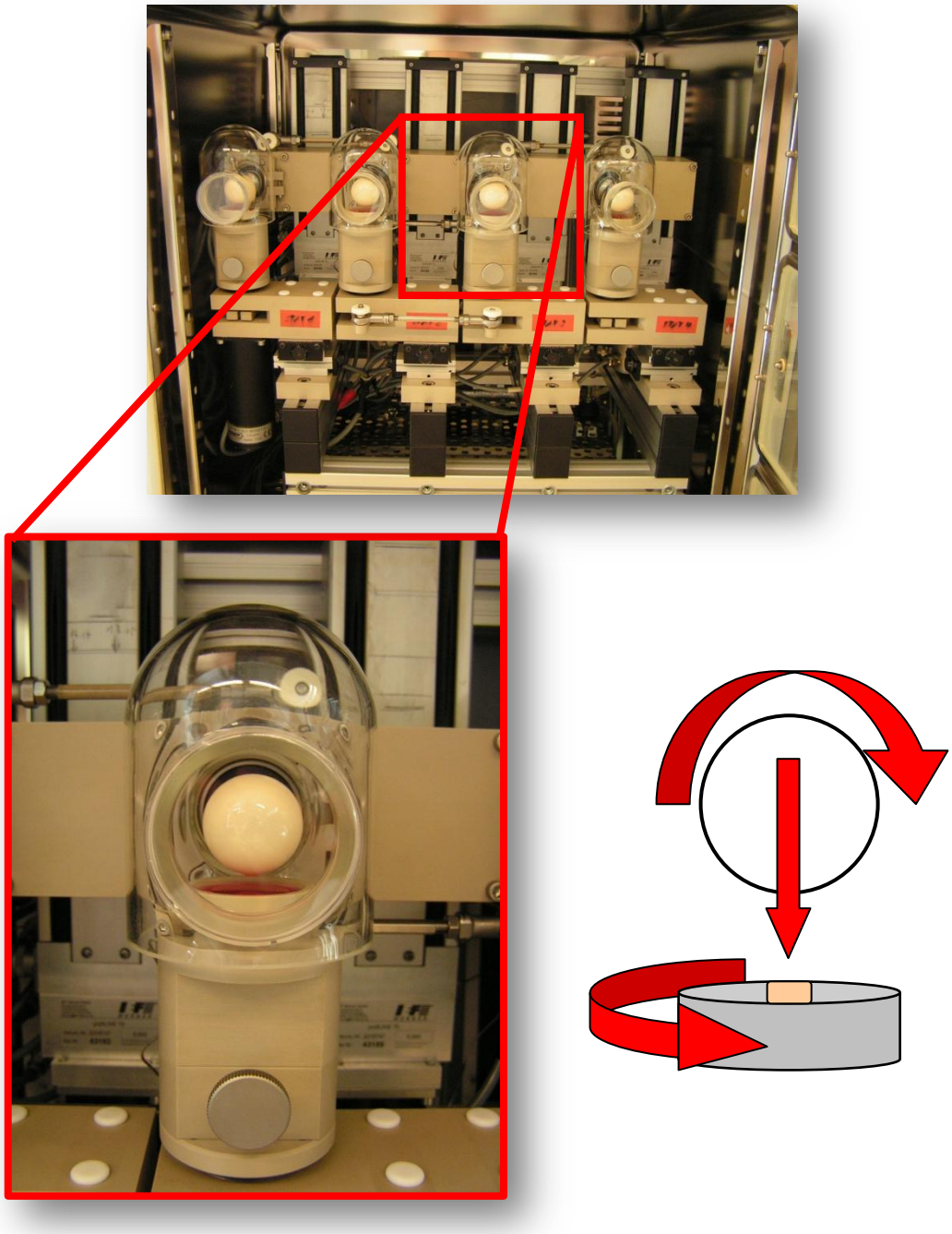


Image 11: Bioreactor in Incubator with four Loading Stations



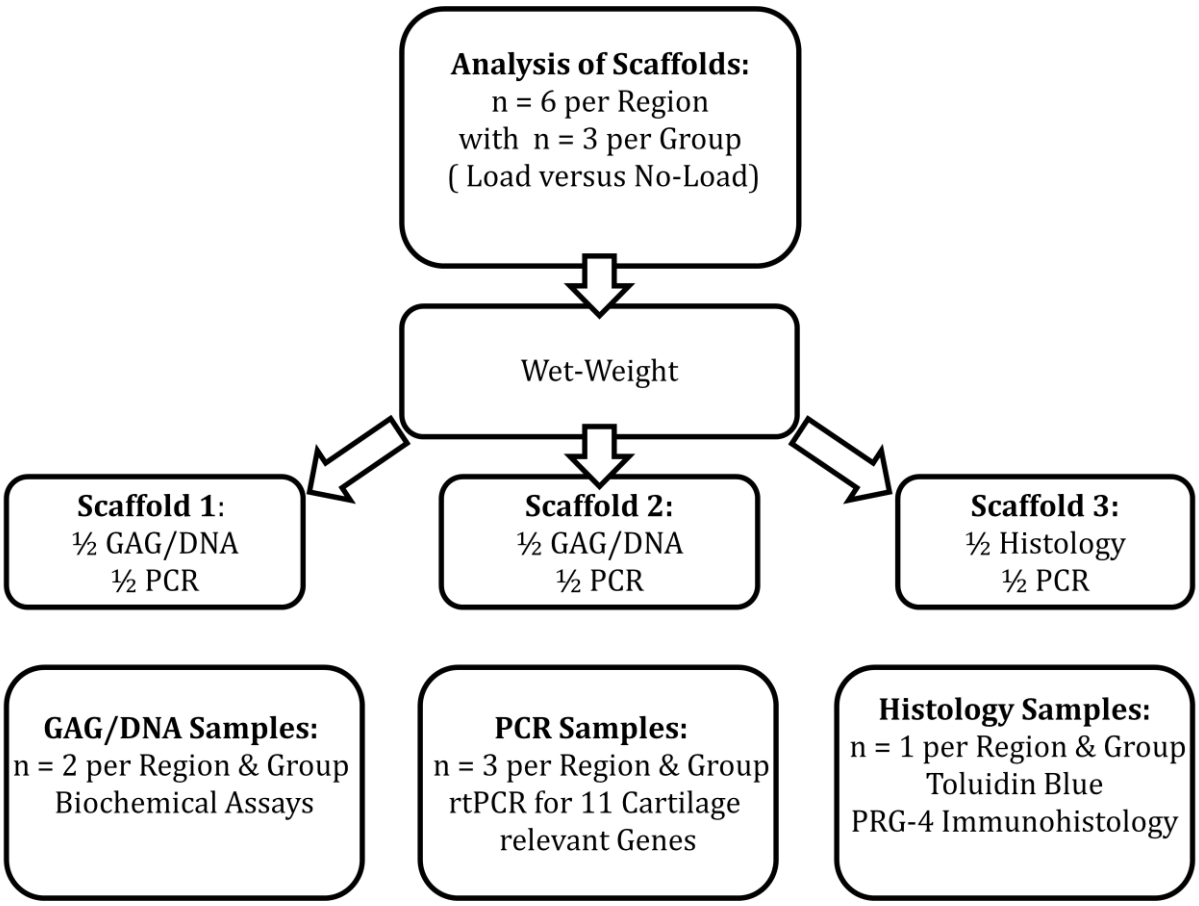
Loaded groups were loaded for 1 h twice a day with 8 h rest in between every second day, for a total of 16 h loading after 21 days of culture. Between loading cycles, constructs remained without contact with the ball. Unloaded groups served as controls cultured under equal free-swelling conditions. All constructs were cultured for a total of 21 days in vitro.

3.10. Scaffold Harvest

After a total of 16 days of loading (every second day for two hours) cell-scaffold constructs were harvested after one day to prepare them for following analysis.

The work was performed under the laminar flow to secure a sterile working process. Medium samples were preserved in cryotubes and frozen at -20°C for GAG analysis. Scaffolds were weighted for wet weight documentation and subsequently divided for further processing for analysis.

Image 12: Schematic Procedure of Scaffold Harvest after 21 days of culture

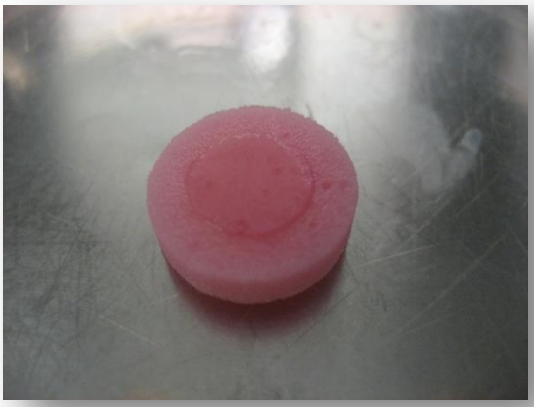


For PCR analysis, scaffold halves were put into RNase free Eppendorf Tubes with 1ml of TRI-Reagent. Samples were lysed in the Tissue Lyser at 25Hz with a sterile steel ball for 3 min. After centrifugation for 10 min at 1200g, clear supernatant was frozen at –80°C in RNase free cryotubes.

For GAG and DNA analysis, samples were washed twice with 1ml PBS, and 1 ml Proteinase K (0.5mg/ml) was added. The samples were digested over night at 56°C and Proteinase K was afterwards inactivated at 95°C for 10 min. Samples were analyzed immediately or frozen at -20°C.

Histology samples were conserved in 100% methanol and stored at 4°C for further analysis.

Image 13: Cell-Seeded PU Scaffold with PU Ring



3.11. Biochemical Analysis

Total DNA and GAG content of the scaffold halves were analyzed after samples were digested in Proteinase K (0.5mg/mL) at 56°C overnight. Proteinase K was then deactivated in the Thermostat 5320 at 95°C for 10 min.

3.11.1. DNA

Total DNA content was analyzed using the Hoechst 33258 dye assay. Calf thymus DNA served as standard.

Standards for the extended range assay (DNA samples: 100ng/well – 2000ng/well) were prepared from the stock solution to obtain a concentration of 2000ng DNA in 40µl volume for the first standard. The standards were diluted in DPBS in a row by two down to a concentration of 62.5ng in 40µl. The two blanks consisted of DPBS.

40µl of standards and samples were pipette into designed wells of a 96 well white plate. Samples and the standard were run in duplicates.

160µl assay solution were added to each well and the plate was wrapped in alumina foil to let the stain react for 20 min.

Afterwards the plate was gently tapped and read at 360nm (excitation) / 465nm (fluorescence emission). A linear standard curve E (465nm) vs. DNA amount (ng/well) was designed and samples were calculated according to their fluorescence values.

3.11.2. GAG

The total amount of sulphated GAG in the scaffold and in the medium samples of the 3-D culture was measured using the dimethylmethylene blue dye-binding assay. The conditioned culture medium was analyzed for total GAG content to determine the amount of proteoglycan released from the

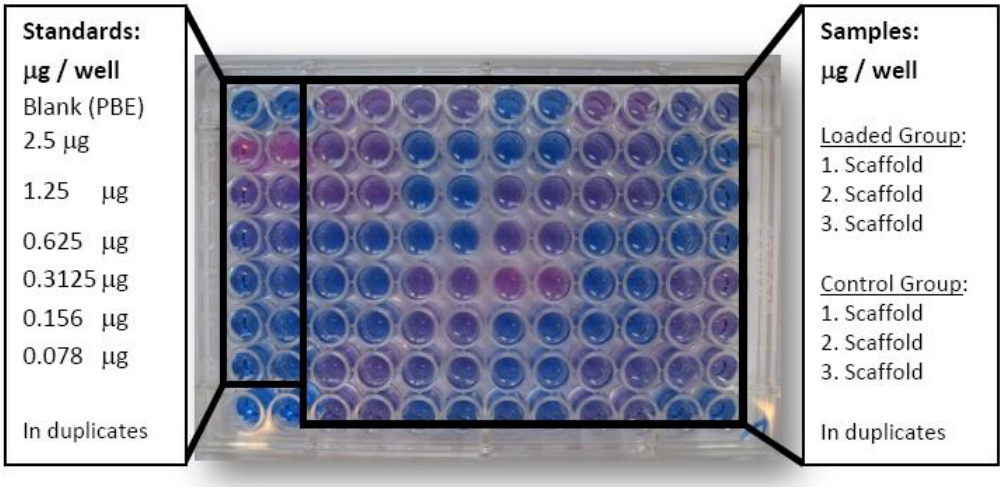
scaffolds into the medium. Chondroitin-4-sulfate sodium salt from bovine trachea served as a standard.

Dimethylethyleneblue was first introduced by Taylor and Jeffree as a strongly metachromic dye for histochemical detection of sulphated glycosaminoglycans. This analytical method was further improved and refined by Farndale et al., Saklatvala et al. and Jubb et al.

Standards were prepared from the stock solution to obtain concentrations of 2.5 / 1.25 / 0.625 / 0.3125 / 0.156 / 0.078µg/well. Dilution was prepared with PBE (since Proteinase K is also prepared with PBE) and two blanks consisted also of PBE.

20µl (or 50µl, depending on the expected concentration) of standards and samples were pipette into designed wells of a 96 well assay plate in duplicates. 200µl DMMB color reagent was added to each well, the plate tapped gently and immediately read at 535nm. A linear standard curve A_{535} vs. GAG content (µg/well) was designed to calculate samples from their A_{535} values.

Image 14: Schematic Arrangement for GAG Analysis



3.12. PCR

To evaluate gene (mRNA) expression levels of chondrocyte specific genes, mRNA was isolated from lysed cells, reverse transcribed into copyDNA and subsequently analyzed with the polymerase chain reaction (rtPCR).

3.12.1. RNA Isolation

For gene expression analysis a constant number of cells of each group and after each passage were lysed and RNA was extracted using TRI Reagent according to the manufacturer's specifications using the high salt precipitation method. Scaffolds were homogenized in TRI REAGENT (1ml / 50-100mg tissue) using the tissue lyser. To gain the mRNA of chondrocytes from the native cartilage biopsies,

50-150mg tissue of each group was grounded to a fine powder with liquid nitrogen before the samples were lysed with TRI Reagent and RNA isolation was performed using SV Total RNA Isolation System according to the manufacturer`s instructions.

Note: The following steps have to be followed with special attention and care. Time frames must be followed in detail to ensure sufficient results. All the following steps were done on ice to prevent the denaturation of RNA.

To facilitate the isolation of RNA, 1ml of TRI REAGENT was supplemented with 2-8µl of Polyacryl Carrier™.

After homogenization (or lysis) samples can be stored at –80°C for at least one month.

Native Cartilage Biopsies were grounded to fine powder using liquid nitrogen and a special hammering device. For native cartilage biopsy, a slightly different protocol was followed to extract RNA from the tissue than from scaffolds or monolayer cultured cells. Samples with high contents of protein, fat, polysaccharides or extracellular material, such as cartilage, need an additional isolation step to secure sufficient amounts of RNA and thus, better results.

After pulverization of the cartilage pieces, the powder was lysed in TRI Reagent by strongly vortexing several times. Insoluble material was then removed from the homogenate by centrifugation at 1200g for 10 min at 4°C. The clear supernatant was transferred to an RNase free reaction tube and it was either processed with RNA isolation or samples were stored at -80°C.

To the aqueous phase from the separation step (see details below) 0.7 volumes of ethanol (96-100%) were added and the solution subsequently loaded onto RNA binding column. After that we proceeded according to the protocol of the manufacturer.

Image 15: : Hammering Device to pulverize native Cartilage Biopsy Pieces

Cartilage pieces were placed in the tube, liquid nitrogen was added, the metal pole was fitted in the tube and with a hammer, strongly beating the metal pole, cartilage pieces were pulverized.
Scaffolds were homogenized in TRI REAGENT (1ml / 50-100mg tissue) using the tissue lyser. Sample volume should not exceed 10% of the volume of TRI REAGENT used for homogenization. Following homogenization, insoluble material was removed from the homogenate by centrifugation at 12000g for 10 min at 4°C. The clear supernatant was then transferred to a fresh tube and processed for the phase separation.

Chondrocytes grown in monolayer culture were lysed directly after passaging. Approximately 10^6 cells were added to TRI REAGENT (1ml) and passed several times through a pipette to lyse the cells. The homogenate was stored for 5 min at room temperature (if samples were frozen, stored until they unfroze and subsequently waited 5 min). 0.1ml of BCP per 1ml of TRI REAGENT was added, the samples covered tightly and shaken vigorously for 15 sec. The resulting mixture was stored at room temperature for 2–15 min on a shaker platform and centrifuged at 12000g for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, whereas DNA and proteins are in the interphase and organic phase.

Exclusively the aqueous phase was carefully transferred to a fresh tube. It was made sure, that no other material was transferred. RNA was precipitated from the aqueous phase by adding isopropanol. Since the RNA was isolated from sources rich in polysaccharides and proteoglycans, 0.25ml of isopropanol was added to the mixture, additionally followed by 0.25ml of a high salt precipitation solution (0.8M sodium citrate and 1.2M NaCl) per 1ml of TRI REAGENT used for the homogenization.
Samples were subsequently stored at room temperature on a shaker platform for 5-10min and centrifuge at 12000g for 8min at 4-25°C.

The supernatant was removed and RNA pellets washed (by vortexing and pipetting) with at least 1ml of 75% ethanol per 1ml TRI REAGENT used for the initial homogenization. Tubes were then centrifuged at 7500g for 5 min at 4-25°C. If the RNA pellet accumulates on the side of the tube and has a tendency to float, the pellet could be sedimented at 12000g.

After the ethanol wash was removed, the RNA pellet was air-dried for 3-5 min. It is important to avoid any rest of ethanol, since it might disturb the measurements of RNA content as well as other procedures.

RNA was dissolved in DEPC treated water (40µl / 10µl if the pellet was not visible) by passing the solution a few times through a pipette tip and incubating for 10 min at 56°C.

After this step, RNA concentration and purity was measured at an absorbance of A_{260} and A_{280} using the Nano-Drop before diluting the samples with TE-buffer. Dilution was performed to obtain an absorbance at A_{260} between 0.1 and 1.0, corresponding to 4 -40µg/ml RNA.

To assess the purity of RNA, the A_{260}/A_{280} ratio was also determined. An A_{260}/A_{280} ratio of 1.6-1.9 is expected. Protein contamination results in an A_{260}/A_{280} ratio <1.6.

3.12.2. Reverse Transcription

To be able to ananlyze the gene expression (mRNA) levels, isolated RNA was reversed transcribied into copyDNA in order to run PCRs.

All reagents were thawed except the Reverse Transcriptase and the RNase inhibitor since these enzymatic reagents are stored differently. After thawing, all reagents were kept on ice, like the RNA samples. The reaction mix was prepared for a 40µl reaction volume, for the sample number +1 by combining all nonenzymatic components.

Reaction mix:

10x TaqMan RT Buffer (PCR Buffer II)	4.0 µl
25 mM Magnesium chloride	8.8 µl
DeoxyNTPs mixture (2.5 mM each dNTP)	8.0 µl
Random Hexamer (50 µM)	2.0 µl

After mixing the components throurougly by pipetting up and down, the reverse transcriptase and the RNase inhibitor were added.

RNase Inhibitor (20 U/µl)	0.8 µl
MultiScribe Reverse Transcriptase (50 U/µl)	1.0 µl

Components were mixed by inverting the tube (no vortexing!) and 24.6µl of reaction mix were placed into 200µl mico-reaction tubes.

Then, RNA sample (1µg total RNA) and RNAse free (DEPC) water were added (15.4µl DEPC water - RNA sample volume) and the reaction mix was centrifuged down using a 1200g impulse for 15 sec.

Samples were then placed in the Thermal Cycler 9600. At 25°C, primers were incubated for 10 min, then at 48°C the reverse transcription took place for 30 min before the reverse transcriptase was inactivated at 95°C for 5 min.

3.12.3. rt-PCR

Polymerase chain reaction (PCR) was performed with a 7500 real-time PCR system. For collagens IαII, IIαI and X, aggrecan, cartilage oligomeric matrix protein (COMP), superficial zone protein (SZP/ PRG-4), matrix metalloproteinase (MMP)-3 and 13, oligonucleotide primers and TaqMan probes were

designed with Primer Express Oligo Design software, versions 1.5/2.0 (AB) and produced by Microsynth, Balgach, Switzerland**.** The nucleotide sequences were taken from the GenBank database. For MMP-1 and parathyroid hormone related peptided (PTHrp), TaqMan Gene Expression Assays (non-inventoried small scale, AB) were used. 18S ribosomal RNA was used as the housekeeping gene with primers and probe from AB.

Table 3: Gene Sequences for rtPCR designed for forward (fw) Primer, reverse (rv) Primer & Probe.

Gene	Primer fw (5'-3')	Primer rev (5'-3')	Probe (5'FAM/3'TAMRA)
Aggrecan	CCA ACG AAA CCT ATG ACG TGT ACT	GCA CTC GTT GGC TGC CTC	ATG TTG CAT AGA AGA CCT CGC CCT CCA T
Collagen 1A2	TGC AGT AAC TTC GTG CCT AGC A	CGC GTG GTC CTC TAT CTC CA	CAT GCC AAT CCT TAC AAG AGG CAA CTG C
Collagen 2A1	[2]AAG AAA CAC ATC TGG TTT GGA GAA A	TGG GAG CCA GGT TGT CAT C	CAA CGG TGG CTT CCA CTT CAG CTA TGG
Collagen X	ACT TCT CTT ACC ACA TAC ACG TGA AAG	CCA GGT AGC CCT TGA TGT ACT CA	TGC CGT TCT TAT ACA GAC CTA CCC AAG CAT G
COMP	CCA GAA GAA CGA CGA CCA GAA	TCT GAT CTG AGT TGG GCA CCT T	ACG GCG ACC GGA TCC GCA A
MMP-3	GGC TGC AAG GGA CAA GGA A	CAA ACT GTT TCG TAT CCT TTG CAA	CAC CAT GGA GCT TGT TCA GCA ATA TCT AGA AAA C
MMP-13	CCA TCT ACA CCT ACA CTG GCA AAA G	GTC TGG CGT TTT GGG ATG TT	TCT CTC TAT GGT CCA GGA GAT GAA GAC CCC
SOX9	AAC GCC GAG CTC AGC AAG	ACG AAC GGC CGC TTC TC	TTC AGC AGT CTC CAG AGC TTG CCC A
SZP(PRG4)	GAG CAG ACC TGA ATC CGT GTA TT	GGT GGG TTC CTG TTT GTA AGT GTA	CTG AAC GCT GCC ACC TCT CTT GAA A

The reaction mixture for (2n + 2) reactions (n=sample number) was prepared by combining TaqMan Universal Master Mix, primers / probes, and DEPC-treated H_2O and distributed into the optical 96-well plates.

<u>For 20 µl reaction volume per well the following components were mixed:</u>

TaqMan Universal Master Mix	2x10 µl	→ 1x final concentration
Forward Primer 45 µM	0.4 µl	→ 900 nM
Reverse Primer 45 µM	0.4 µl	→ 900 nM

TaqMan Probe 12.5 µM	0.4 µl	→ 250 nM
cDNA + DEPC-treated H_2O	8.8 µl	

For 18S rRNA (endogenous control) and Assays-on-Demand the following components were mixed:

TaqMan Universal Master Mix	2x10 µl	→ 1x final concentration
Primer/Probe Mix 20x	1.0 µl	
cDNA + DEPC-treated H_2O	9.0 µl	

2µl/reaction of the cDNA samples were added into the 96-well plate in duplicates. 1 NTC (no template control) reaction was run without cDNA for each reaction mixture.
The plates were then covered with the adhesive cover and centrifuged briefly to remove air bubbles from the bottom of the tubes.
Real time PCR was performed under standard conditions with AB 7500 Real Time PCR System running 40 cycles of 15 sec at 95°C, 60 sec at 60°C after an initial 10 min at 95°C to activate the DNA polymerase. For a relative quantification of the target mRNA the comparative CT method was used with 18S ribosomal RNA as the housekeeping gene and a calibrator (baseline) sample. Using 18S ribosomal RNA as the housekeeping gene (endogenous control), variations in the number of cells per sample were rendered insignificant, since the expression of 18S RNA is assumed to be constant from chondrocyte to chondrocyte and the gene of interest ´s relative expression can be quantified this way independently from the number of cells in the sample.

Quantity is expressed relative to a calibrator (baseline) sample. The amount of mRNA relative to the calibrator is calculated as $\mathbf{2^{-\Delta\Delta CT}}$; $\Delta\Delta C_T$ is the difference between $C_{T(target)} - C_{T(control)}$ of sample and $C_{T(target)} - C_{T(control)}$ of calibrator.
The threshold ΔR_n for each gene and experiment was evaluated and compared after the last experiment. To make all experiments the best possible comparable, the threshold was reset into the exponential phase of the logarithmic scale amplification curve and adapted for all experiments and CT values were recalculated to avoid slight differences between experiments.

3.13. Histology

Histological staining was performed to visualize and support biochemical and pcr observations/finding. Scaffolds were stored in 100% methanol at 4°C until further histological preparation.

Before cutting, the samples were rinsed in a 5% sucrose–PBS solution for at least 24 h and then embedded in cryocompound ‘‘Jung Gel’’ for four hours. Twelve µm serial cryosections were prepared using a microtome, and samples were stored at –20°C.

3.13.1. Toluidin Blue Stain

Toluidin Blue stain is an overview stain, designed to stain cartilage matrix, mast cell granules, some connective tissue and mucopolysaccharides.

Slides were rehydrated for 5 min in distilled H_20. Afterwards, slides were stained with Toluidin Blue solution for 5 min. The solution contained 110ml Toluidin Blue mixture with 1% Toluidin Blue and 1% Natriumtetraborat and 110ml H_20. After rinsing the slides with distilled H_20, they were dipped shortly in 70% ethanol, 96% ethanol and absolute ethanol to differentiate. After another minute of absolute ethanol, slides were rinsed and dried. Before mounting the slides under a coverslip with DPX, they were shortly incubate in Xylene for 2 min, twice.

3.13.2. Immunohistochemistry for PRG-4 / Superficial Zone Protein

Sections on the slides were circled with a Dako Pen an rehydrated 5 min in PBS-Tween. Slides were then incubated with the methanol-mix for 30 min to block the endogeneous peroxidase. After three washing steps with PBS-Tween for 5 min each, the slides were incubated with the horse serum, diluted 1:20 with PBS, at room temperature for 60 min.

Following this step, slides were knocked on the table to free the samples from the horse serum. The primary antibody was then pipetted onto the slide for an overnight incubation at 4°C. The monoclonal mouse antibody 3A4 was generously provided by Dr. Milz, AO Research Institut, and diluted 1:400 in PBS. Control slides were covered only with PBS. After approximately 12-16 h of incubation, the slides were washed three times in PBS-Tween for 5 min and incubated with the secondary antibody, an anti-mouse antibody from Vectastain in a dilution of 1:200 for 30 min at room temperature.

The ABC-Complex to stain the antibodies was prepared with 1ml PBS (without Tween), 20µl ABC-A and 20µl ABC-B and stored at 4°C for at least 30 min before it was used. It was then applied for 30 min at room temperature, after another three washing steps with PBS-Tween for 5 min each. After the ABC-Complex was applied, slides were washed again (3x5 min with PBS-Tween) and incubated in the dark for 4 min with DAB.

Afterwards, the slides were rinsed three times with water and counterstained with Meyer´s hematoxylin for 15 sec. Slides were then extensively rinsed for 5 min in running water before they were dehydrated with ascending (50% → 70% → 96% → 100%) alcohol for 5 min each. After two times 5 min of treatment with Xylene, slides were mounted under coverslips with DPX.

3.14. Statistics

The data was analyzed statistically using the software package SPSS (Version 17, SPSS Inc., Chicago, IL). All data were tested for normal distribution using the Kolmogorov–Smirnov test. When skewed distributions were observed in a data set, transformation by natural logarithm was applied to normalize the plotting and controlled afterwards by another set of Kolmogorov–Smirnov test.

Cellnumber per gram biopsy and Population Doubling data was analyzed using the Kruskal-Wallis test.

Subsequent 2-D monolayer culture data were compared using a mixed linear model which properly reflects the structure of repeated data and takes correlation between measurements within the same individuals into consideration. Pairwise comparison between the distinct locations was performed using the Bonferroni test for proper results.

For the statistical analysis of the 3-D scaffold data , the GEE model (generalized estimation equation) was chosen to properly take into account not only the structure of repeated data and correlation between measurements within the same individual but also dependencies between measurements when comparing loaded with control scaffolds from the same location. Pairwise comparison was also tested, using Bonferroni sequential testing.

Correlations were performed using Spearman's correlation coefficient (*r*). All descriptive results within the text were demonstrated as the mean-standard deviation or error of mean. Descriptive results were demonstrated graphically as the mean ± standard deviation. The significance level was defined at $p < 0.05$ for all tests.

No outliers were removed to keep the results as exact as possible and display the true nature of distribution.

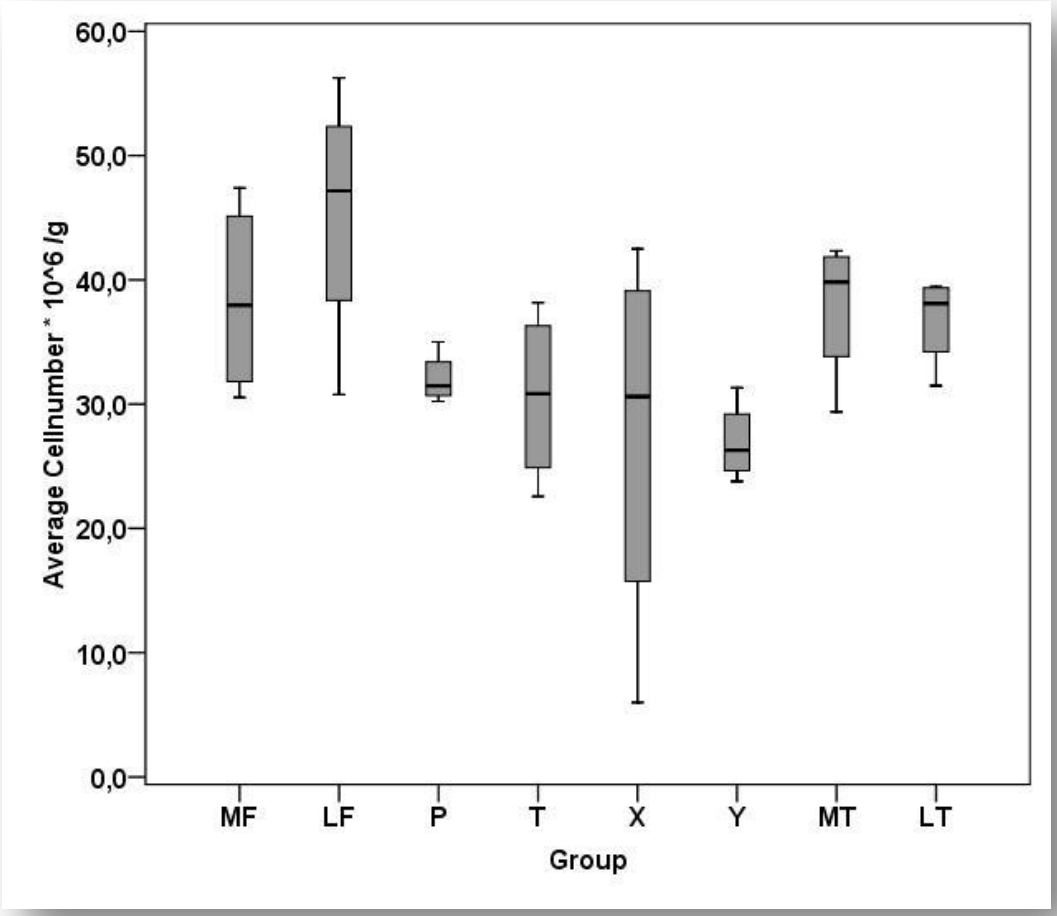
4. Results

4.1. Cartilage Biopsy: Cell Number / Gram Cartilage

The initial number of cells that was enzymatically isolated from the fresh articular cartilage biopsies showed significant regional differences (p= 0.044). The mean average cell count in $1x10^6$ per gram cartilage biopsy was 40.27 ± 8.72 (MF), 50.19 ± 5.39 (LF), 40.66 ± 2.11 (MT), 35.90 ± 3.99 (LT), 33.28 ± 5.56 (T), 32.13 ± 2.53 (P), 34.57 ± 8.56 (X), and 26.87 ± 3.95 (Y).

Peak cellular densities were found at the femoral condyles and the medial tibia plateau, followed by the lateral tibia plateau. The lowest cell density was found at the proximo-medial femoral condyle.

Graph 1: Average Number of Isolated Cells per Gram Native Cartilage Biopsy



n = 3 per region; statistical results within the text;

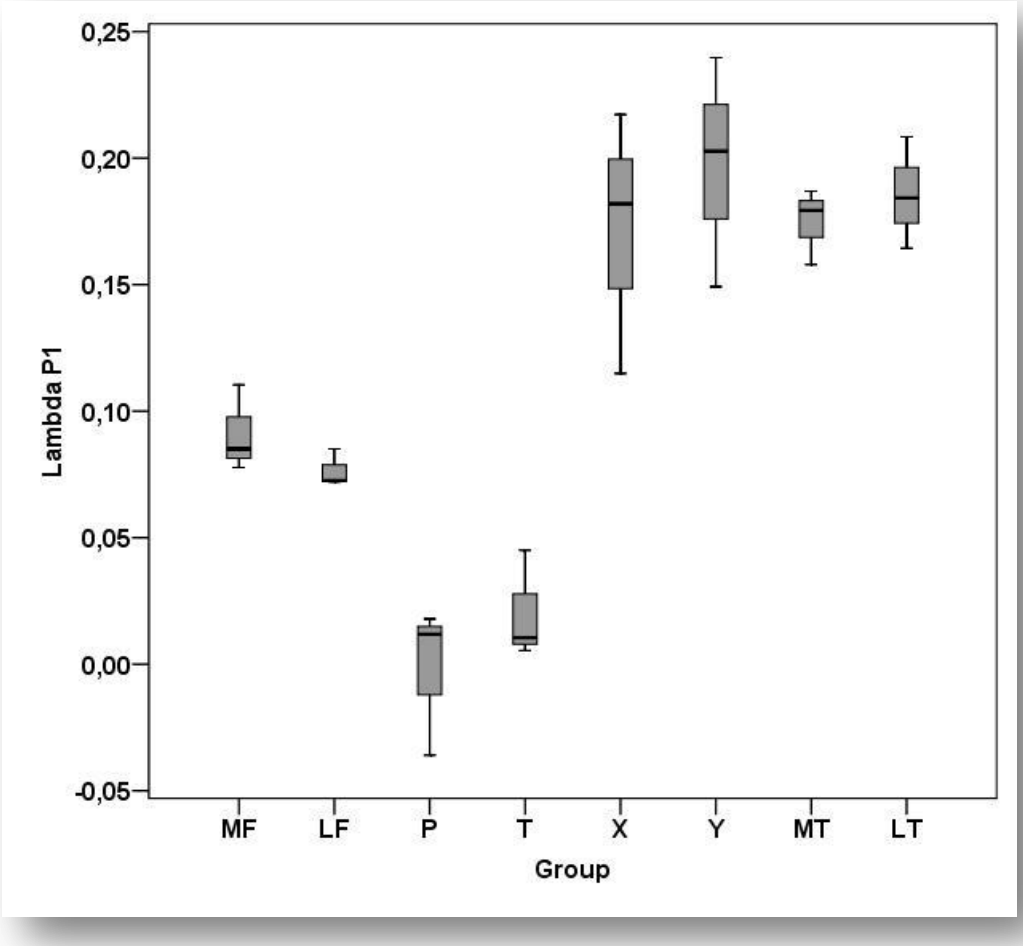
4.2. Monolayer Culture

4.2.1. Population Doubling

The overall population doublings between the eight subpopulations did not differ significantly for the time of culture (p= 0.926). But a trend could be detected, ranking LT highest, followed by MT (87% of LT`s PDs), MF (86%), LF (79%), X (72%), Y and P, both reaching 66% of the PDs of LT, with the trochlea cells performing poorest with 59% for the overall culture period.

Only for P1 a striking difference was seen (p= 0.006)**.** The cells of the non weight bearing area biopsy sites, X (PD= 2.14) and Y (PD= 2.49), showed the highest cell proliferation activity in P1, together with the tibial groups MT (PD= 2.19) and LT (PD= 2.33), followed by the condyle groups, MF (PD= 1.14)and LF(PD= 0.96). The chondrocytes from the patellofemoral joint showed the poorest cell dividing activity (PD P= -0.01, PD T= 0.26).

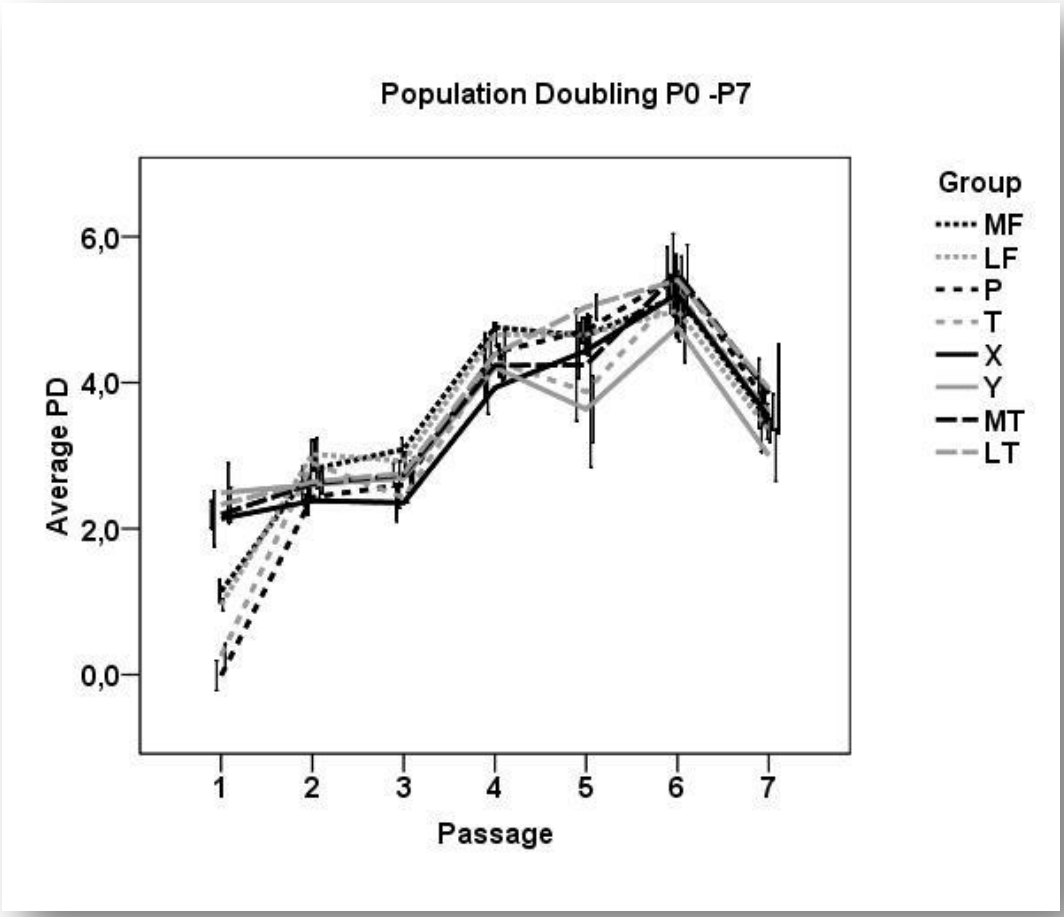
Graph 2: Factor of Growth (Lambda) during First Passage (P1)



n = 3 per region; statistical results within the text;

After this initial passage, differences between the eight subpopulations diminished and the growing curves started to synchronize. The passages themselves showed significant differences in their population doublings (p= 0.000), resulting in a characteristic growth curve when averaged, with an initial lag-phase, followed by a log-phase, reaching a plateau before starting to decline. On average 24.37 PDs (22.6-26.5 PDs) were observed during P0 to P7.

Graph 3: Population Doubling P0 - P7



n = 3 per region; statistical results within the text;

4.2.2. PCR

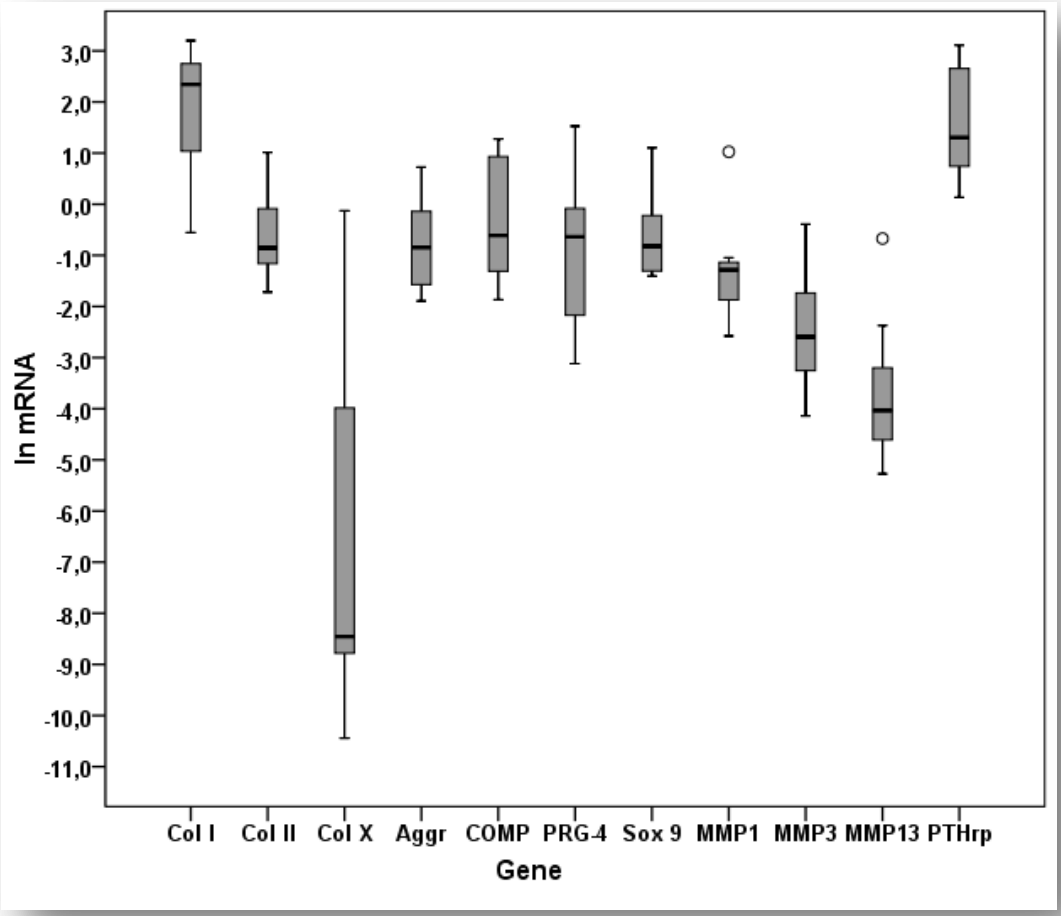
Differences in the gene expression levels between the eight topographically different populations were found depending on the gene and the passage number. Freshly isolated, P0 cells were taken as the reference group for the following findings described unless stated otherwise.

In general when these P0 cells were compared with the initial expression levels for the native cartilage biopsies, changes in the expression levels could already be detected. P0 cells showed downregulated Col I levels for all groups, slightly upregulated Col II for most groups, and

downregulated COMP, SOX-9, PRG-4 and PTHrp level for the majority of groups. Aggrecan and MMP-1, -3, and -13 levels were upregulated, as well as Col X for mostly all groups.

The following graphGraph 4: Differences in mRNA Levels between Native Cartilage Biopsies and freshly isolated Cells shows the difference between native cartilage mRNA levels compared to freshly isolated P0 cells. Already the processing of the cartilage biopsies and isolation protocol seems to have an impact on gene expression levels. It displays the general behavior concerning the varying genes not taking the regional differences between subpopulations into account.

Graph 4: Differences in mRNA Levels between Native Cartilage Biopsies and freshly isolated Cells



n = 3 per gene; P0 freshly isolated cells were taken as the reference group; Displayed are the levels of mRNA relative to the native cartilage; statistical results within the text;

In the following graphs concerning the monolayer gene expression levels, P0 values were chosen as the baseline expression level. Since cartilage biopsies showed greater distribution in values and had no detection for MMP-1 levels for the biopsies of experiments two and three.

4.2.2.1. Collagens

Collagen I

For Col I expression levels, the eight subpopulations differed significantly for P1 throughout P7 ($p < 0.025$). When comparing the expression levels detected in the native cartilage biopsies with P1, the femoral condyles showed the highest increase (MF 261 fold, LF 384 fold), while the cells of the classical NWB biopsy locations showed low or even no increase (X 2.93 fold, Y 0.82 fold). After the first passage, all groups stayed at the level of gene expression they had reached and no dramatic further change in the expression levels was detected. Overall, there was no significant difference between the passages themselves ($p = 0.891$), while group differences were detected ($p = 0.000$). Differences between passages, showing the dynamic of gene regulation, were significant merely for group LF ($p = 0.011$) while there was no significance but only trends reached for MF ($p = 0.071$), P ($p = 0.070$), T ($p = 0.168$), X ($p = 0.075$), Y ($p = 0.627$), MT ($p = 0.069$) LT ($p = 0.481$).

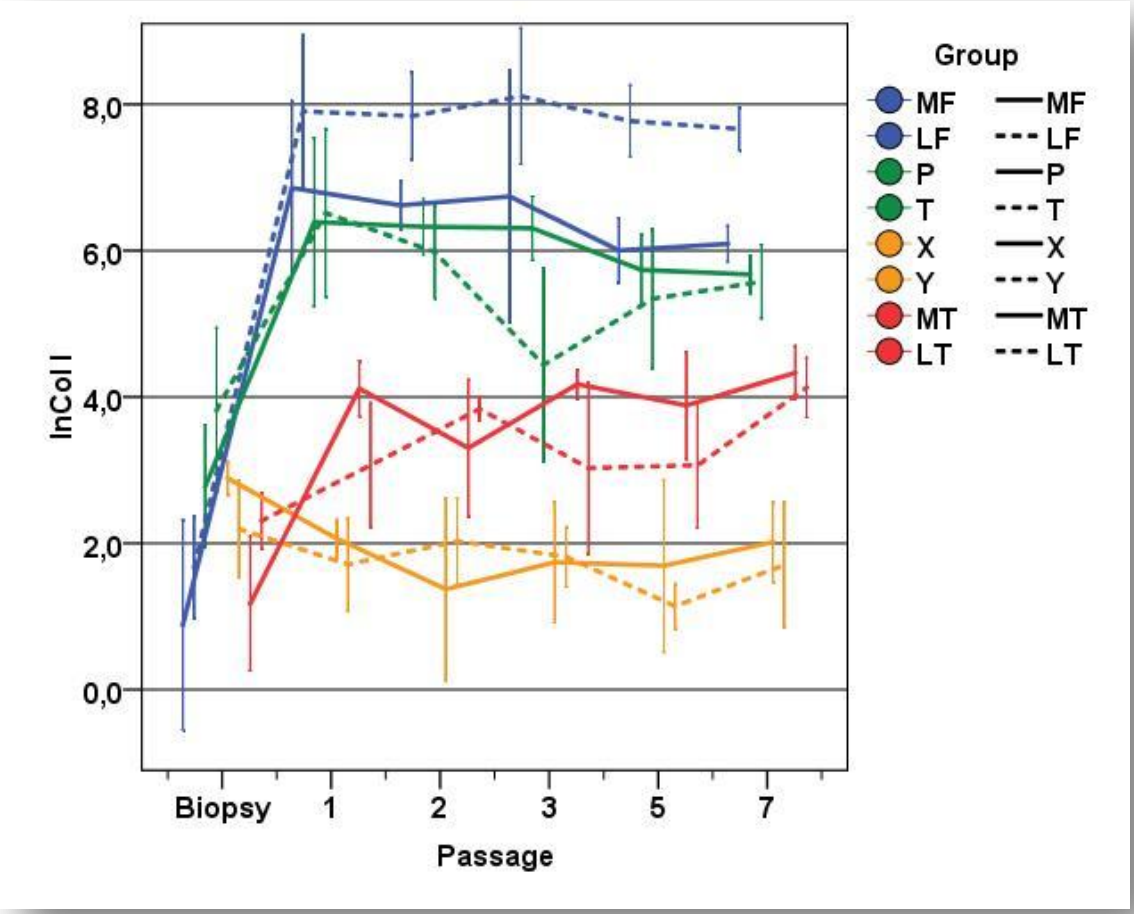
The cells of the NWB origin showed significant differences to the cells from femoro-patella origin as well as the femoral condyle groups. No significant difference was detected compared to the tibial cells**.**

Table 4: Collagen I Bonferroni Pairwise Comparison from Biopsy to P7

Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	0.021	0.026	1.000	0.134
LF	1.000		1.000	1.000	0.000	0.001	0.106	0.015
P	1.000	1.000		1.000	0.000	0.000	0.036	0.006
T	1.000	1.000	1.000		0.000	0.001	0.183	0.048
X	0.021	0.000	.000	0.000		1.000	1.000	1.000
Y	0.026	0.001	0.000	0.001	1.000		1.000	1.000
MT	1.000	0.106	0.036	0.183	1.000	1.000		1.000
LT	0.134	0.015	0.006	0.048	1.000	1.000	1.000	

n = 3; p-values < 0.05 considered to be significant;

Graph 5: Collagen I mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;

<u>Collagen II</u>

The differences in the Col II expression level were not very uniform. Significant differences for the eight subpopulations were found (p= 0.001) especially between the medial tibia and the femoral condyles as well as the femoro-patella cells (p< 0.035). No significant differences were detected for the cells of the femoral notch as well the lateral tibiaplateau compared to the other groups. The cells of the NWB proximo-medial condyle showed significant differences to the groups of the trochlea and lateral condyle (p= 0.005, p= 0.018).

The passages differed significantly from each other, native cartilage biopsies expression levels being 5600 times higher than P7 levels, showing the most pronounced averaged drop in the gene expression level between the native cartilage and passage 1 (25 fold). This drop was biggest for the cells of NWB origin (X= 90 fold, Y= 126 fold) and the lateral tibiaplateau (LT= 94 fold), followed by the medial tibiaplateau (MT= 60 fold) with the femoral condyles and the femoro-patella cells showing

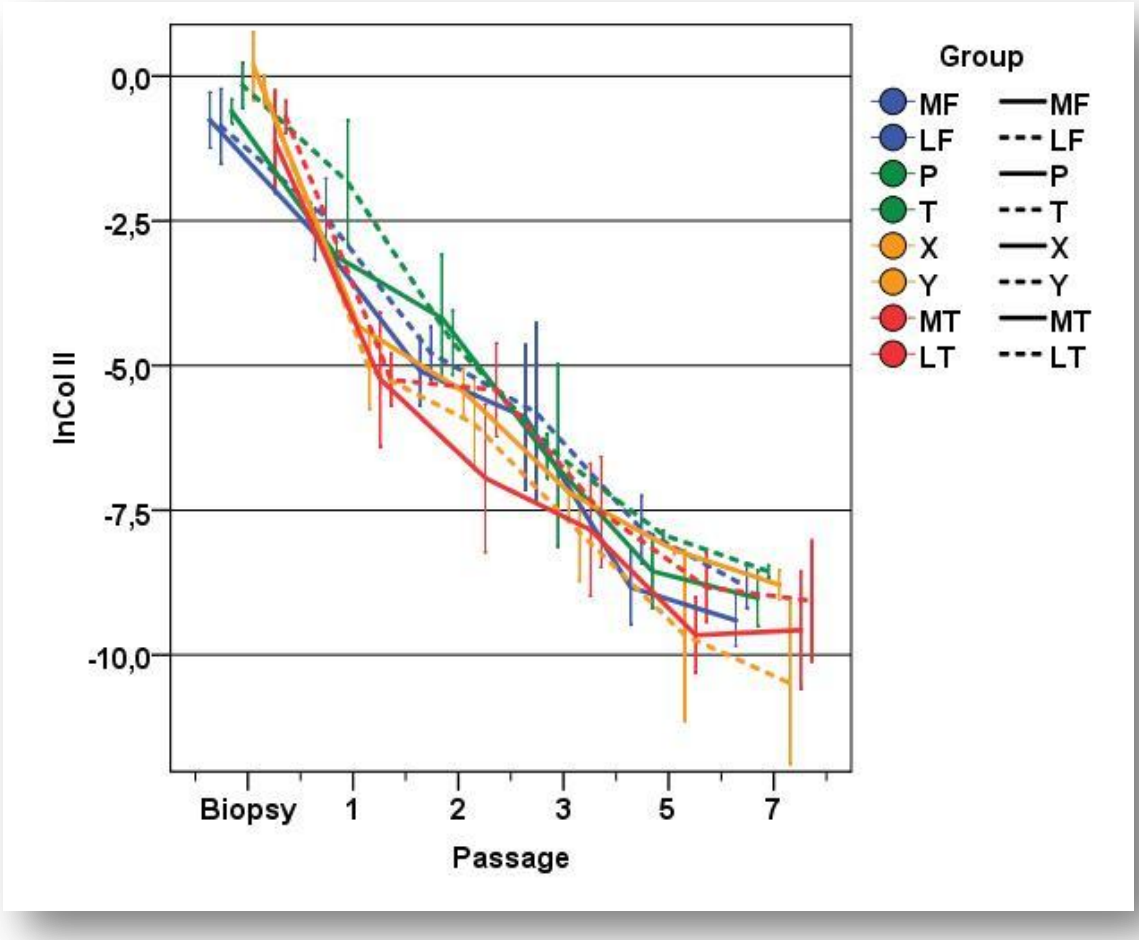
the least drop with only 5-12 fold changes. Overall, only between P5 and P7 no overall significant difference between passages was observed (p= 0.201). For each group, the overall gene expression showed significant differences between the passages for all subpopulations, MF (p= 0.000), LF (p= 0.001), P (p= 0.000), T (p= 0.000), X (p= 0.000), Y (p= 0.000), MT (p= 0.000) LT (p= 0.000).

Table 5: Collagen II Bonferroni Pairwise Comparison from Biopsy to P7

Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	1.000	0.192	0.034	0.988
LF	1.000		1.000	1.000	1.000	0.018	0.003	0.218
P	1.000	1.000		1.000	1.000	0.110	0.032	1.000
T	1.000	1.000	1.000		1.000	0.005	0.002	0.199
X	1.000	1.000	1.000	1.000		0.196	0.165	1.000
Y	0.192	0.018	0.110	0.005	0.196		1.000	1.000
MT	0.034	0.003	0.032	0.002	0.165	1.000		1.000
LT	0.988	0.218	1.000	0.199	1.000	1.000	1.000	

n = 3; p-values < 0.05 considered to be significant;

Graph 6: Collagen II mRNA Levels from Native Cartilage Biopsies up to Passage 7 (P7)

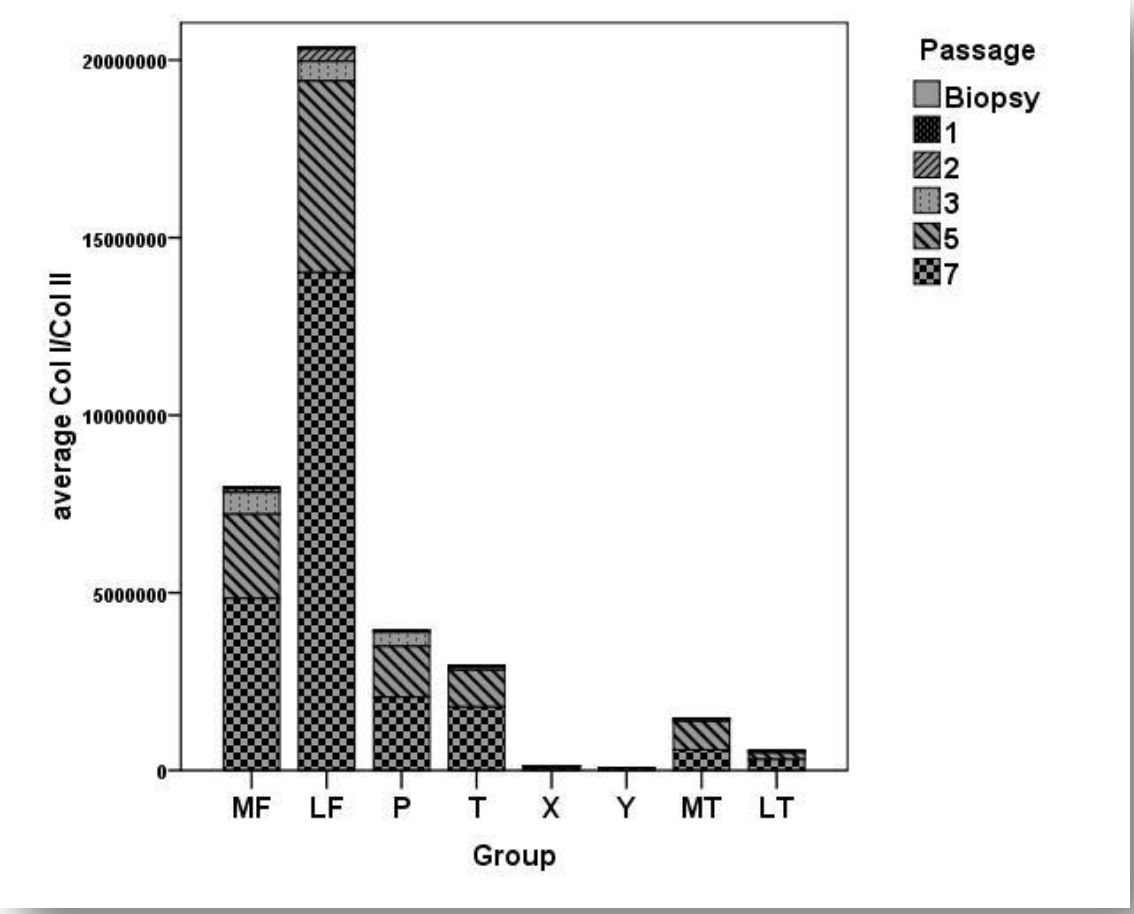


n = 3 per region; statistical results within the text;

Ratio Collagen I/Collagen II

The Col I to Col II level showed a clear trend and grouping depending on the anatomical compartment of origin over the entire cultivation period. Starting with the first passage, the ratio between Col I/II was lowest for the cells of NWB origin (X and Y), followed by the tibia plateau, the femoro-patella joint and the femoral condyles, showing the highest, and thus least favorable Col I/ Col II ratio. For the passages P3, P5 and P7, the ranking of expression levels was constant even within the anatomical partners, showing a Col I/II ratio highest for LF>MF>P>T>MT>LT>X>Y.

Graph 7: Collagen I /Collagen II Ratio of mRNA Expression Levels from Biopsies to P7



n = 3 per region; statistical results within the text;

Collagen X

An averaged 155 fold overall decrease was found for the Col X expression levels, with the biggest drop on average between P1 and P2 (10.3 fold). The cells of the femoral condyle as well as the patella showed an increase in the expression level of P1 when compared with the native cartilage values, while all other groups decreased in their detected levels. The biggest overall drop was found

for the chondrocytes of the trochlea (2390 fold drop), while the cells of the patella lost only 2.74 times of their initial native cartilage levels (65.5 fold drop, compared to P1).

Significant differences between passages were detected for the subpopulations of MF (p= 0.011), LF (p= 0.003), P (p= 0.004), T (p= 0.000), X (p= 0.000) and MT (p= 0.000) but not for LT (p= 0.063) and Y (p= 0.201). Although the subpopulation of the patella showed the least overall drop compared to their original cartilage levels, the difference between passages was still significant since it showed the highest upregulation of the Col X mRNA levels between the native cartilage and P0.

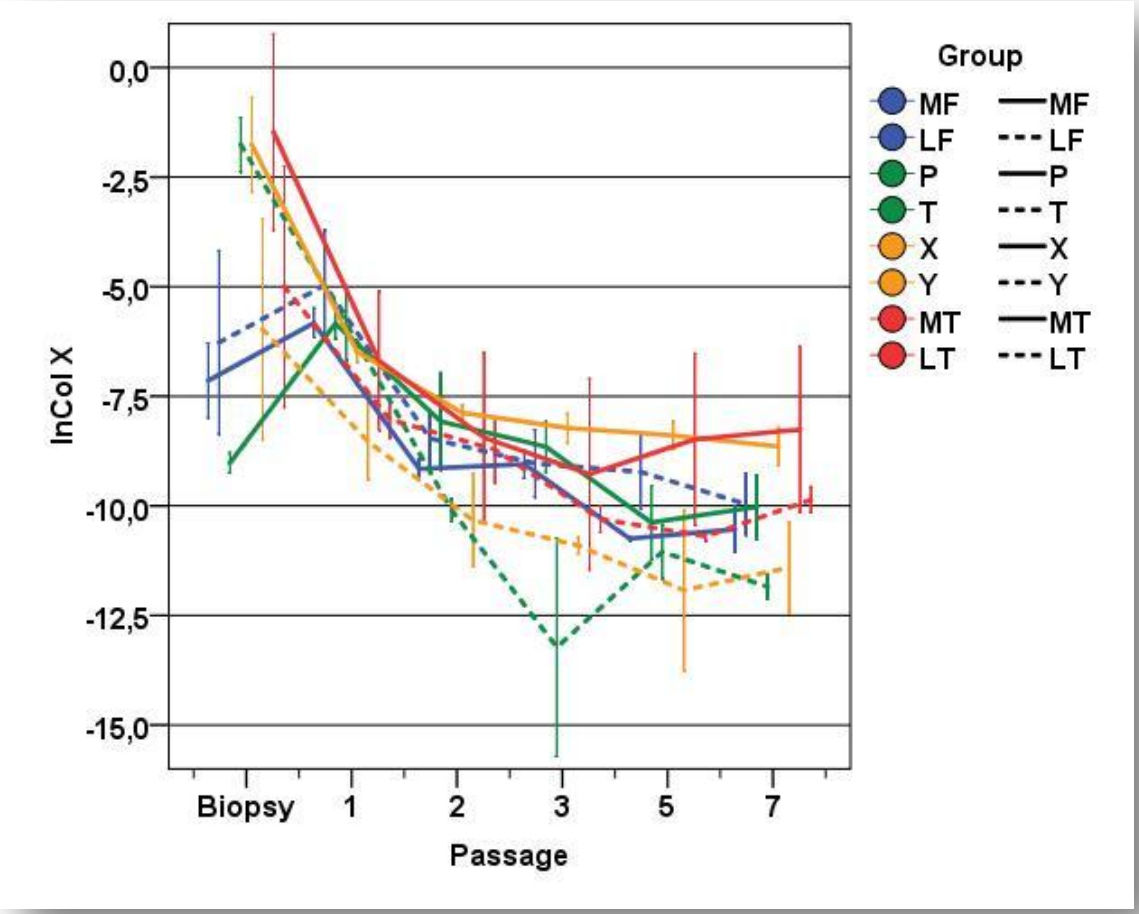
No overall significant group differences were detected for the femoral condyles, the femoro-patella joint groups as well as the lateral tibiaplateau. Despite their same NWB origin, the cells of the femoral notch (X) had an on average significantly different Col X expression level than the cells of the proximo-medial condyle (Y) (p= 0.000). The chondrocytes of the medial tibiaplateau also showed a significant differences compared to Y (p= 0.004). The difference between all groups was significant (p= 0.000).

Table 6: Collagen X Bonferroni Pairwise Comparison from Biopsy to P7

Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	0.243	1.000	1.000	1.000
LF	1.000		1.000	1.000	1.000	0.210	1.000	1.000
P	1.000	1.000		1.000	0.283	1.000	1.000	1.000
T	1.000	1.000	1.000		0.118	1.000	1.000	1.000
X	0.243	1.000	0.283	0.118		0.000	1.000	0.163
Y	1.000	0.210	1.000	1.000	0.000		0.004	1.000
MT	1.000	1.000	1.000	1.000	1.000	0.004		0.513
LT	1.000	1.000	1.000	1.000	0.163	1.000	0.513	

n = 3; p-values < 0.05 considered to be significant;

Graph 8: Collagen X mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)

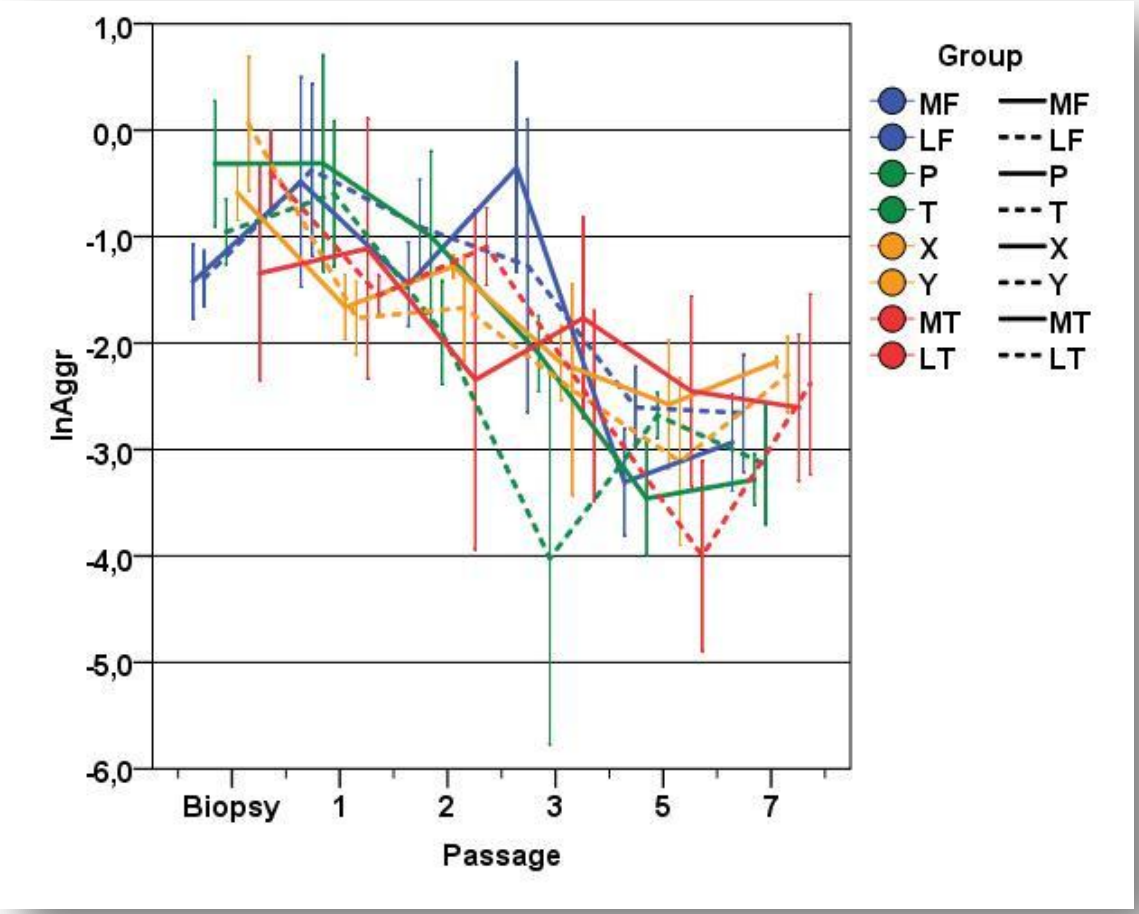


n = 3 per region; statistical results within the text;

4.2.2.2. Aggrecan

Aggrecan expression levels showed no significant difference between the eight groups over the entire cultivation period (p= 0.709). Between the passages themselves, only some groups showed significant differences in gene expression levels. Significant differences were found for both non weight bearing locations X (p= 0.022), Y (p= 0.007) as well as the patella (p= 0.029) and the lateral tibia plateau (p= 0.024). All other groups showed no significant differences between the passages MF (p= 0.064), LF (p= 0.321), T (p= 0.139) and MT (p= 0.744). The average changes were 1.6 fold only, with an overall 6.2 fold drop between the native cartilage biopsies and P7.

Graph 9: Aggrecan mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;

4.2.2.3. COMP

The cells of the tibia plateau showed overall significant differences to all other groups for the COMP gene expression levels ($p < 0.048$), being between 4.3 and 10.4 times lower. From passage 1 throughout P7, an overall significant difference between the eight groups could be detected ($p= 0.000$) as well as between the passages for each subpopulations, MF ($p= 0.000$), LF ($p= 0.001$), P ($p= 0.000$), T ($p= 0.002$), X ($p= 0.000$), Y ($p= 0.000$), MT ($p= 0.002$) and LT ($p= 0.000$).

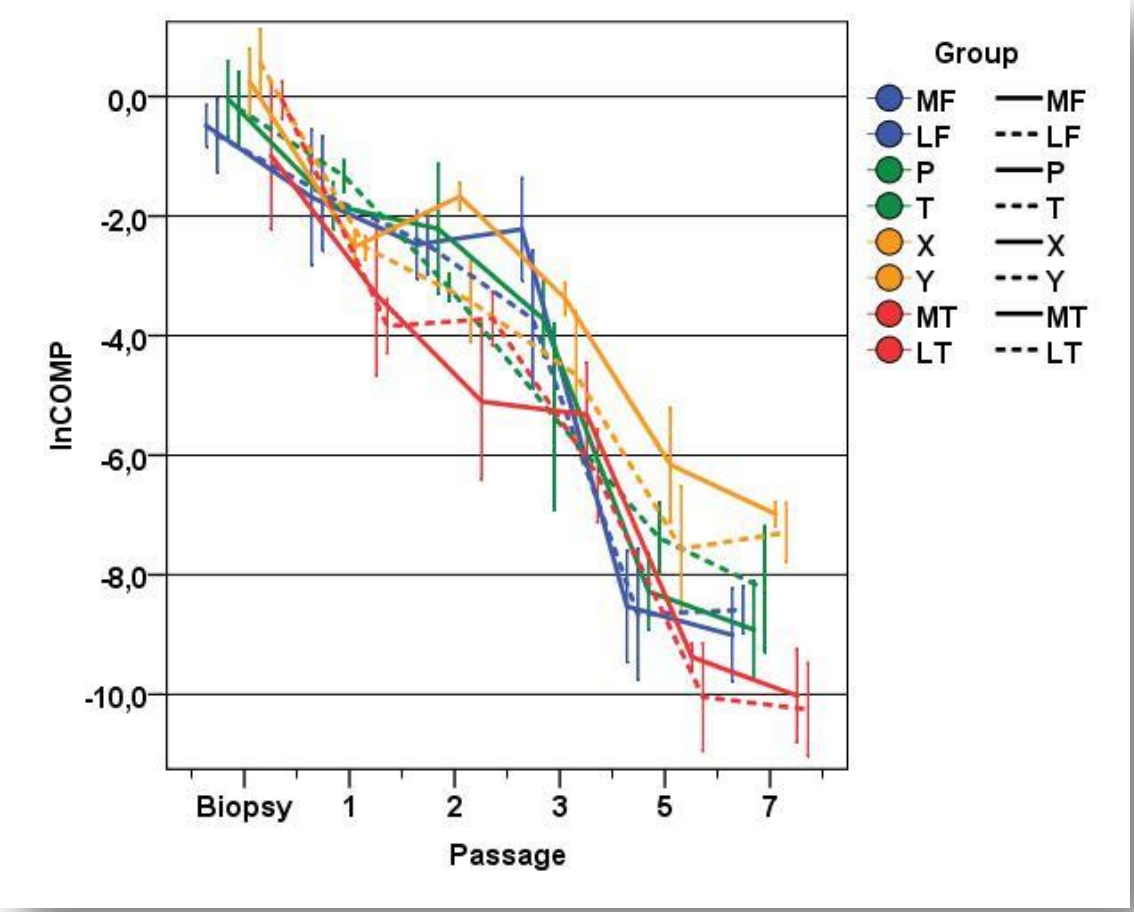
Like in the Col II expression level the most dramatic drop in gene expression was found between the native cartilage biopsies and the first passage (10.4 fold), being most pronounced for the lateral tibia (44 fold), followed by the classical biopsy site cells (X 16 fold, Y 22 fold) and the medial tibia (10 fold). Compared to the native cartilage, P7 cells showed a 5200 times lower gene expression for COMP and overall, the only passages not differing significantly were P5 and P7 ($p= 0.335$).

Table 7: COMP Bonferroni Pairwise Comparison from Biopsy to P7

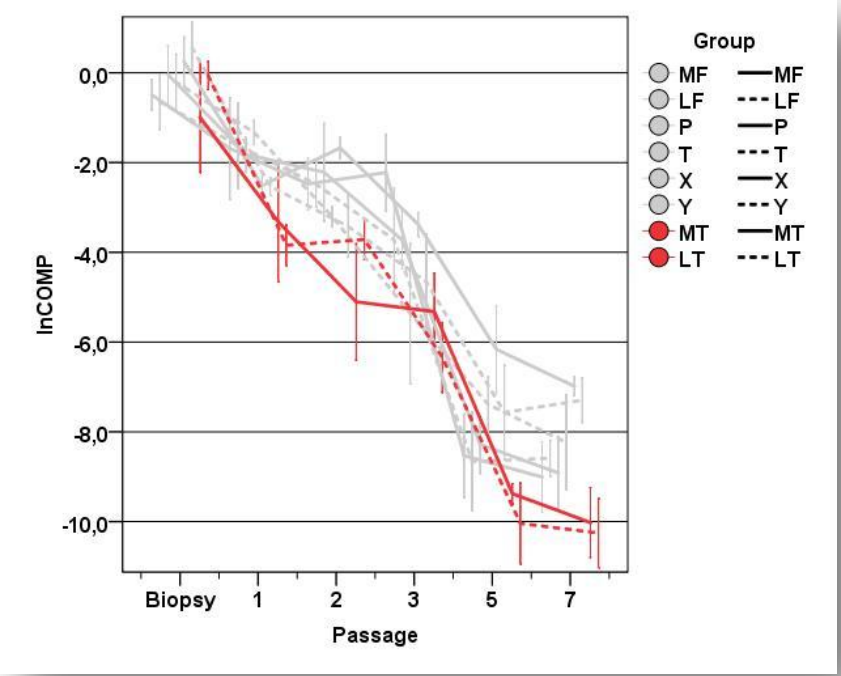
Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	1.000	1.000	0.005	0.000
LF	1.000		1.000	1.000	1.000	1.000	0.047	0.021
P	1.000	1.000		1.000	1.000	1.000	0.021	0.012
T	1.000	1.000	1.000		0.437	1.000	0.044	0.034
X	1.000	1.000	1.000	0.437		1.000	0.000	0.000
Y	1.000	1.000	1.000	1.000	1.000		0.001	0.006
MT	0.005	0.047	0.021	0.044	0.000	0.001		1.000
LT	0.000	0.021	0.012	0.034	0.000	0.006	1.000	

n = 3; p-values < 0.05 considered to be significant;

Graph 10: COMP mRNA Expression Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;

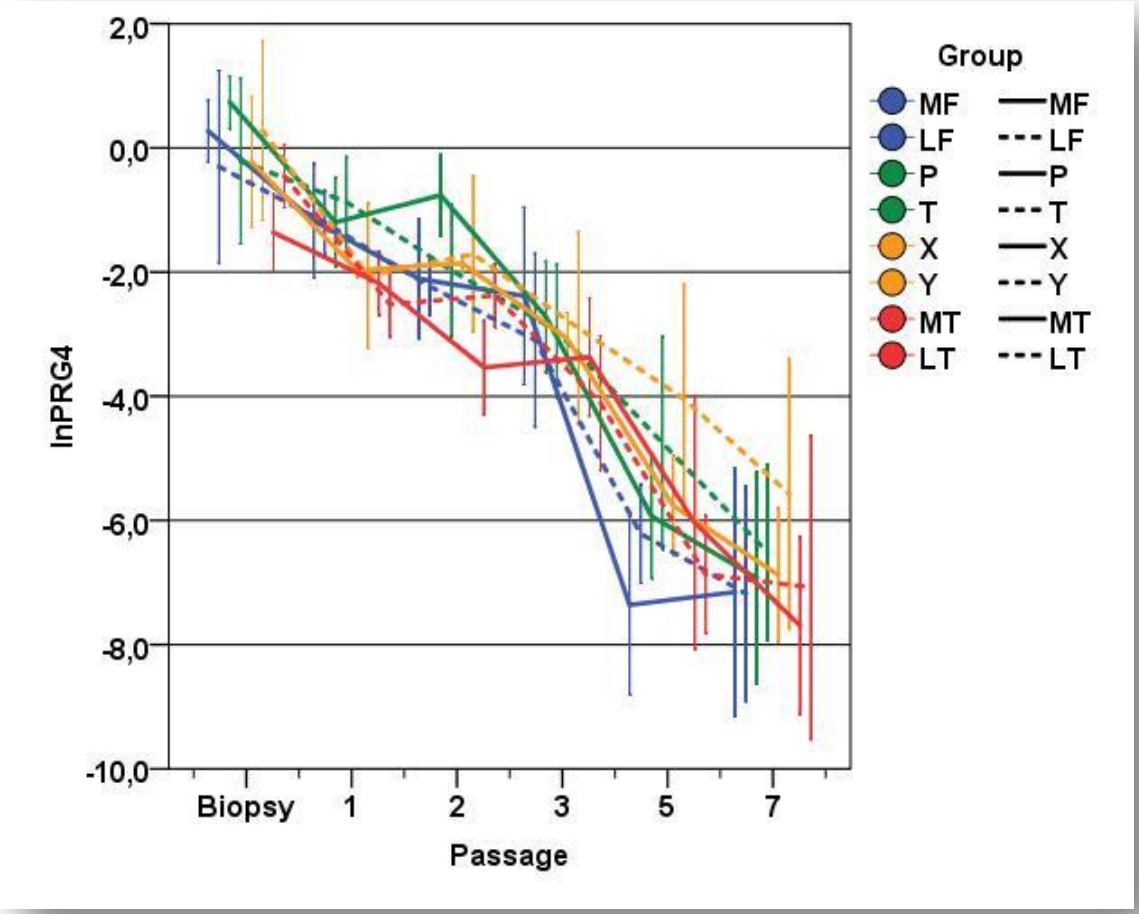


4.2.2.4. PRG-4 / Superficial Zone Protein

The gene expression levels for PRG-4 showed no statistical differences between the eight subpopulations for the overall cultivating period (p> 0.093) but a clear trend for the tibial groups to show the lowest expression levels ranging from 1.8 up to 3.48 fold differences to the other subpopulations was detected. Expression levels showed a steady decline (on average overall 800 fold) with interestingly the most dramatic drop (23.7 fold) between P3 and P5 and not, like detected and expected in most other cartilage specific gene expression levels between native cartilage and P1. For all groups put together the only non significant difference between the passages was between P5 and P7 (p= 0.277). The least decline was detected for the groups Y (347 fold), T (545 fold) and MT (563 fold). Results for PRG-4 showed no obvious grouping depending on the anatomical compartment of origin.

Differences between passages, reflecting the dynamics of gene expression down regulation were also significant for the all subpopulations but the trochlea (p= 0.089). [MF (p= 0.002); LF (p= 0.042), P (p= 0.007), X (p= 0.003), Y (p= 0.042), MT (p= 0.031) and LT (p= 0.035)]

Graph 11: PRG-4 mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



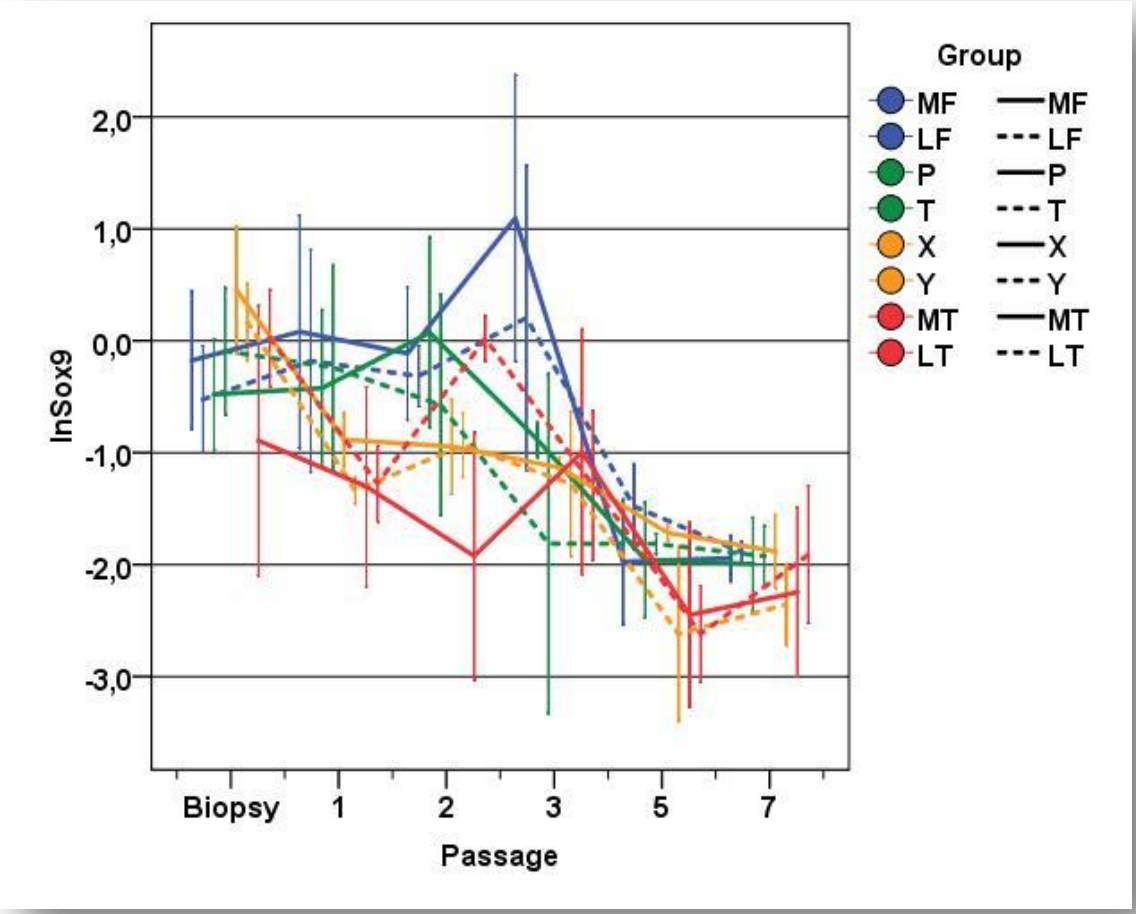
n = 3 per region; statistical results within the text;

4.2.2.5. Sox 9

The Sox 9 expression levels showed no significant differences between all eight subpopulation for the entire time of culturing (p= 0.128). The biggest change in the gene expression level was, like for PRG-4, interestingly also detected between P3 and P5 (3.9 fold). Overall, P7 levels were only 6 times lower when compared to the native cartilage, showing a very modest dynamic compared to other gene expression levels like collagenes or COMP.

Differences between passages were thus not significant for the subpopulations of MF (p= 0.121), LF (p= 0.405), P (p= 0.194), T (p= 0.506) and MT (p= 0.171) but only for LT (p= 0.021) as well as the NWB biopsy sites X (p= 0.022) and Y (p= 0.007) showing again a trend towards anatomical grouping.

Graph 12: Sox9 mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;

4.2.2.6. MMP-1, -3, -13

MMP-1

The MMP-1 expression level didn´t show any drastic changes during the entire cultivation period, thus there was no overall statistically significant deference between passages detectable (p= 0.850). Differences between passages for the subpopulations were thus not significant for all of them. [MF (p= 0.663), LF (p= 0.168), P (p= 0.711), T (p= 0.388), X (p= 0.751), Y (p= 0.780), MT (p= 0.663) and LT (p= 0.869)].

Changes between passages ranged from 0.69 up to 1.2 fold increases or decreases, revealing an overall 1.5 fold, slight increase in the expression between P1 and P7. In the native cartilage biopsies, MMP-1 wasn´t detectable for all groups throughout all experiments. To be more specific, it was merely detectable for experiment number one but not at all for the two following experiments,

although measurements were repeated and new reagents and primers used to rule out methodical mistakes.

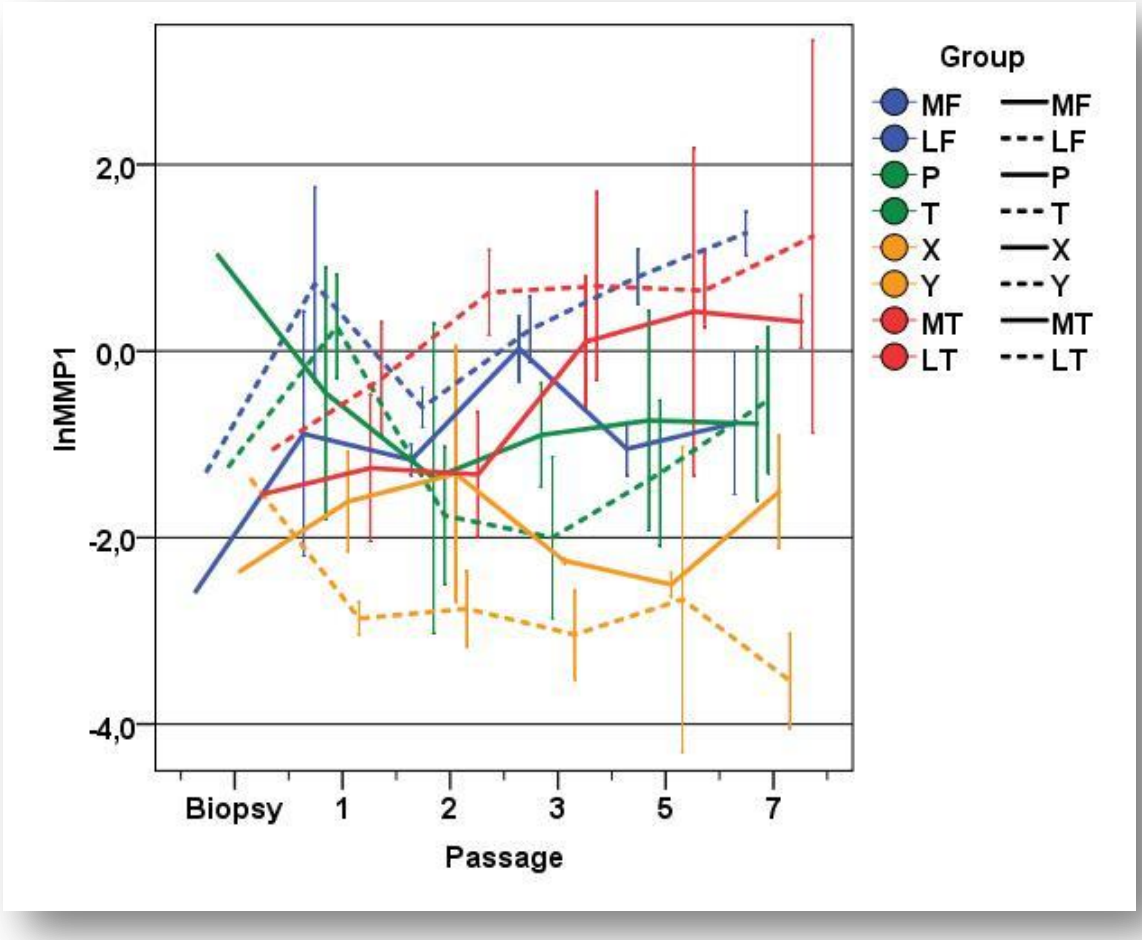
Between the eight subpopulations, significant differences were found (p= 0.000) with up to 29 fold changes (LT compared to Y). The chondrocytes of the NWB proximo-medial condyle (Y) showed a significantly lower MMP-1 expression than all other groups (p< 0.018) excluding X (femoral Notch). The femoral notch NWB biopsy site X itself was only significantly different from the lateral condyle as well as the lateral tibia plateau (p< 0.01) as shown in table 8. Furthermore the medial femoral condyle expression levels differed significantly from the lateral tibial plateau (p= 0.039).

Table 8: MMP-1 Bonferroni Pairwise Comparison from Biopsy to P7

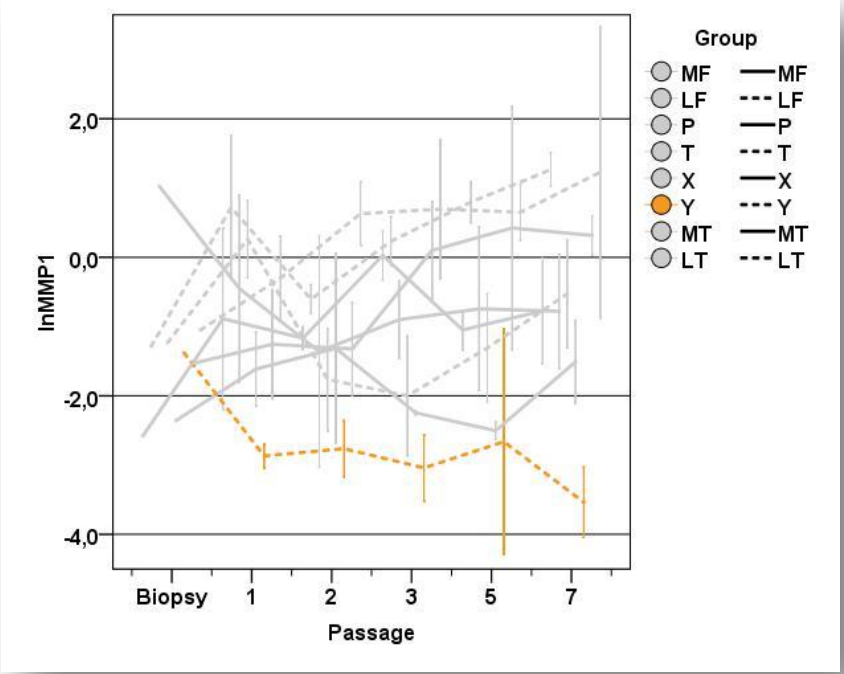
Groups	MF	LF	P	T	X	Y	MT	LT
MF		0.258	1.000	1.000	1.000	0.003	1.000	0.039
LF	0.258		1.000	0.411	0.009	0.000	1.000	1.000
P	1.000	1.000		1.000	1.000	0.002	1.000	0.895
T	1.000	0.411	1.000		1.000	0.017	1.000	0.132
X	1.000	0.009	1.000	1.000		0.617	0.248	0.001
Y	0.003	0.000	0.002	0.017	0.617		0.000	0.000
MT	1.000	1.000	1.000	1.000	0.248	0.000		0.816
LT	0.039	1.000	0.895	0.132	0.001	0.000	0.816	

n = 3; p-values < 0.05 considered significant

Graph 13: MMP-1 mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;



MMP-3

Similar findings were made for the MMP-3 mRNA expression levels. As with MMP-1, the cells of the NWB proximo-medial condyle (Y) showed significant differences ($p < 0.022$) to all other groups including its anatomical partner X, but P, the patella ($p = 0.085$) this time. Thus, like in the MMP-1 mRNA expression levels the NWB proximo-medial condyle were showing again constantly the lowest expression level of all groups (overall 10.6 fold smaller than MF, the group with the highest expression) thus, the statistical difference between overall group was $p = 0.000$.

No other significant differences were detected between the eight subpopulations as shown in table 9 below. Overall a slight decrease in the MMP-3 expression level was detected over the cultivating period compared to the initial, native cartilage expression (5.7 fold). The biggest change in the expression level was detected between P3 and P5 (10.2 fold drop) as for PRG-4 and Sox 9, ranging from a 19.55 fold drop for MF to a 6.2 fold, slighter decrease for T and X. The differences between passages for each subpopulation were thus not statistically significant for P ($p = 0.158$), T ($p = 0.737$), Y ($p = 0.135$), MT ($p = 0.176$) and LT ($p = 0.206$) but the dynamic of the mRNA expression levels between passages was significant for X ($p = 0.014$), MF ($p = 0.026$) and LF ($p = 0.034$).

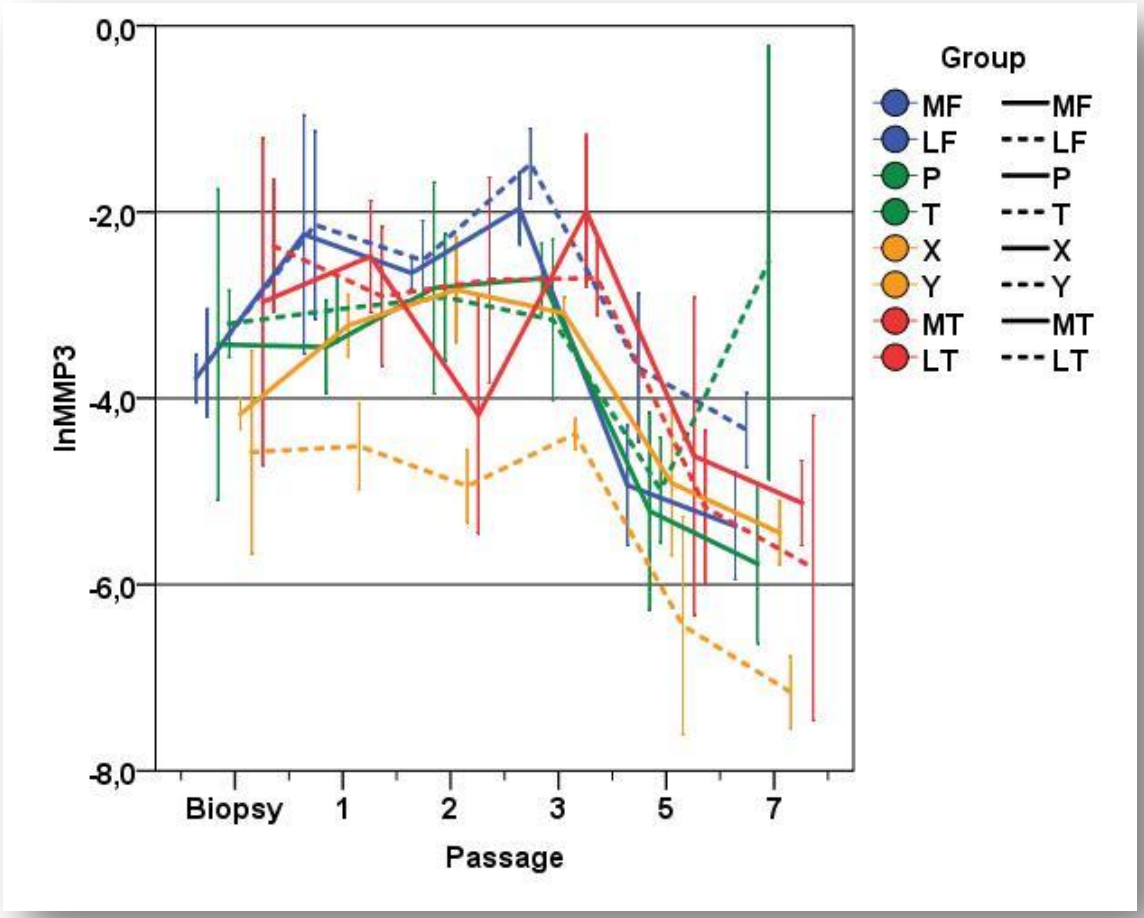
Especially for P1, a clear grouping based on the anatomical compartments was detected, showing lower expression levels from MT/LT over P/T, MF/LF to finally X/Y.

Table 9: MMP-3 Bonferroni Pairwise Comparison from Biopsy to P7

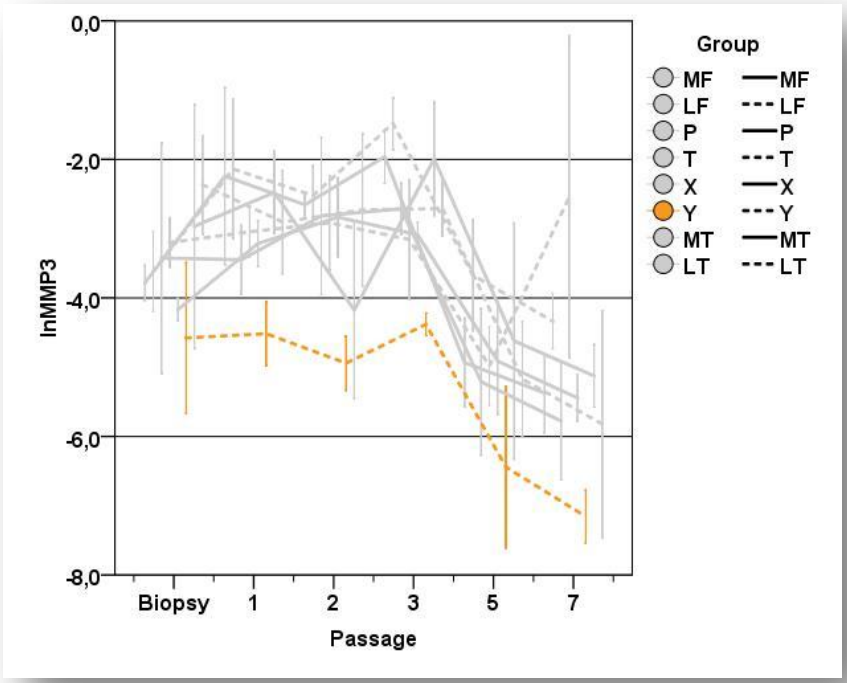
Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	1.000	0.006	1.000	1.000
LF	1.000		0.624	1.000	1.000	0.000	1.000	1.000
P	1.000	0.624		1.000	1.000	0.085	1.000	1.000
T	1.000	1.000	1.000		1.000	0.001	1.000	1.000
X	1.000	1.000	1.000	1.000		0.021	1.000	1.000
Y	0.006	0.000	0.085	0.001	0.021		0.001	0.009
MT	1.00	1.000	1.000	1.000	1.000	0.001		1.000
LT	1.000	1.000	1.000	1.000	1.000	0.009	1.000	

n = 3; p-values < 0.5 considered to be significant;

Graph 14: MMP-3 mRNA Levels from Native Cartilage Biopsy to Passage 7 (P7)



n = 3 per region; statistical results within the text;



MMP-13

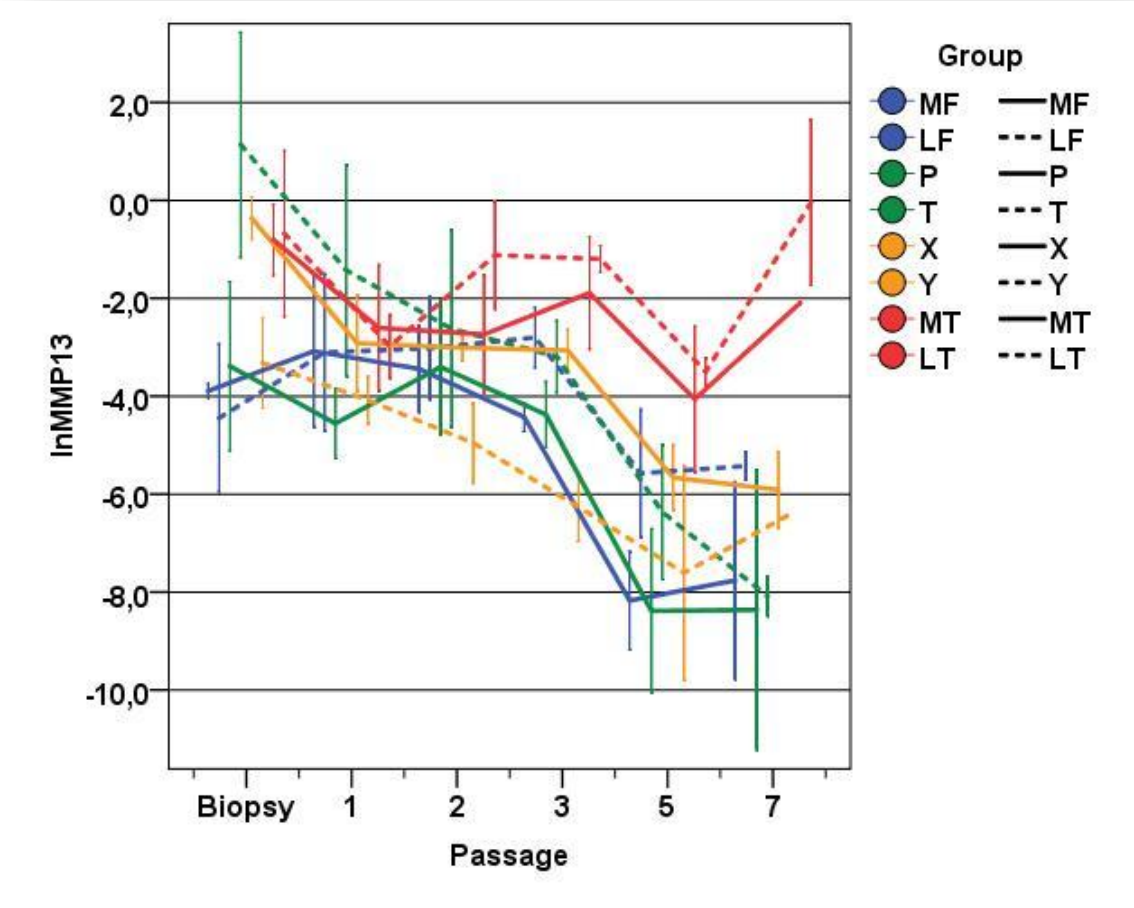
For the MMP-13 mRNA expression levels overall differences were detected between the eight subpopulations only partly in a statistically significant matter. Grouping based on the origin depending on the anatomical compartment was possible for the results of the tibia plateau and the femur condyles. The cells from femoro-patella as well as NWB origin showed significant differences within (p (T/P) =0.005, p (X/Y) =0.007)). The on average 30-fold drop in the MMP-13 expression level displayed clear differences between the subpopulations, showing for example a decrease in a much lesser extent, thus statistically not significant for the groups LF (overall 14 fold, 2.7 fold compared to the native cartilage, p= 0.141) and MT (4 fold, p= 0.298), with LT (p =0.266) even showing an overall slight increase. MF (p= 0.051), P (p= 0.063) and Y (p= 0.195) showed also no significant dynamic in gene expression values. Only two statistically significant difference in expression dynamic were detectable between passages for T (p= 0.045) and X (p= 0.003).The overall biggest drop in the expression level could be detected between P3 and P5 (19.5 fold) again , ranging from a 55-fold (P) to a 5-fold (Y) decrease. The only non significant development or change in gene expression levels for all groups was between P5 and P7 (p= 0.646).

Table 10: MMP-13 Bonferroni Pairwise Comparison from Biopsy to P7

Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	0.770	1.000	1.000	0.017	0.000
LF	1.000		0.296	1.000	1.000	0.770	0.958	0.028
P	1.000	0.296		0.005	0.109	1.000	0.003	0.000
T	0.770	1.000	0.005		1.000	0.023	1.000	0.968
X	1.000	1.000	0.109	1.000		0.007	1.000	0.407
Y	1.000	0.770	1.000	0.023	0.007		0.000	0.000
MT	0.017	0.958	0.003	1.000	1.000	0.000		1.000
LT	0.000	0.028	0.000	0.968	0.407	0.000	1.000	

n = 3; p-values < 0.05 considered to be significant;

Graph 15: MMP-13 mRNA Levels from Native Biopsies to Passage 7 (P7)

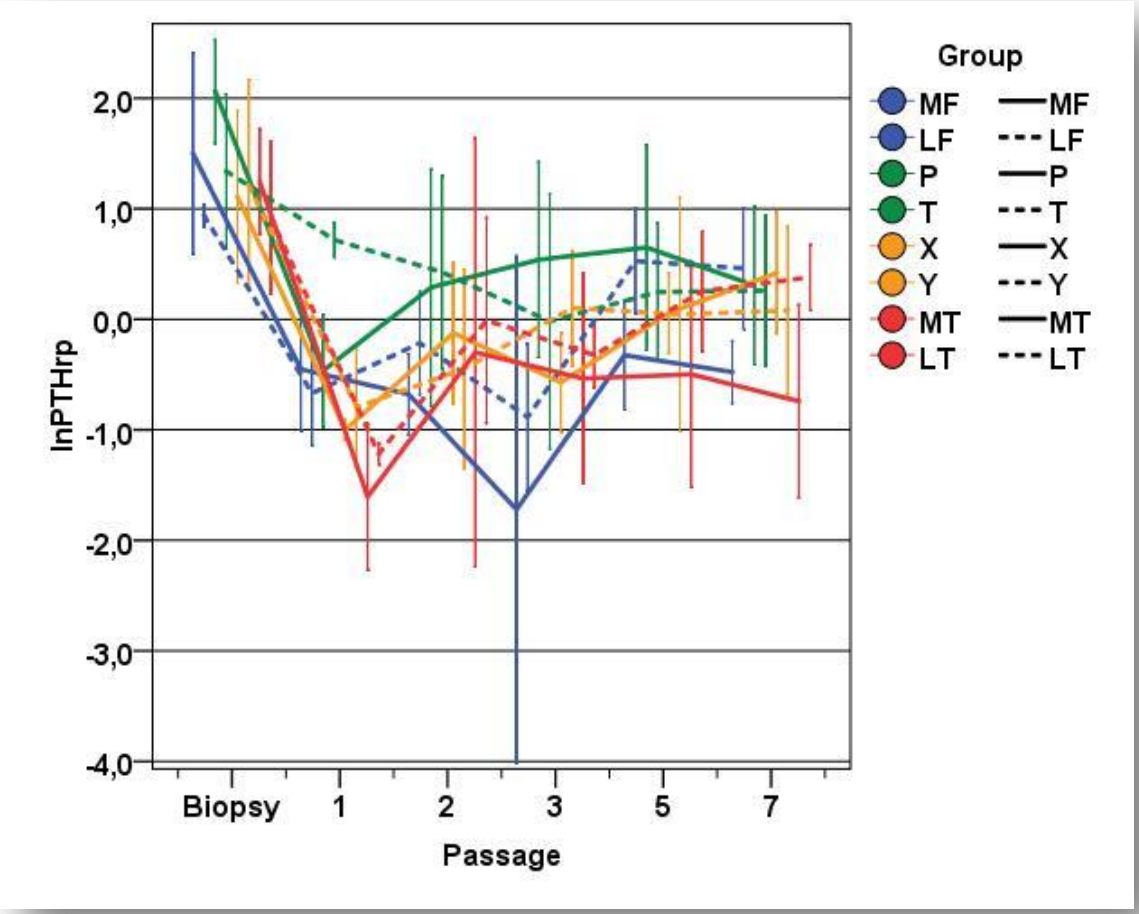


n = 3 per region; statistical results within the text;

4.2.2.7. PTHrp

No overall significant differences between the eight subpopulations were detected for the PTHrp expression (p> 0.473) and an anatomical grouping was not possible. The only dramatic drop in the expression levels were found for all groups between the native cartilage and P1 (6.1 fold on average), while all other passages showed slight de- and increases, with an on average, a 3.15 fold drop in the overall expression level over the entire cultivation period. Since the mRNA expression levels showed only little dynamic, significant differences could only be detected for the patella P (p= 0.002) and the NWB femoral notch X (p= 0.050). All other subpopulations showed no statistically significant differences between the passages. [MF (p= 0.478), LF (p= 0.109), T (p= 0.860), Y (p= 0.147), MT (p= 0.106) and LT (p= 0.258)]

Graph 16: PTHrp mRNA Levels from Native Cartilage Biopsies to Passage 7 (P7)



n = 3 per region; statistical results within the text;

4.3. 3-D Culture

4.3.1. Wet Weight

There was no statistically significant difference between loaded and non-loaded, free swell control groups in concerns of the wet weight of scaffolds at the end of the experiment when they were harvested (p= 0.665).

Loaded scaffolds (n= 9) of each regionally different subpopulation did not reveal any differences in their weights (p= 0.665), although a trend towards a higher wet weight for group T could be observed. Same was true for all regions of the not mechanically loaded, free swell control scaffolds (p= 0.559). Loaded scaffolds weight on average 199.75mg, ranging from 165mg to 250mg with a standard deviation of 17.5mg. Non-loaded scaffolds weight on average 200.03mg, ranging from

156mg to 235mg with a standard deviation of 16.5mg. In a pairwise comparison between each region for loaded and non loaded scaffolds no difference could be detected for all loaded scaffolds. In the bonferroni test the only detectable difference was for the non loaded scaffolds between MF and Y (p= 0.000)

4.3.2. DNA and GAG

When comparing mean overall DNA (Graph 17), mean overall GAG (Graph 18) and GAG/DNA (Graph 19) among all scaffolds that were cultured under static conditions with constructs that were cultured within the bioreactor (group No Load vs. group Load), the difference was significant for all parameters. Total mean DNA content was higher for the loaded groups (p= 0.047) as well as average GAG production (p< 0.001), thus the ratio of GAG/DNA (p< 0.001) was significantly higher for the bioreactor groups as well.

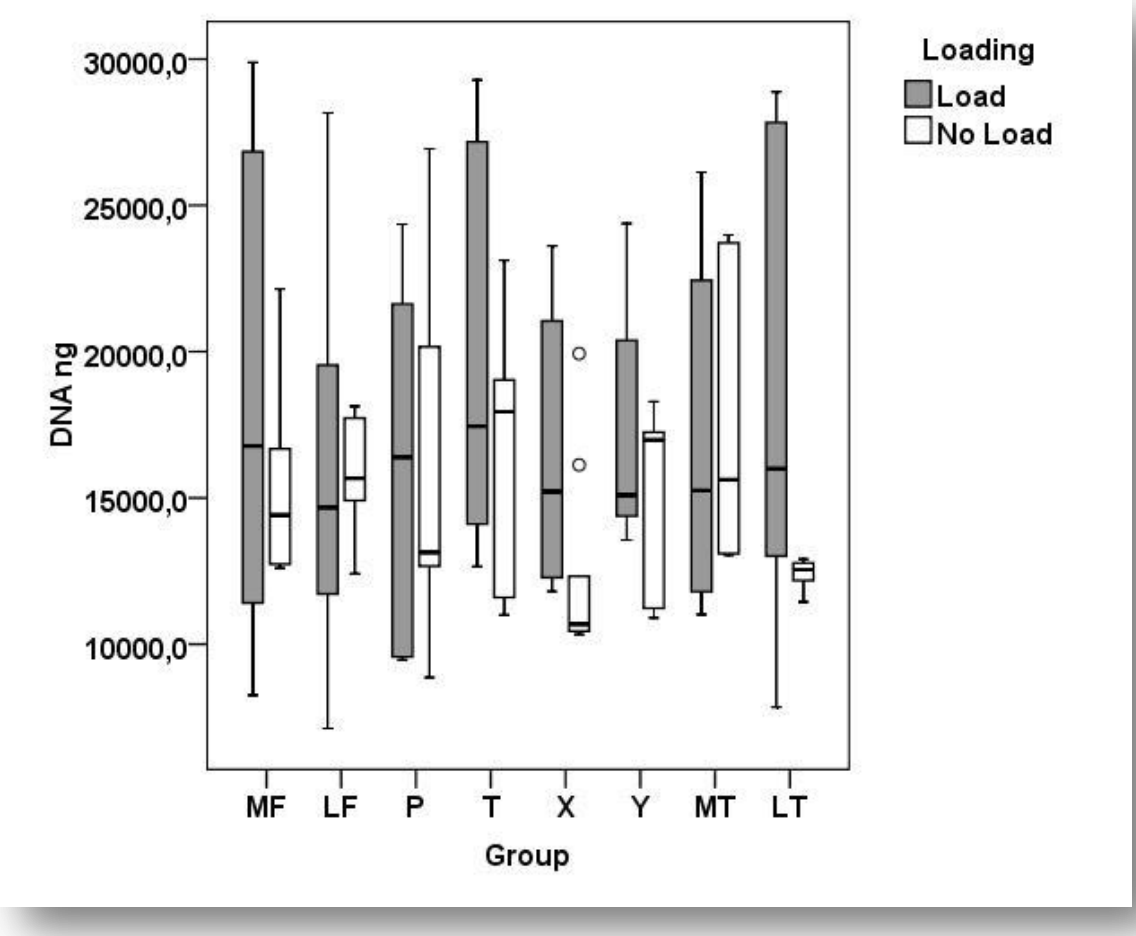
Mean overall DNA content was significantly different between the regional subpopulations that were cultured under static conditions (group No Load) (p= 0.012), while this differences were not significant between regions that were cultured within the bioreactor (group Load) (p= 0.958). The mean overall GAG production (content per scaffold plus released amount into the culture medium) among statically cultured constructs was on average 0.09 ± 0.03mg/construct, while it was on average 0.43 ± 0.11mg/construct among dynamically cultured scaffolds. There was no significant (p= 0.095) difference between the subpopulations in the mean overall GAG production among constructs that were cultured under static conditions, while mean overall GAG production among the distinct regions subjected to load was significantly different (p= 0.036) for the distinct regions, especially between the patella region and the NWB proximo-medial femoral condyle (p= 0.016). This significant difference was not based on varying GAG content in the scaffolds itself (p= 0.106) but on the distinct amounts released into the medium (p= 0.042). For the static control scaffolds this relationship was vice versa. The released amount of GAG in the medium was not differing (p= 0.375) while the content of the scaffolds was significantly varying between the regions (p= 0.000). When pairwise compared between load and no load for each varying region scaffolds of all locations showed significant differences in the GAG content but MF (p= 0.153) and Y (p= 0.062). [LF (p= 0.032), P (p= 0.000), T (p= 0.031), X (p= 0.004), MT (p= 0.000). When the released amount into the medium was compared for each location (load vs. no load), all groups showed a significant difference (p= 0.000). The overall GAG amount was also significantly different for all subpopulations when load vs. no load was compared (p= 0.000).

Between static, free swell control samples there was no significant difference for GAG/DNA ratio when comparing the regional subpopulations (p= 0.103), although the difference for the DNA content had been statistically significant as described above. There were significant differences when

calculating particular region to region differences. Highest GAG/DNA was found at weight-bearing medial femoral condyle. The lowest values were at weight-bearing medial tibia plateau (MT). The only statistically significant difference was detected between MT and the NWB femoral notch (X) in the bonferroni pairwise comparison (p= 0.008).

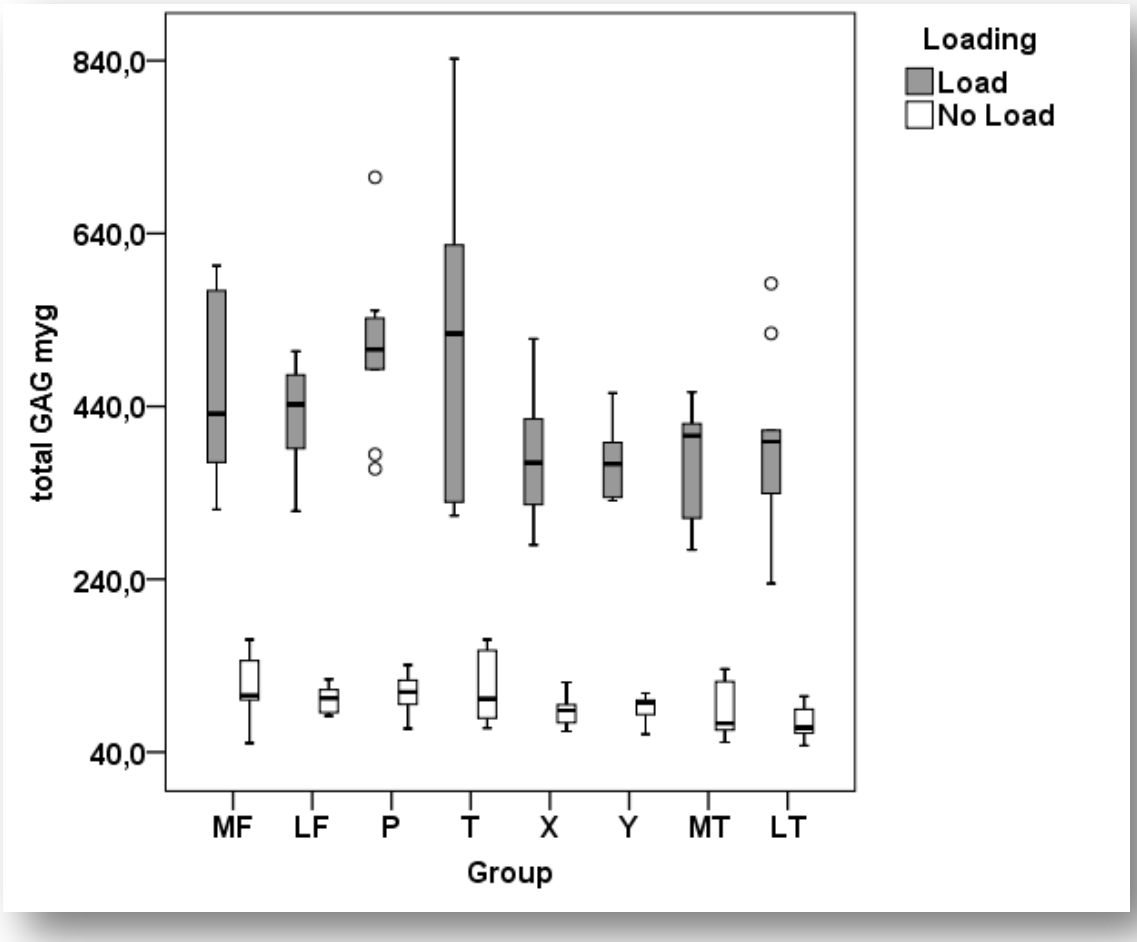
Among the loaded constructs that were cultured within the bioreactor there was as well no significant difference when comparing all regions for GAG/DNA (p= 0.43), although the GAG content has been significant different between the varying regions as shown above. The highest GAG/DNA value was found at the patello-femoral joint. The lowest values were found at both biopsy regions which had one fourth of GAG/DNA when compared to the patella region.

Graph 17: DNA Levels in ng of Loaded and Control Scaffolds



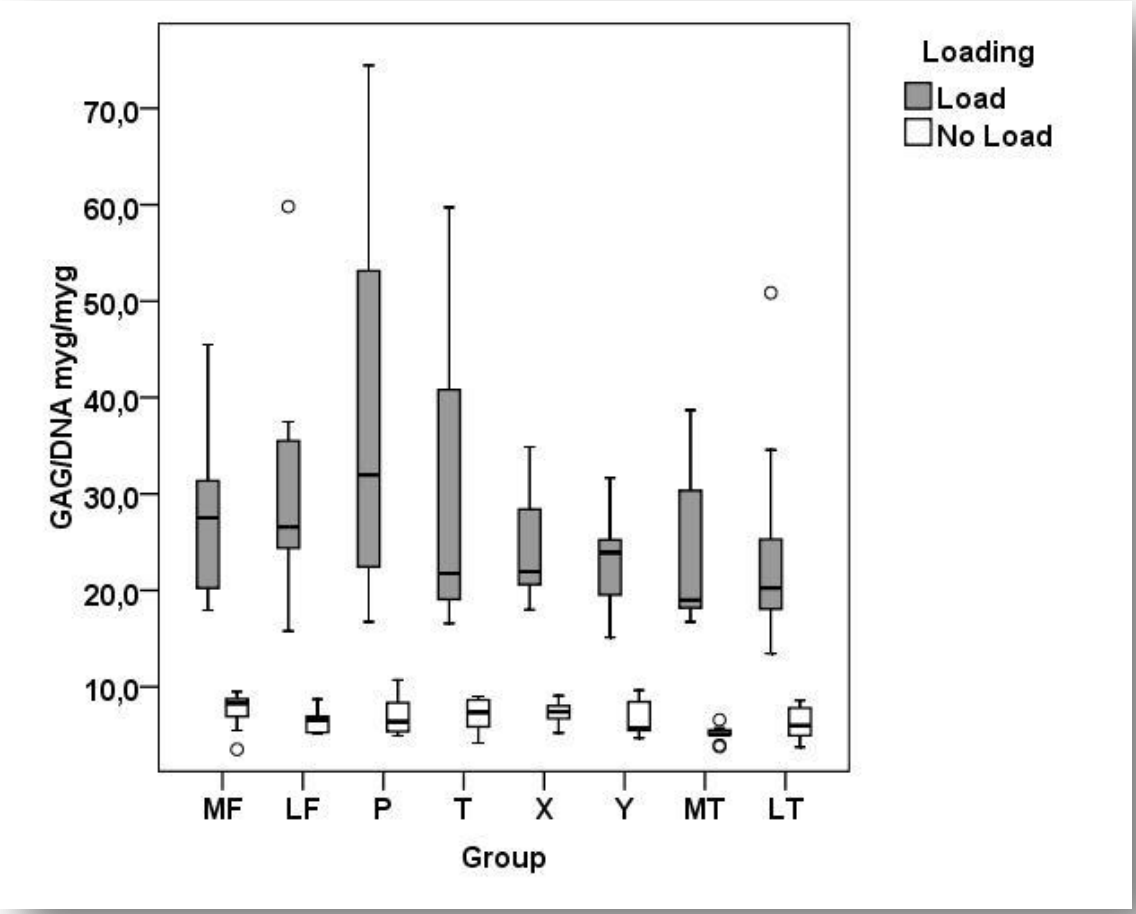
n = 6 per region; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text; The No Load group LT was significantly different from LF (p = 0.008)

Graph 18: Total Amount of GAG (scaffold plus medium) in µg for Loaded and Control Scaffolds



n = 6 per region; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

Graph 19: GAG per DNA in µg for Loaded and Control Scaffolds



n = 6 per region; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.3. PCR

The mRNA gene expression levels detected were significantly different between the topographically varying regions for all analyzed parameters except for the expression of Aggrecan and PTHrp for loaded constructs as well as the static control scaffolds. No distinct variations were also found for PRG-4 for the static control constructs as well as for Sox9 for the mechanically loaded scaffolds. When expression levels were compared between load and no load in general, all mRNA expression levels seemed to respond significantly to mechanical stress but Collagen I, X, MMP-1 and -3, although trends could be detected here. Thus, this comparison was significant for the remaining genes Collagen II, Aggrecan, COMP, Sox9, PRG-4, PTHrp and MMP-13. Among those, the expression of genes was constantly higher in response to the mechanical load except for the expression of PTHrp

and MMP-13, which was more strongly expressed among static controls and seemed to be down regulated in response to mechanical stress.

In the static, free swell 3-D culture Collagen I, MMP-1 and PTHrp were upregulated, while expression of Collagen II and X, Aggrecan, Sox9, COMP and MMP-3 were down-regulated when compared to freshly isolated chondrocytes, P0. Levels for PRG-4 and MMP-13 showed no big differences when compared to P0 cells.

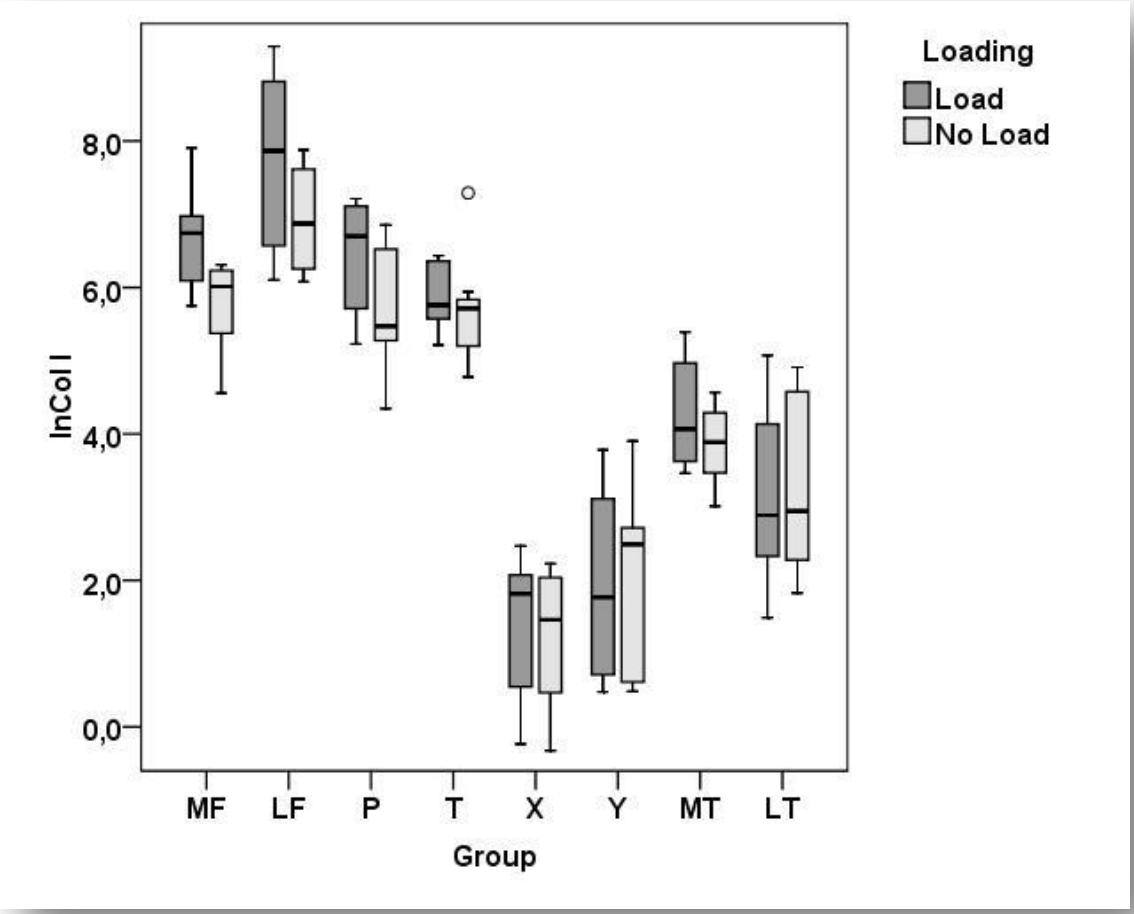
In the mechanically stressed 3-D cultures Collagen I, PRG-4, MMP-1 were upregulated while expression for Collagen II and X, COMP, Sox9, and MMP-3 were downregulated when compared to freshly isolated chondrocytes, P0. Aggrecan, MMP-13 and PTHrp displayed more or less the same values as P0 cell.

4.3.3.1. Collagens

Collagen I

Collagen I showed no difference between overall loaded and non-loaded, static groups (p= 0.253). A clear anatomical grouping and significant differences between the topographically distinct regions for each group, mechanically loaded and static control was possible as seen below in graph 20 (p= 0.000 for both load and no load). The groups of the femoral condyle as well as patello-femoral region showed higher collagen I mRNA expressions than the NWB regions or the tibia plateau. In the weight-bearing femoral groups there was no significant difference for the loaded groups but the trochlea from the femoral condyles, respectively the trochlea from the lateral femoral condyle in the non loaded scaffolds as shown in the table below. The weight bearing femoral groups were all significantly different from the NWB groups as well as the tibial groups, in loaded and control groups. Collagen I expression was thus clearly lowest among both biopsy regions, with approximately 680 times lower levels in real numbers in mRNA levels for the non weight-bearing femoral notch when compared to weight-bearing lateral femoral condyle among the loaded constructs.

Graph 20: Collagen I mRNA Levels for Loaded and Control Scaffolds



n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

Table 11: Collagen I Bonferroni Pairwise Comparison of Groups for Loaded and Control Scaffolds

	Load							
Groups	MF	LF	P	T	X	Y	MT	LT
MF		0.608	0.933	0.013	0.000	0.000	0.000	0.000
LF	0.608		0.535	0.036	0.000	0.000	0.000	0.000
P	0.933	0.535		0.841	0.000	0.000	0.004	0.000
T	0.013	0.036	0.841		0.000	0.000	0.002	0.000
X	0.000	0.000	0.000	0.000		0.933	0.000	0.185
Y	0.000	0.000	0.000	0.000	0.933		0.037	0.841
MT	0.000	0.000	0.004	0.002	0.000	0.037		0.608
LT	0.000	0.000	0.000	0.000	0.185	0.841	0.608	
	No Load							
Groups	MF	LF	P	T	X	Y	MT	LT
MF		0.058	1.000	1.000	0.000	0.000	0.000	0.001
LF	0.058		0.129	0.009	0.000	0.000	0.000	0.000

P	1.000	0.129		1.000	0.000	0.000	0.001	0.003
T	1.000	0.009	1.000		0.000	0.000	0.000	0.000
X	0.000	0.000	0.000	0.000		1.000	0.000	0.038
Y	0.000	0.000	0.000	0.000	1.000		0.038	0.547
MT	0.000	0.000	0.001	0.000	0.000	0.038		1.000
LT	0.001	0.000	0.003	0.000	0.038	0.547	1.000	

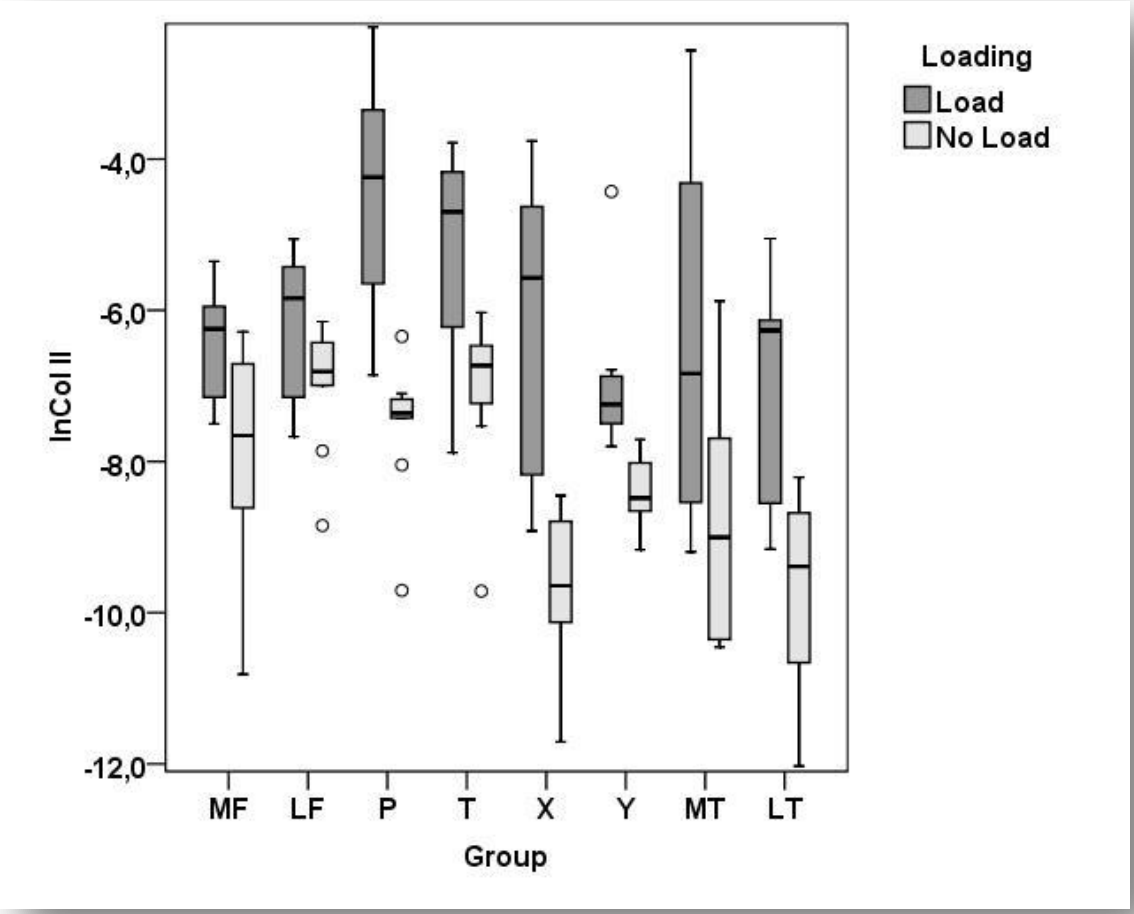
n = 9 per region; p-values of < 0.05 considered to be significant;

Collagen II

Collagen II showed a significant difference between overall loaded and non-loaded, static groups (p= 0.000). A clear anatomical grouping and significant differences between the topographically distinct regions for each group, mechanically loaded and static control was possible as seen below in graph 21 (p= 0.024 for loaded groups and p= 0.000 for static groups). The only significant difference between regional subpopulations for the loaded scaffolds could be found between the patella and the NWB proximo-medial femoral condyle (p= 0.016) when pair wise compared. Lowest mRNA values were found for the weight-bearing lateral tibia plateau group, similar to the non weight-bearing proximo-medial femoral condyle, being approximately 14 times lower compared with the highest values at weight-bearing patella followed by the weight-bearing trochlea femoris group.

For the static free swell scaffolds the pair wise comparison is shown in the table below. The anatomic grouping was more obvious than in the loaded constructs and more significant differences could be found when subpopulations were compared with each other. MF and MT were the only groups showing no significant difference to other groups, due to the wide range of expression levels between the different sample of the subpopulation. The lateral femoral condyle samples as well as the patellar-femoral joint scaffolds were mostly significantly distinct from the NWB groups as well as the lateral tibia plateau (see table below). The NWB areas displayed a significant difference to the femoral condyles and partly to the patella- and trochlea-group but not compared to the tibia plateau. The weakest Collagen II expression was found in the non weight-bearing femoral notch and similar in the weight-bearing lateral tibia plateau, while the highest expression could be detected in trochlea femoris group, followed by the patella and weight-bearing lateral femoral condyle.

Graph 21: Collagen II mRNA Levels for Loaded and Control Scaffolds



n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

Table 12: Collagen II Bonferroni Pairwise Comparison between Control Scaffolds

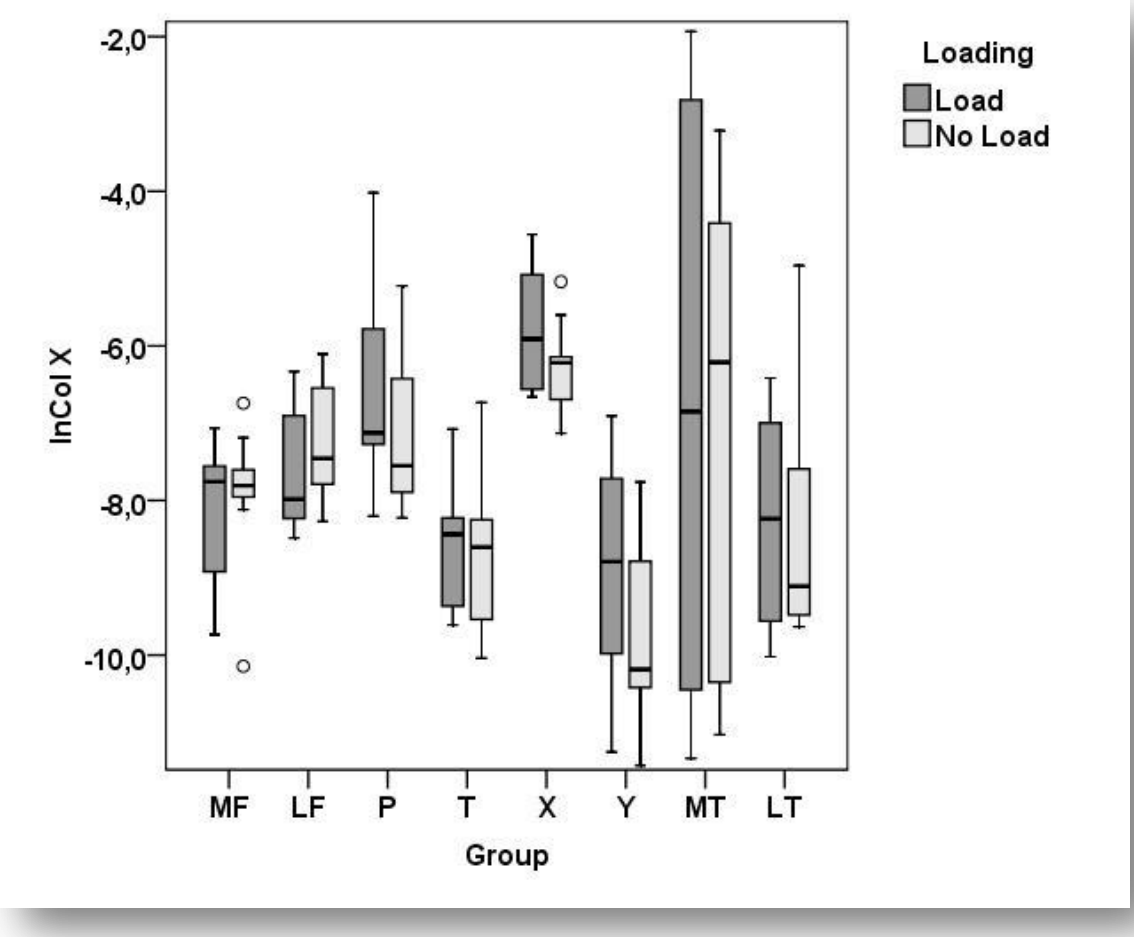
	No Load							
Groups	MF	LF	P	T	X	Y	MT	LT
MF		1.000	1.000	1.000	0.211	1.000	1.000	0.961
LF	1.000		1.000	1.000	0.000	0.003	1.000	0.024
P	1.000	1.000		1.000	0.001	0.180	1.000	0.160
T	1.000	1.000	1.000		0.000	0.009	1.000	0.030
X	0.211	0.000	0.001	0.000		0.051	1.000	1.000
Y	1.000	0.003	0.180	0.009	0.051		1.000	1.000
MT	1.000	1.000	1.000	1.000	1.000	1.000		1.000
LT	0.961	0.024	0.160	0.030	1.000	1.000	1.000	

n = 9 for each region; p-values < 0.05 considered to be significant;

Collagen X

Collagen X showed no difference between overall loaded and non-loaded, static groups (p= 0.680). A clear anatomical grouping was not as easily observed as for collagen I and II. Significant differences between the topographically distinct regions for each group, mechanically loaded and static control were found as seen below in Graph (p= 0.000 for both load and no load). In mechanically induced constructs the only strong distinction was between the femoral notch biopsy site X and the femoral condyles (p< 0.038), the trochlea (p= 0.000) and the NWB proximo-medial femoral condyle when pair wise compared (p= 0.009). Almost the same applied for the static control scaffolds. The femoral notch group X was expressing higher levels of collagen X mRNA significantly when compared to MF (p= 0.009), T (p= 0.000) and the anatomical NWB partner Y (p= 0.001).

Graph 22: Collagen X mRNA Levels for Loaded and Control Scaffolds

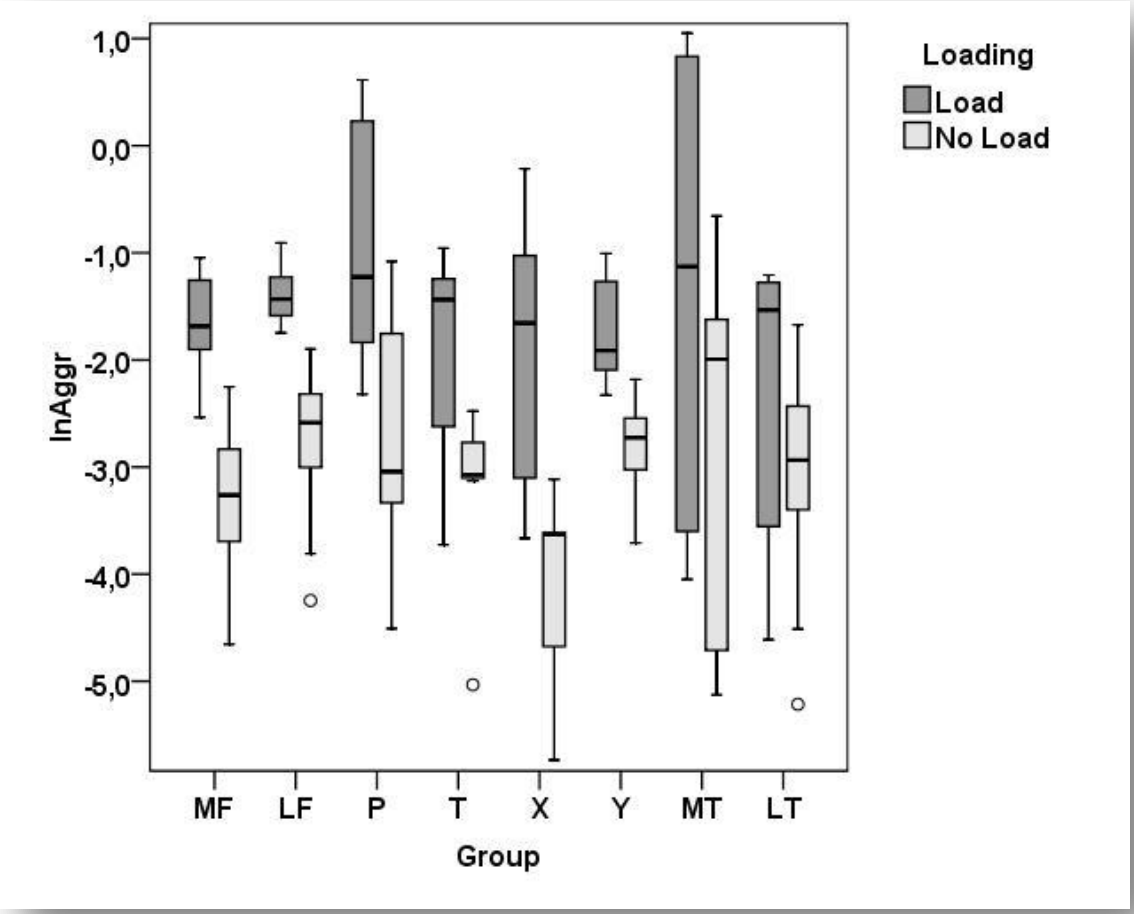


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.3.2. Aggrecan

Aggrecan showed a significant difference between overall loaded and non-loaded, static groups (p= 0.000) as shown in Graph . A clear anatomical grouping could not be observed and there were no significant differences between the topographically distinct regions for the mechanically loaded scaffolds (p= 0.103), also when pair wise compared. The highest mRNA levels for Aggrecan were reached by the group of the medial tibia plateau, showing an upregulation together with the patella subpopulation that was even slightly higher than the reference P0 cells due to the mechanical induction. The same findings applied for the static control scaffolds. There were no regional differences detectable (p= 0.190) and the pair wise comparison was not statistically significant for any subpopulations. The trend towards the least down-regulation of the expression levels for Aggrecan was as for the loaded scaffolds seen for MT and P, whereas the NWB group of the femoral notch showed the most severe down-regulation.

Graph 23: Aggrecan mRNA Levels for Loaded and Control Scaffolds

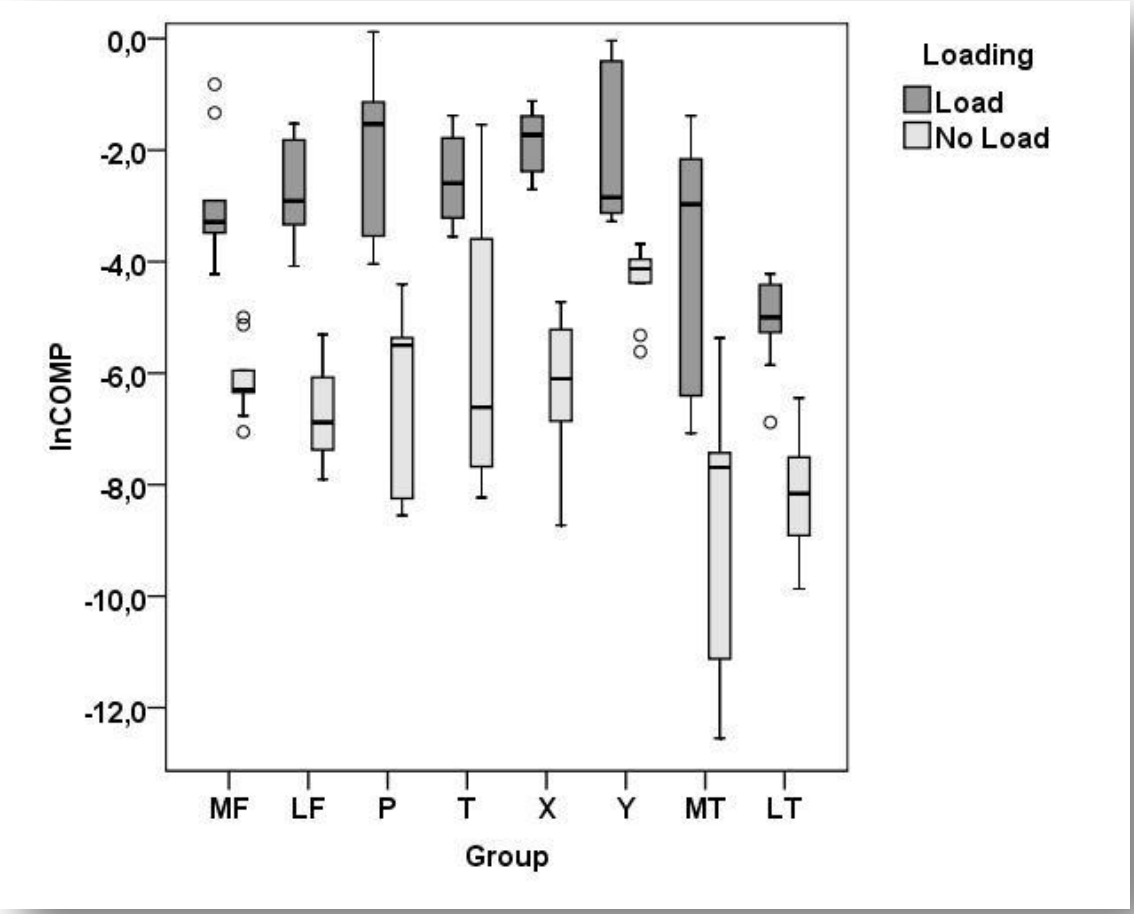


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.3.3. COMP

COMP showed a significant difference between overall loaded and non-loaded, static groups (p= 0.000). A clear anatomical grouping could not be observed but significant differences between the topographically distinct regions for each group, mechanically loaded and static control were possible to detect as seen below in Graph (p= 0.000 for both load and no load). The group of the lateral tibia plateau had a measurable lower mRNA expression level in loaded scaffolds than all other groups (p< 0.009) but the anatomical partner MT when pairwise compared. The results for the static scaffolds differed from the loaded group findings. Interestingly the NWB proximo-medial femoral condyle Y showed significantly higher expression levels than the weight-bearing groups of the femoral condyles as well as the tibia (MF, LF, MT, LT p< 0.013). The other statistically significant difference revealed was between MF and LT with LT showing the overall lowest expression levels of all groups (p= 0.001).

Graph 24: COMP mRNA Levels for Loaded and Control Scaffolds

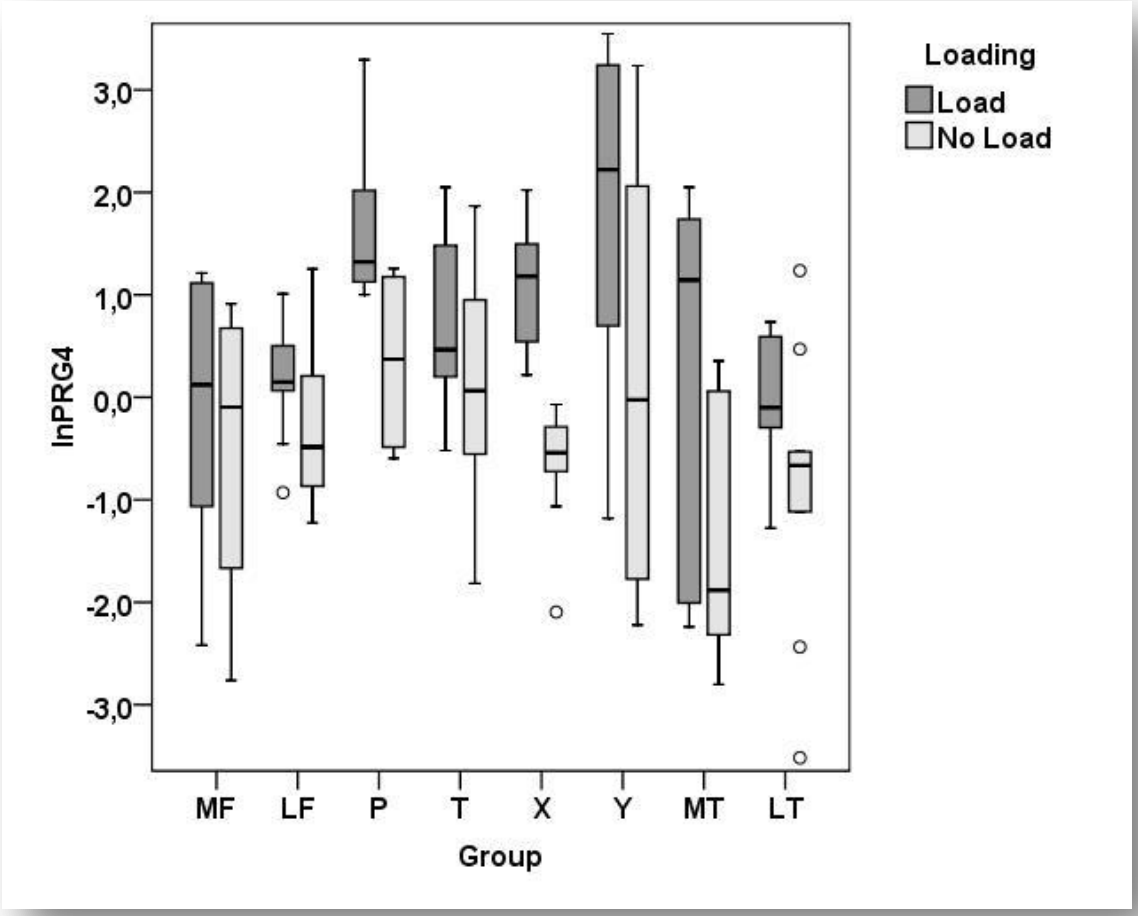


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.3.4. PRG-4

PRG-4 showed a significant difference between overall loaded and non-loaded, static groups (p= 0.000). A clear anatomical grouping could not be observed but significant differences between the topographically distinct regions for each mechanically loaded subpopulation were found as shown in Graph *11* 25 (p= 0.000). In the pair wise comparison distinct differences were found for the two lateral weight bearing areas of the condyle and the tibia compared to the patella (LF p= 0.029; LT p= 0.002) as well as between the femoral notch X and the lateral tibia plateau (p= 0.009). In the static control group no differences were found between the regions (p= 0.062). Interestingly all loaded groups showed a clear upregulation due to the mechanical induction when compared to the freshly isolated P0 cells whereas the static free swell scaffolds seemed to oscillate around the same levels as the P0 cells. The cells of the NWB femoral notch X showed the biggest difference between static and loaded culture with a two times higher mRNA expression level.

Graph 25: PRG-4 mRNA Levels for Loaded and Control Scaffolds

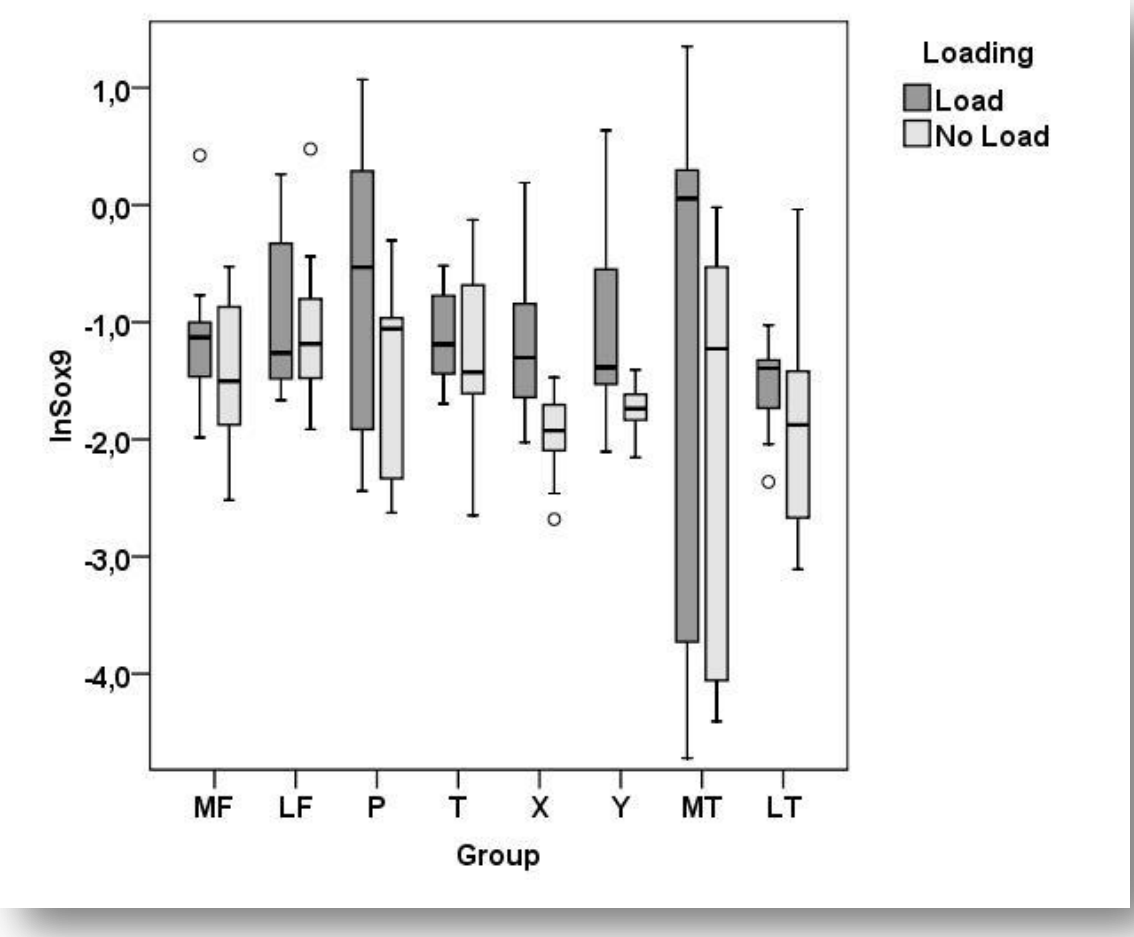


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.3.5. Sox 9

Sox9 mRNA expression levels showed a significant difference between overall loaded and non-loaded, static groups (p= 0.001) as seen below in Graph 26. For the mechanically loaded scaffolds a clear anatomical grouping could not be observed and significant differences between the topographically distinct regions were not found (p= 0.379), also when pairwise compared. In the static control a trend towards an anatomical grouping was detected and significant differences were found between the topographically distinct regions (p= 0.000). As displayed in the graph below, the NWB biopsy sites showed a greater down regulation when compared to P0 cells which was significant when compared between the femoral notch X with LF (p= 0.011) and the T (p= 0.000) and the proximo-medial femur condyle Y and T (p= 0.000).

Graph 26: SOX9 mRNA Levels for Loaded and Control Scaffolds



n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

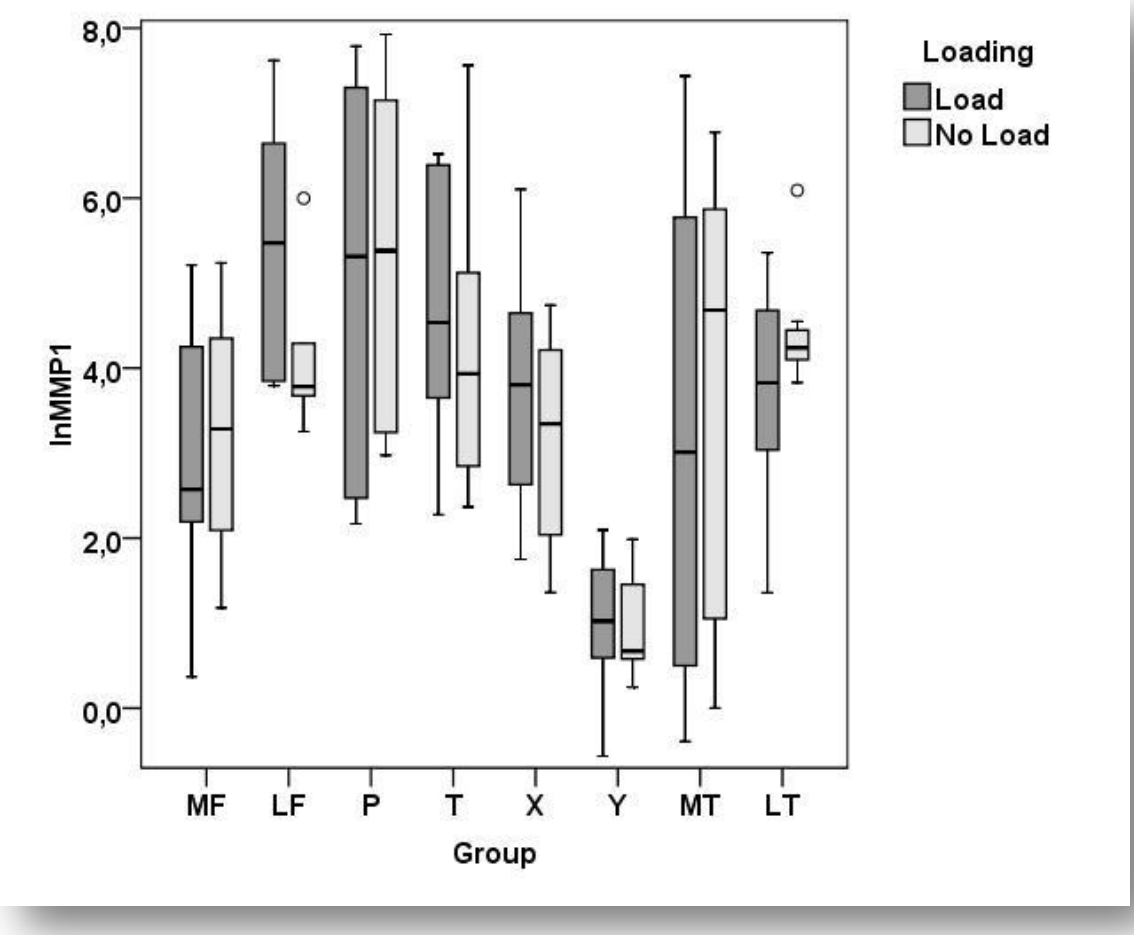
4.3.3.6. MMP-1, -3, -13

All MMPs showed a significant difference for all topographically distinct regions in loaded as well as non-loaded, static groups ($p < 0.005$). Besides that, only MMP-13 mRNA expression levels were significantly different when the loaded groups were compared with the non-loaded scaffolds ($p = 0.000$) while MMP-1 and -3 showed similar levels of expression apparently not inducible by mechanical load (MMP-1 $p = 0.865$; MMP-3 $p = 0.281$).

MMP-1

MMP-1 mRNA levels showed an overall upregulation when compared to the freshly isolated P0 cells. A clear anatomical grouping could not be observed but significant differences between the topographically distinct regions for each group, mechanically loaded and static control were possible to detect as seen below in Graph 27 ($p = 0.000$ for loaded and non-loaded scaffolds). The least upregulation in MMP-1 mRNA levels was found for the subpopulation of the NWB proximo-medial femoral condyle Y which was significantly distinct from the lateral tibia plateau LT and the lateral weight bearing condyle LF for load and control ($p = 0.000$ for loaded and non-loaded scaffolds) as well as the trochlea for the loaded scaffolds ($p = 0.001$) when pairwise compared.

Graph 27: MMP-1 mRNA Levels for Loaded and Control Scaffolds

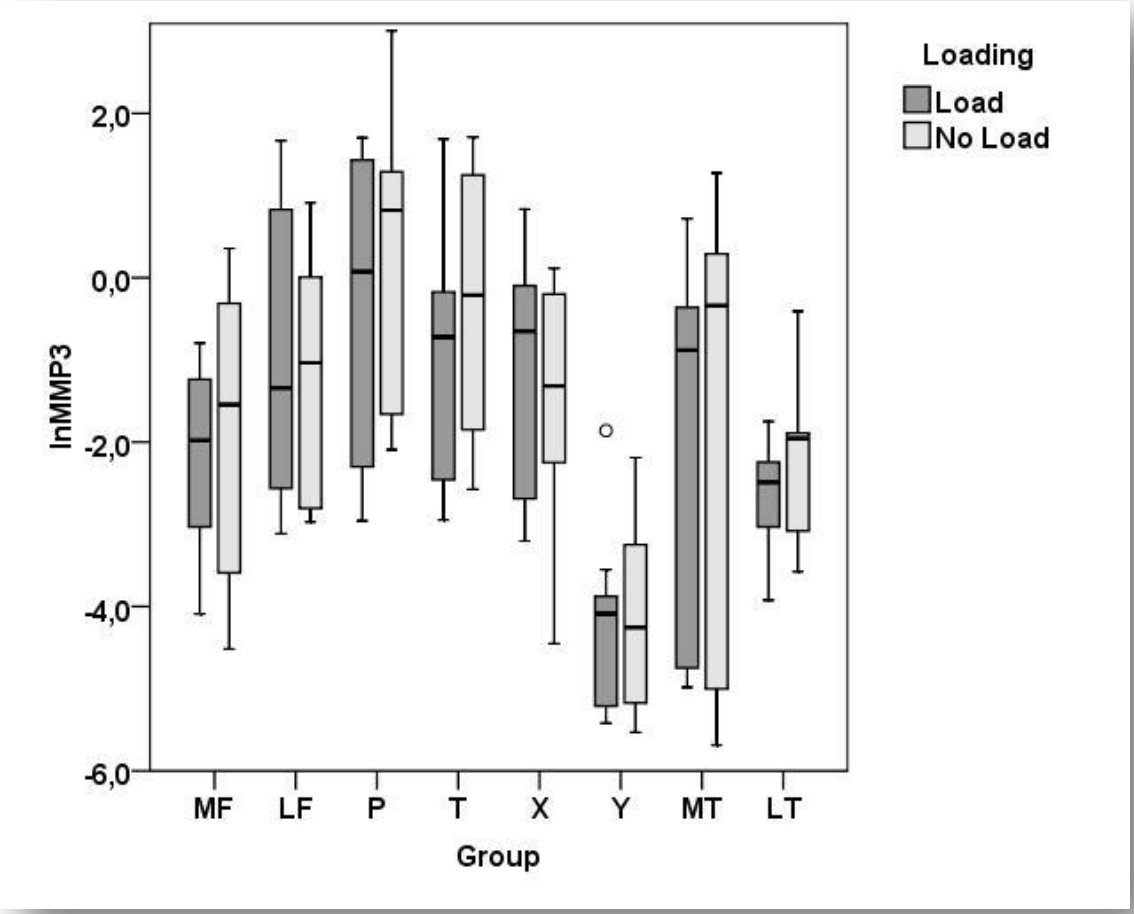


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

MMP-3

MMP-3 mRNA levels showed an overall down regulation when compared to the freshly isolated P0 cells, contrary to the MMP-1 level. A clear anatomical grouping could not be observed but significant differences between the topographically distinct regions for each group, mechanically loaded and static control were possible to detect as seen below in Graph 28 (p= 0.000 for loaded scaffolds and p= 0.004 for non-loaded scaffolds). The highest down regulation in MMP-3 mRNA levels was found for the subpopulation of the NWB proximo-medial femoral condyle Y which was significantly distinct from the patella-femoral regions P and T for both groups, load and control (P p= 0.008 for loaded and p= 0.006 for non-loaded scaffolds; T p= 0.006 for loaded and p= 0.01 for non-loaded scaffolds) when pairwise compared. Interestingly in the mechanically induced scaffolds, the anatomical related groups X and Y differed significantly in their mRNA levels (p= 0.012) since the femoral notch biopsy group seemed to behave very similar to the patella-femoral regions.

Graph 28: MMP-3 mRNA Levels for Loaded and Control Scaffolds

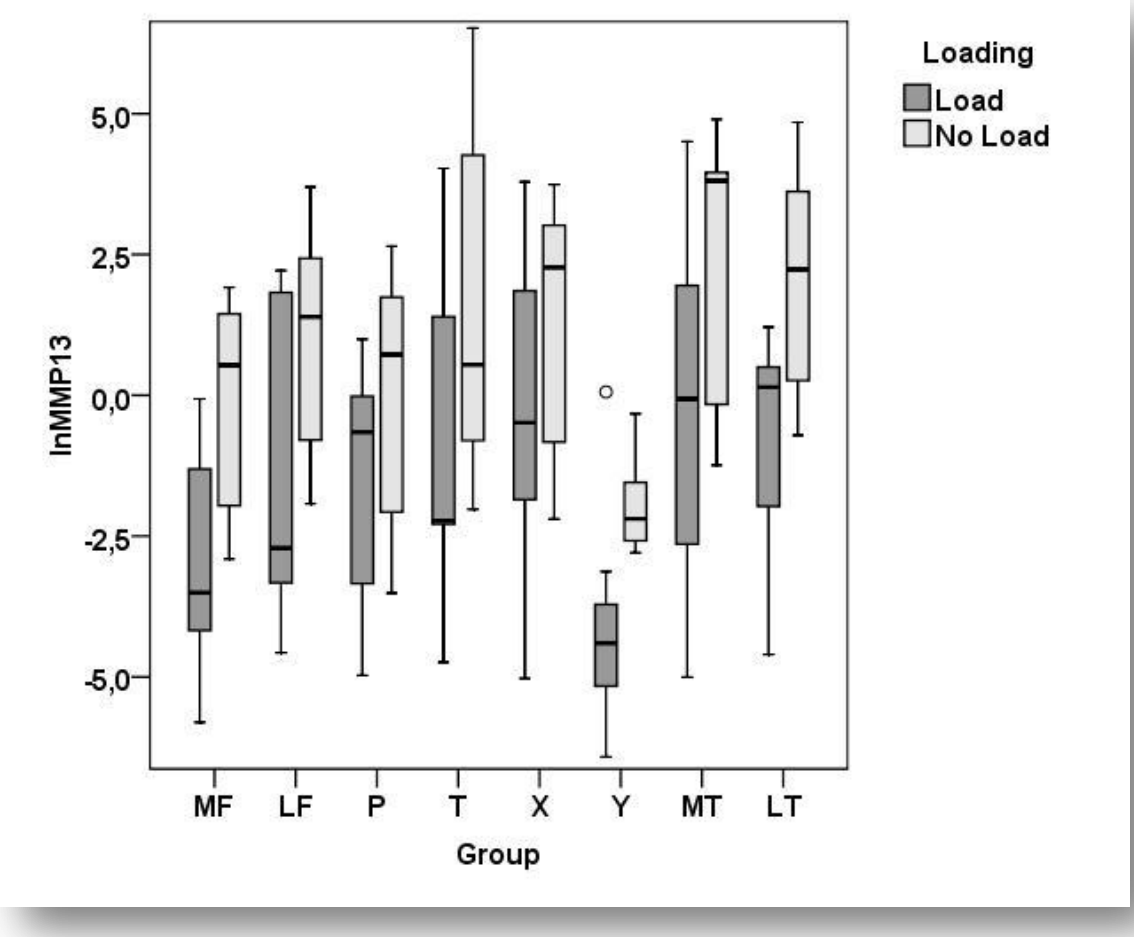


n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

MMP-13

MMP-13 mRNA levels showed an overall significant down regulation when mechanically induced compared to the static control. A clear anatomical grouping could again not be observed but significant differences between the topographically distinct regions for each group, mechanically loaded and static control were possible to detect as seen below in Graph 29 (p= 0.004 for loaded scaffolds and p= 0.000 for non-loaded scaffolds). As for MMP-1 and -3 mRNA levels the proximo-medial NWB condyle showed the most distinct variation in mRNA levels and statistically significant differences in the pair wise comparison. Loaded constructs as well as static controls showed a down-regulation when compared to the freshly isolated P0 cells. Y showed a distinct lower amount of mRNA for MMP-13 than the lateral tibia plateau for both load (p= 0.004) and control (p= 0.005) as well as the medial tibia plateau for the control scaffolds (p= 0.04).

Graph 29: MMP-13 mRNA Levels for Loaded and Control Scaffolds



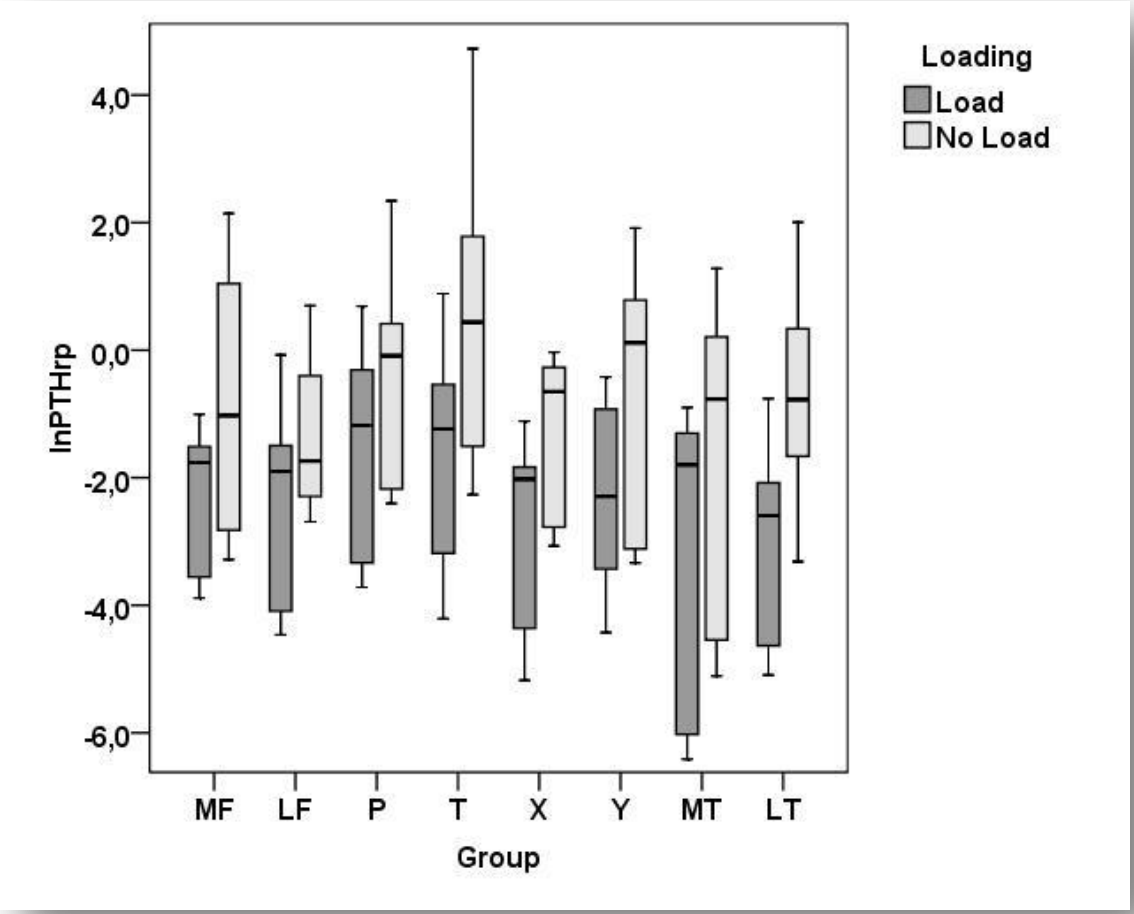
n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

Overall the non weight bearing proximo-medial femur condyle Y showed an explicitly different behavior concerning the mRNA expression levels for all three MMPs compared to all other groups, showing a distinct pattern with the highest down regulation of MMP-13 and -3 as well as the least upregulation of MMP-1.

4.3.3.7. PTHrp

PTHrp showed a significant difference between overall loaded and non-loaded, static groups (p= 0.000). No anatomical grouping could be observed as well as no significant differences between the topographically distinct regions for each group, mechanically loaded and static control were possible to detect as seen below in Graph 30 (p= 0.902 for loaded scaffolds and p= 0.882 for non-loaded scaffolds). The expression levels seemed to be quite homogenous between the varying regional subpopulations when pair wise compared. Mechanical load down regulated mRNA expression levels for all subpopulations when compared to the reference group P0, the freshly isolated cells while the static control showed a slight upregulation compared to P0. Thus, loaded scaffolds interestingly behaved more like P1 cells than the native cartilage analyzed when compared to the expression level for the monolayer analysis (4.2.2.7) while the static 3-D constructs behaved more like the native cartilage with a trend towards an upregulation of PTHrp mRNA levels when compared to P0.

Graph 30: PTHrp mRNA Levels for Loaded and Control Scaffolds



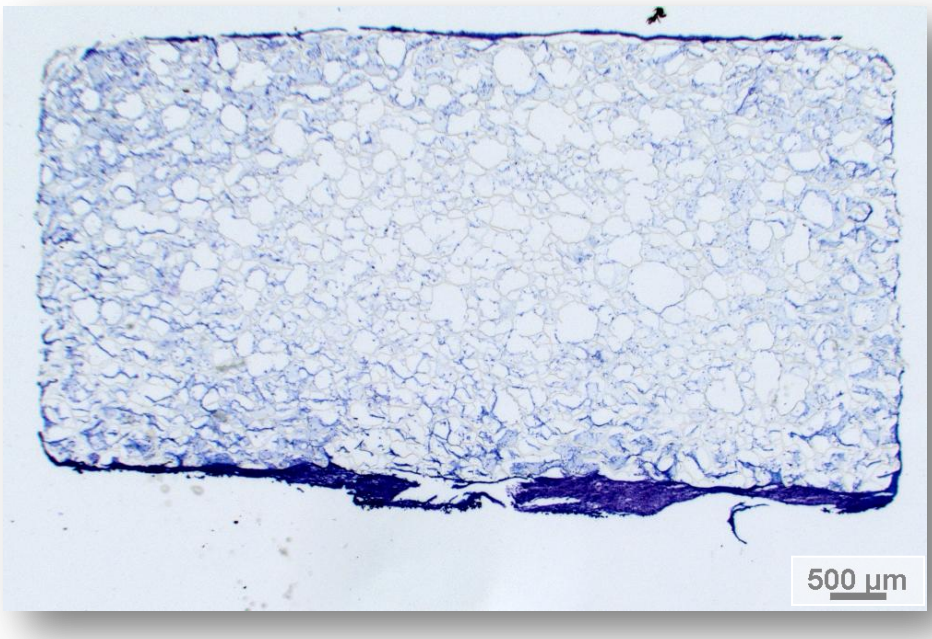
n = 9 per region and group; ○ symbolizes outliers which were not taken into account for the box plot calculations; statistical results within the text;

4.3.4. Histology

To visualize the biochemical and rtPCR findings exemplary histological analysis was done for one subpopulation each of the anatomically related groups.

In general, the three-dimensionally cultured chondrocytes maintained their native morphology, representing the rounded chondrocytic phenotype throughout culture. Spindle-shaped fibrinocytic like cells, a sign of dedifferentiation were occasionally noted but due to the 3-D culture (and mechanical load) most cells regained the rounded, native morphology. There was denser extracellular matrix at the construct edges while fewer cells and less ECM were seen in the central areas of the matrices. This observation was true for all groups with no major differences between load and control or regions. However, there was a clearly visible difference in concern of matrix density and also in part for staining intensity between regions. Also, for the loaded constructs the highest ECM density was always found at the surface, facing the motion-ball, as shown below.

Image 16: Overview of Structural Differences between Construct Edges and Center



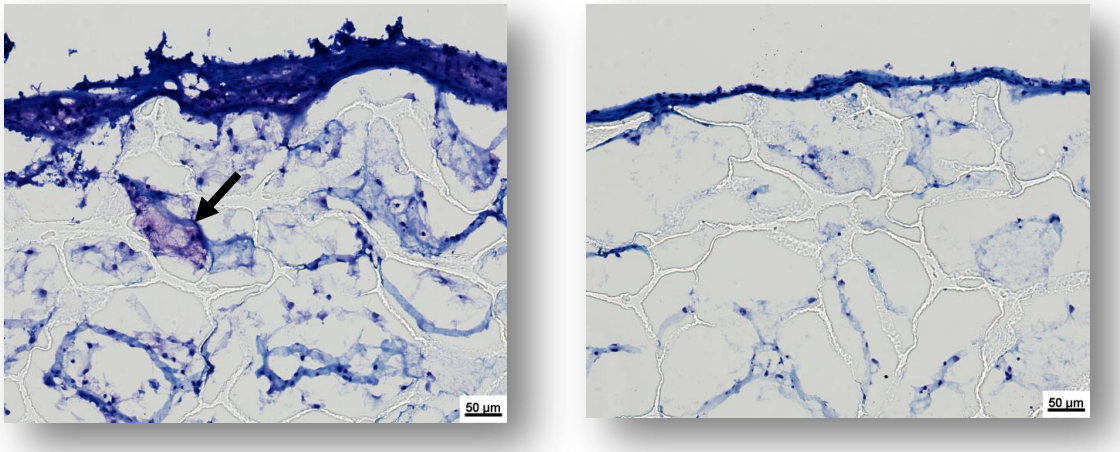
4.3.4.1. Toluidin Blue

In the toluidin blue stain cell shape as well as extracellular matrix produced by the cells during cultivation in static or mechanically loaded conditions can be observed and compared.
Under static conditions, the thickest matrix and strongest ECM staining among all regionally different constructs cultured were found for T, the trochlea of the patello-femoral cells. The Femoral condyles, with comparable results at the biopsy regions, were representative of a denser matrix in comparison to tibial regions.

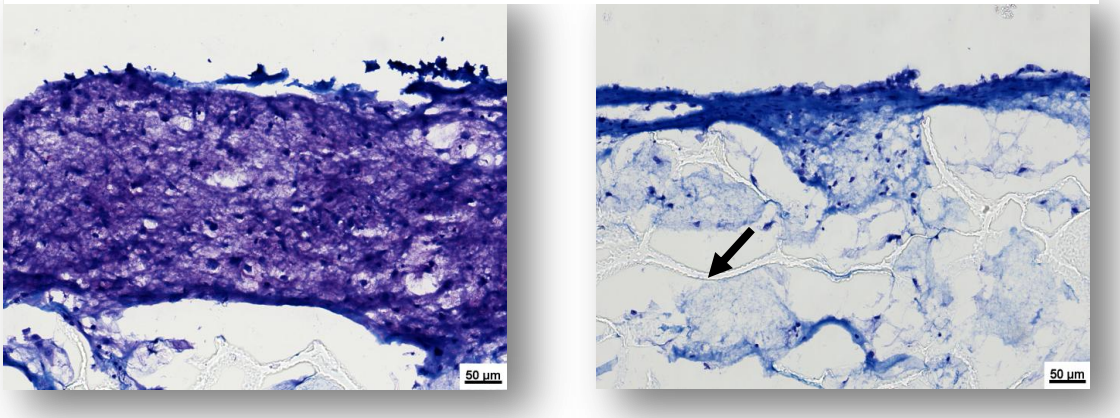
Under mechanical load, biopsy regions were more responsive to load than femoral and tibial regions while the strongest response among cell-scaffold constructs was seen among cells from the patello-femoral joint.

Image 17: Toluidin Blue Stain (20x Objective)

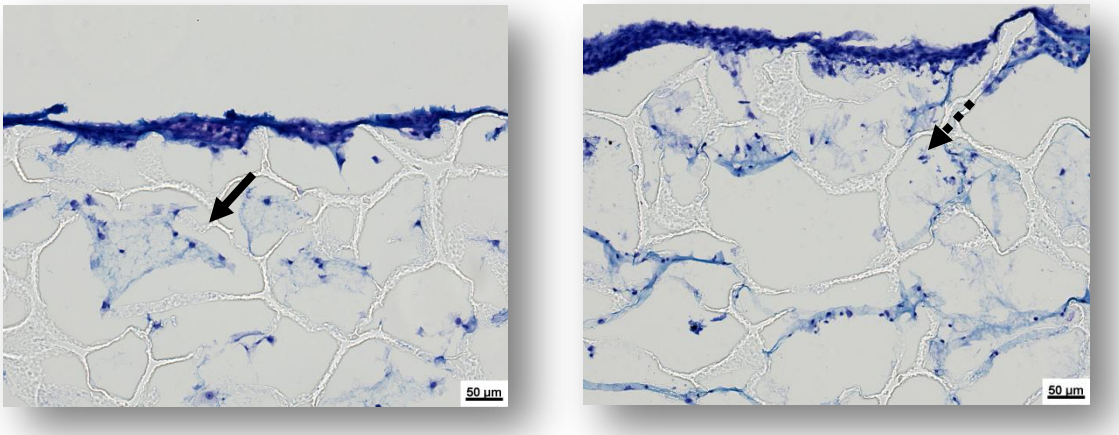
a, MF loaded (left) and static (right) scaffold
b, T loaded (left) and static (right) scaffold
c, X loaded (left) and static (right) scaffold
d, Y loaded (left) and static (right) scaffold
e, MT loaded (left) and static (right) scaffold



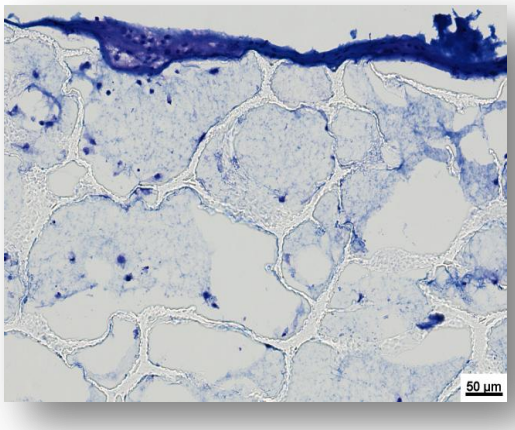
a, Medial Femur Condyle (MF) with loaded (left) and static (right) scaffold; → ECM between PU-Scaffold pores

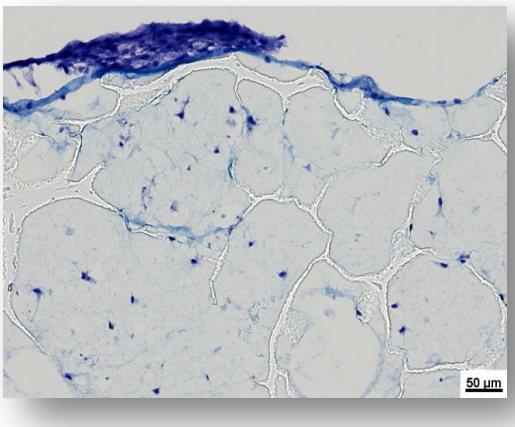


b, Trochlea (T) with loaded (left) and static (right) scaffold ; → PU- Scaffold Material;

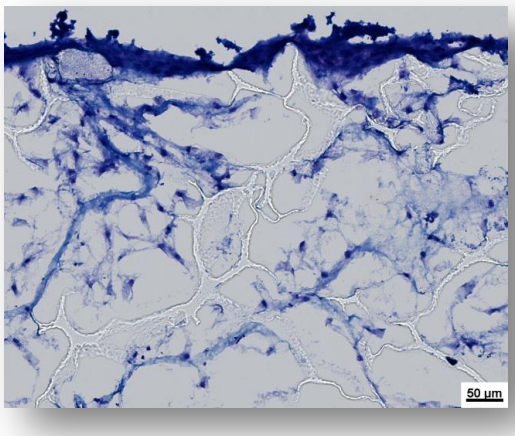


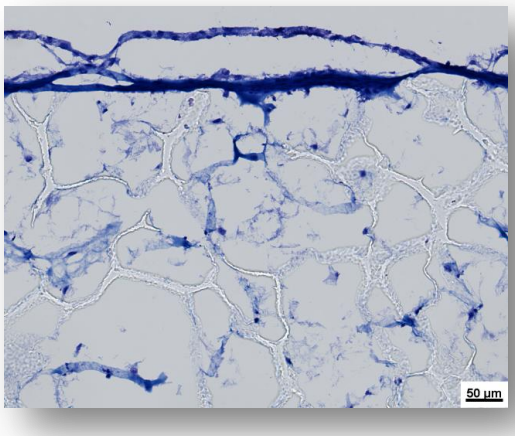
c, NWB Femoral Notch (X) with loaded (left) and static (right) scaffold; → Chondrocyte-like cell with round morphology, versus spindle-shaped like cell with fibroplastic features -->





d, NWB Proximo-femoral Condyle (Y) with loaded (left) and static (right) scaffold





e, Medial Tibia Plateau (MT) with loaded (left) and static (right) scaffold

4.3.4.2. PRG-4 Immunohistological Stain

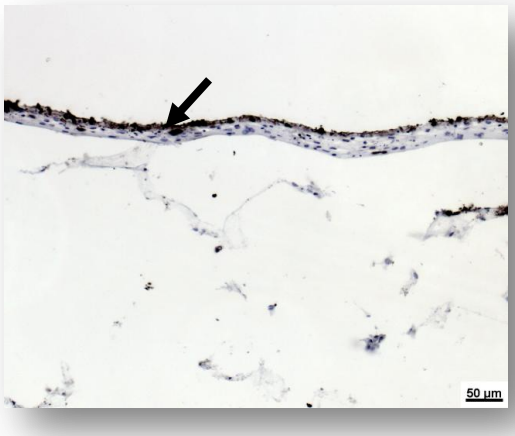
In this stain antibodies against the superficial zone protein SZP were used to visualize the production of this typically of the superficial layer of cells expressed lubricating protein.

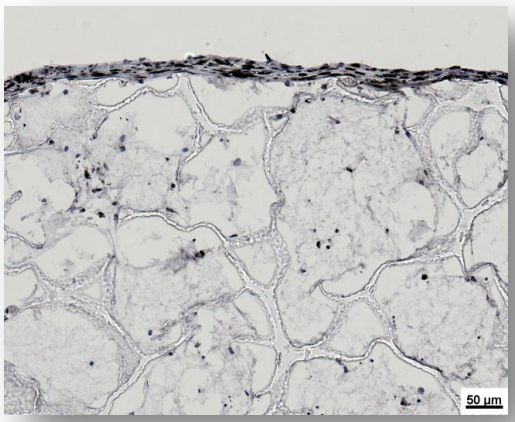
By far the most intense staining was found for the NWB femoral notch X with an expression of SZP even in the center of the scaffold, which was not suspected. SZP detection was slightly lower for the trochlea and tibia group and followed by a clearly less stained condyle group. Only a very faint stain was detected for the other NWB group of the proximo-medial femoral condyle Y. As Image 19 of the control scaffolds shows, the antibodies did not react with the polyurethane material or some other ECM component as one might have suspected in the stained images with SZP detection even in the center of the scaffold but the negative control stains were all negative thus validating the immuno-stain. The fact that SZP was detected also within the scaffold might be due to the fact that the porous

nature of the construct and the by far less dense ECM due to the short period of culture provides a higher superficial layer than initially anticipated.

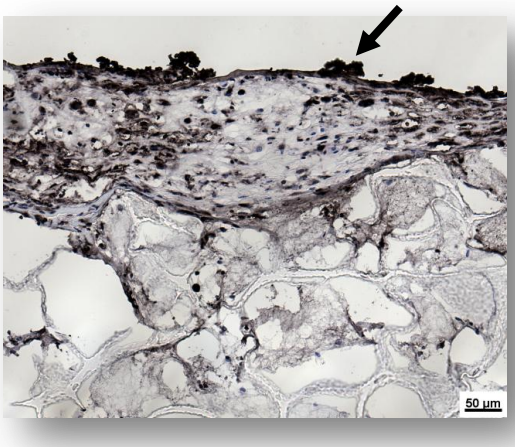
Image 18: PRG-4 Immunohistological Stain (20x)

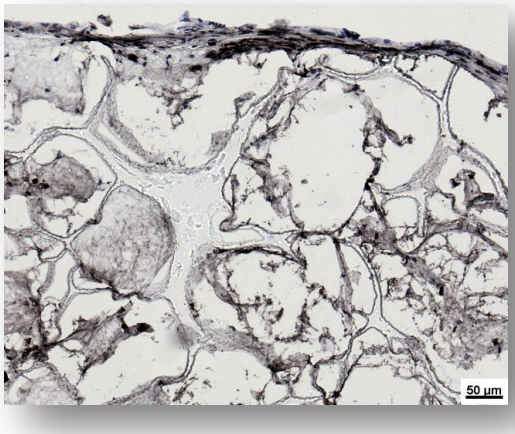
a, MF loaded (left) and static (right) scaffold
b, T loaded (left) and static (right) scaffold
c, X loaded (left) and static (right) scaffold
d, Y loaded (left) and static (right) scaffold
e, MT loaded (left) and static (right) scaffold



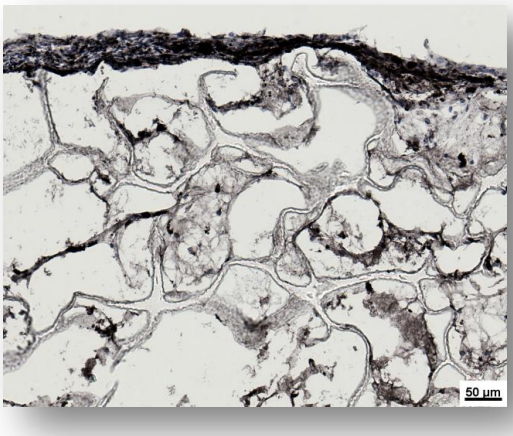


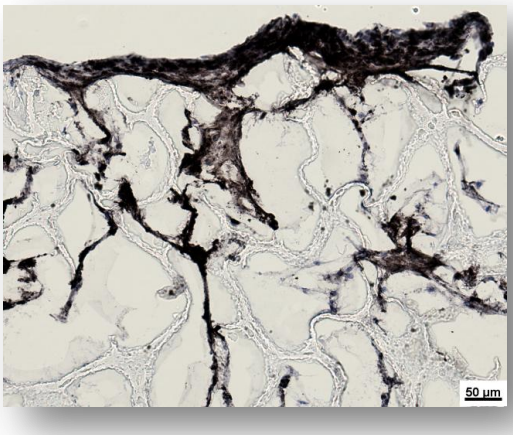
a, Medial Femur Condyle (MF) with loaded (left) and static (right) scaffold; → SZP only in the very superficial layer detecable

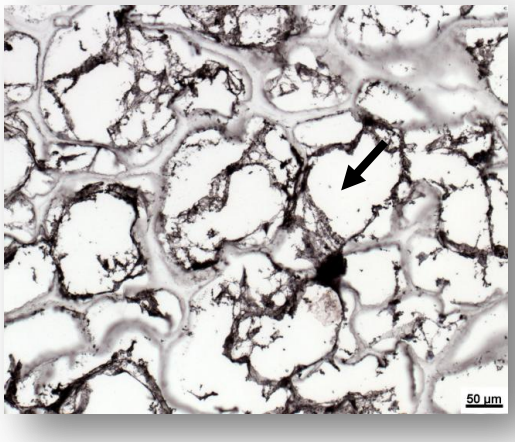




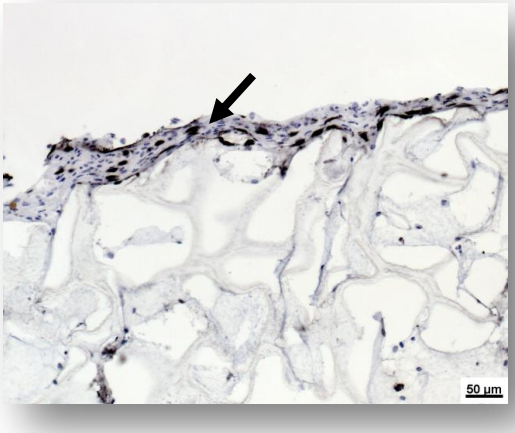
b, Trochlea (T) with loaded (left) and static (right) scaffold; →more SZP detectable than compared to a,

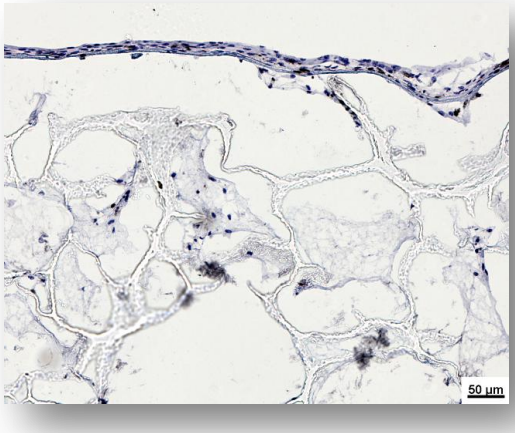




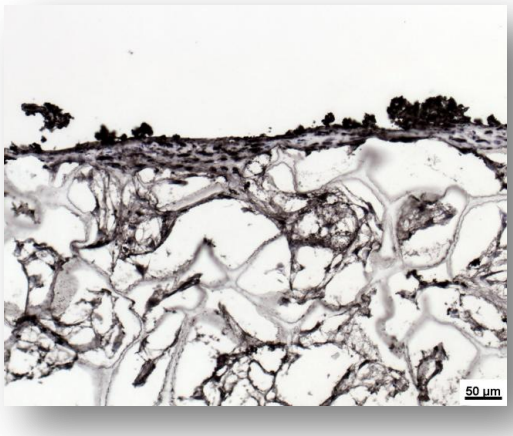


c, NWB Femoral Notch (X) with loaded (left) and static (right) scaffold; Below scaffold center of loaded group; → intense staining even in the center of the scaffold



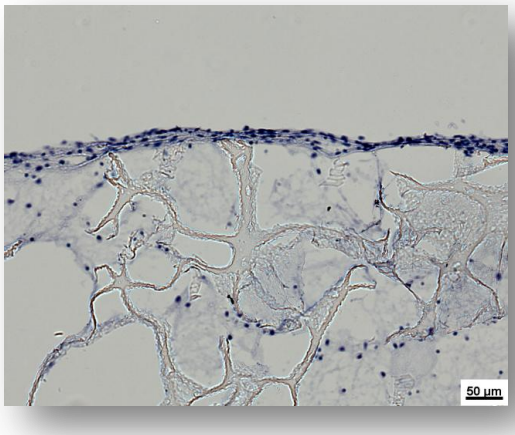


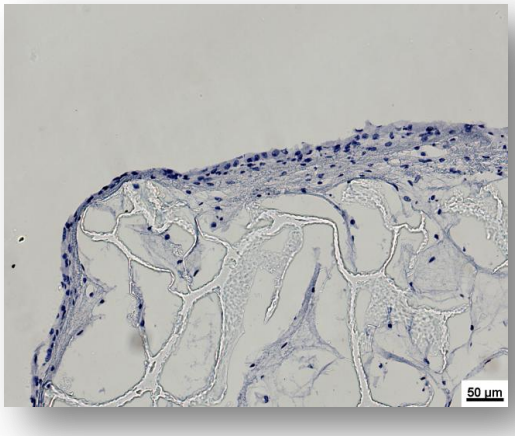
d, NWB Proximo-medial femoral Condyle (Y) with loaded (left) and static (right) scaffold; → very light staining with a rather pericellular distribution



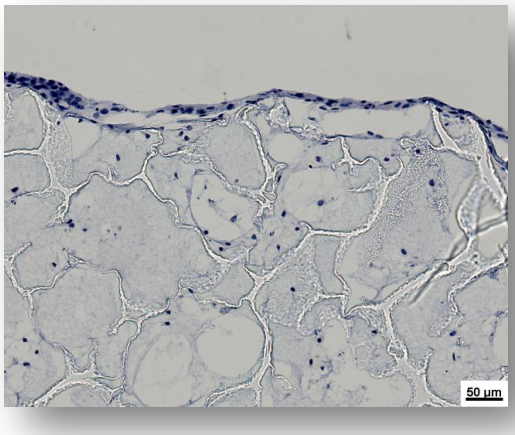
e, Medial Tibia Plateau (MT) with loaded (left) and static (right) scaffold

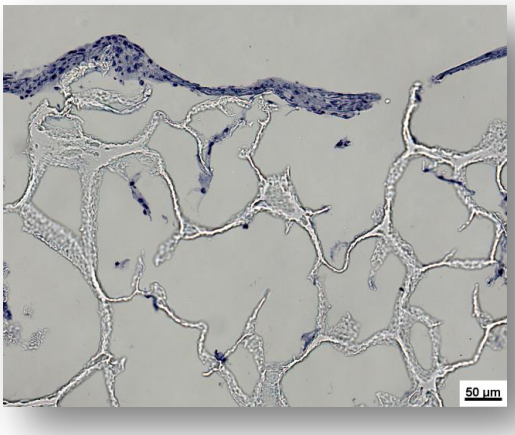
Image 19: PRG-4 Immunohistological Unstained Control Scaffolds (all static culture) (20x)

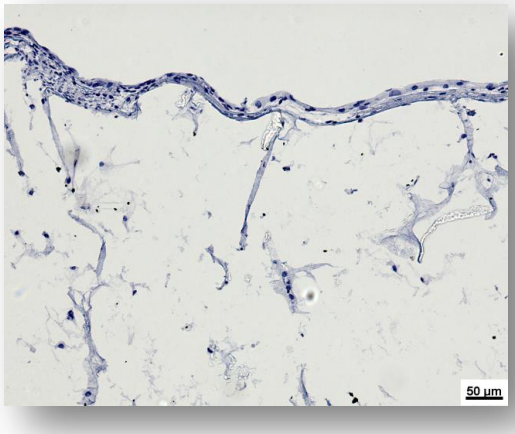




a, Medial Femoral Condyle (MF) left; **Trochlea (T) right;**







b, NWB Femoral Notch (X) left and Proximo-medial Femoral Condyle (Y) right; Weight-bearing Medial Tibia Plateau below.

5. Discussion

Autologous chondrocyte transplantation/ implantation (ACT /ACI) [Brittberg 1994] has been applied for the treatment of cartilage defects of all six joint surfaces in the knee joint for almost 30 years now. Just recently the DGOU, German Society of Orthopaedic Surgery and Traumatology published a review with recommendations for the indications for ACI installing it as a gold standard for large diameter defects [Niemeyer 2013]. Most current randomized-controlled trials comparing ACI with MFX have now been showing ACI superiority [Saris 2009]

Still, results and outcomes, especially long term developments of joint function and patient satisfaction, respectivly quality of life vary from region to region [Crawford 2009; Niemeyer 2008; Peterson 2000], from method to method and author to author [Niemeyer 2014; Van Assche 2010; Kon 2009; Brittberg 2008; Henderson 2007; Jakobsen 2005; Eckstein 2002].The highest frequency of repair is found at the distal femur and to a lesser extent at the patello-femoral joint [Minas 2005]. Crawford et al. implanted Neocart constructs after 3-D culture and ex vivo bioreactor training for example only in defects of the femur condyles in their clinical phase I study with a two year follow up in 8 patients [Crawford 2009]. Since it has been stated that defect repair of the patello-femoral joint has a higher rate of failure [Peterson 2010; Niemeyer 2008; Brittberg 1994] compared to other regions of the knee joint, studies like this might show a selection bias and leaving open the questions why these differences occur.

Heterogeneous outcomes might be due to the topographical variations of the knee joint, which include differences at the cellular, biochemic-compositional and structural level. These differences might influence the integration of repair tissue, mechanical response, matrix composition and structure - possibly even on a cellular level.

In our experimental set-up we were able to show that topographical variances in the knee joint seem to originate from different cellular behavior. Regional differences were observed in monolayer culture, as well as in 3-D culture with and without mechanical stimulation on an intracellular level (rtPCR), biochemically (GAG production) as well as microscopically (histology).

5.1. Articular Cartilage

Articular cartilage is a hyaline cartilage. “Hyaline” originated from the Greek word *ὕαλος* (spoken: "hyalos") describing it´s macroscopic appearance as glassy, glass-like, translucent tissue.

The first documented recognition of cartilage has been attributed to Aristotle (384-322 BC): “Cartilage is found, where it is an advantage that the solid framework should be pliable and glutinous

for the benefit of the flesh that surrounds them" [Benedek 2006 p. 203; Forster 1918]. Aristotle was describing the shape giving cartilage of nose and ears but did not mention articular cartilage and its function. Galen (130-215 AD) on the other hand briefly described the articular cartilage in his major work "On the Usefulness of Various Parts of the Body" as "spread on some part of them [bones], such as joints, to make them smooth…Cartilage serves as a grease for the joint" [May 1968 Book 16 1:683]. Eight centuries later, Avicenna (980-1037) already described quite precisely the function of articular cartilage. "It was made for the purpose of providing a cushion between hard bone and the soft members, so that the latter should not be injured when exposed to a blow or fall, or compression" [Kelley 1970 Chap. 16, p. 94]. Vesalius (1514-1564), the founder of modern anatomy pointed first out, that cartilage changes with age. "In younger people cartilages are soft, but with age they harden and resemble the fragility and friability of bone…". He also better illustrated the function of articular cartilage and of the synovial fluid than Galen did. "… Obviously the purpose of its being so smooth and flat is that the head of the bone may turn easily in its socket and no roughness may impede the ease of movement. In this it is assisted by a viscid and slippery liquid…" [Richardson 1998;Chap. 2, pp.8-11, 39-40].

We learn from this historical digression that articular cartilage is essential to normal diarthrodial joint function, because of its ability to reduce joint stress and surface friction due to the capacity to deform and enlarge its surface contact area to lessen the effect of direct loads by decreasing the applied stress. Besides this functional characteristic, another remarkable one is the exceptional durability of the mechanical-protective ability partly thanks to the biphasic solid-fluid nature. Currently, no synthetic prosthesis will be able to provide a normal joint function for 60 years and more, like a human joint does.

5.2. The Extracellular Matrix

Grossly and histologically, adult cartilage consists primarily of extracellular matrix with apparently one type of cell and no blood vessels, lymphatic vessels or nerves [Buckwalter 2005 p. 466; Buckwalter 1998 p. 192; Stockwell 1978 p. 7]. The tissue is typically composed of 75% to 80% of water and a dense extracellular matrix mainly consisting of 50%-73% collagen II and 15%-30% proteoglycan macromolecules (structural macromolecules contribute 20% to 40% of the wet weight).

Collagen II consists of tightly woven fibers, resulting in insoluble molecules ranging from 30-200nm in diameter. In this framework, water and proteoglycans are dispersed. Proteoglycans consist of a protein core and one or more glycosaminoglycan chains. Glycosaminoglycans that can be found in cartilage include hyaluronic acid, chondroitin sulfate, keratin sulfate and dermatan sulfate.

Concentrations vary among site, zones and also with age, injury and disease. There are two main types of proteoglycans: large aggregating monomers or aggrecans, and small proteoglycans including decorin, biglycan and fibromodulin [Buckwalter 1998 p. 480/ 2005 p. 468-69]. Other components of the ECM are collagens (V, VI, IX, X and XI), link-protein, anchorin, hyaluronate, fibronectin and different lipids [Martinek 2003 p. 167].

The collagen fibril can withstand tension but not compression and provide the matrix with high tensile strengths. Proteoglycans on the other hand assist in the resistance to compression, mostly due to their hydrophilic nature, caused by their extensive carbohydrates attracting and entrapping large amounts of water in the intramolecular and intermolecular space. When load is applied to the tissue, the water becomes pressurized and begins to flow due to a pressure differential through the low permeability extracellular matrix, allowing for energy dissipation through frictional interactions between the solid and fluid phase [Mow 1980; Mansour 1976].

5.3. The Chondrocyte

Articular cartilage is a tissue of relative acellularity. Chondrocytes which reside in lacunae occupy depending on the author between a maximum of 1% [Stockwell 1978 p. 11] up to 2%-5% [Goldring 2006 p. 1006] or less than 10% of the tissue [Jeffery 1991 p. 797] of the volume in adult human articular cartilage. The number of cells rapidly decreases with the maturation of the cartilage. At birth approximately 70 cells/0.22cm^2 can be counted in whole thickness biopsies, droping down to 10 cells/0.22cm^2 in young adults [Stockwell 1967 p. 756]. Cell density, as well as cell shape and metabolic activity vary depending on the zone of cartilage [Aydelotte 1988 A: p. 218, B: p. 230]. The typical human adult knee chondrocyte is found to have a volume of approximately 1650µm^3 and a surface of 730µm^2 [Quinn 2005 p. 676], which would correspond with a cell diameter of 15µm a spherical morphology.

Interaction with the ECM takes places through cells surface receptors like integrins. They are the mechanical link between the cell and its matrix and aid the cell homeostasis. Also, some chondrocytes have short cilia, extending from the cell into the matrix. These structures are thought to have a possible role in sensing mechanical changes in the matrix [Buckwalter 1998 p. 478].

Chondrocytes arise either from mesodermal origin such as the elements of the limp or from the neural crest (for the skeleton of the face). These can be described as primary chondrocytes whereas secondary chondrocytes originate from the periosteal layer surrounding the bone in the head and

clavicular skeleton of higher vertebrates. These secondary chondrocytes develop in response to a mechanical stimulation [Archer 2003 p. 402].

Thus, these chondrocyte can be considered having a primitive precursor that is able to respond to mechanical cues. It was consequently named the mechanocyte by Hall et al. [Hall 2000].

Depending on the cell´s location, the function of chondrocytes is two folded. The prime function of cells occupying the articular cartilage is to synthesize and maintain the ECM. In growth, the major function of chondrocytes is an increase in volume achieved by three mechanisms: Through proliferation, matrix secretion and increased cell volume (in hypertrophy in the terminal differentiation) [Archer 2003 p. 403].

Since the articular cartilage is not vascularized, the tissue nutrition relies on diffusion. Consequently, the entire metabolism of the cell is geared towards functioning at low oxygen tension. Oxygen tension varies from 10% at the surface down to < 1% in the deep layers. The cells meet their energy requirements thus mainly through glycolysis and as a result normally don´t contain mitochondria. The nucleus is usually round or oval with several nucleoli, varying between species. The Golgi apparatus is well developed and vesicles with glycogen as well as lipid droplets can be observed [Lin 2006 p. 1972].

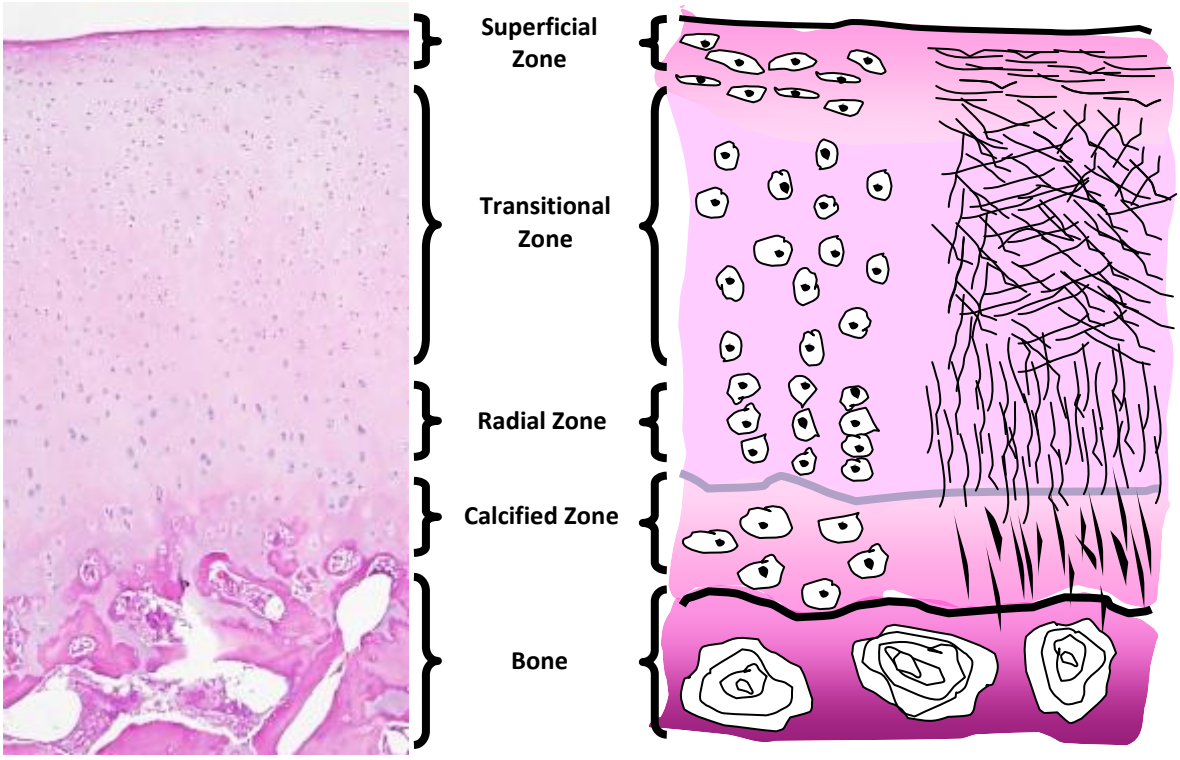
5.4. Tissue Architecture

Architecturally, articular cartilage has four zones of distinct structure from the articular surface to the subchondral bone.

The superficial layer makes up approximately 10% of the cartilage and determines its load bearing ability besides serving as a gliding surface for the joint. The top portion is called lamina splendens and was thought to be a fixed membrane in earlier times (Havers, a London anatomist published this Hypothesis in 1691). It is a clear film consisting of a sheet of small fibrils with little polysaccharide and no cells [Buckwalter 1998 p. 482]. Deeper in this first zone chondrocytes synthesize a matrix with high concentration of collagen and low concentrations of proteoglycan, relative to other zones. Also, water and fibronectin content are highest in this zone. A zone specific protein is the superficial zone protein which is thought to contribute to the lubrication properties of the joint [Flannery 1999]. In this layer, cell density is highest of all four zones (approximately two to three fold) [Hunziker 2002 p. 567; Stockwell 1967 p. 755]. Flat chondrocytes and collagen fibers are arranged tangentially to the articular surface. This orientation imparts in higher tensile strength and stiffness [Roth 1980] as well as resistance against compressive forces [Setton 1997]. Disruption of this superficial layer results in

an increased permeability. Not only structure and mechanical properties are altered but the barrier function of the intact cartilage surface is lost which may result in the release of molecules that stimulate immune or inflammatory responses, possibly an early sign of osteoarthritis [Buckwalter 1998 p.482].

Image 20: Zonal Architecture of Articular Cartilage



Full Thickness Native Cartilage in HE Staining (Hematoxylin Eosin)

Cellular Structure Collagen Fiber Arrangement

Zone two is an intermediate or transitional layer (40%-60% of full thickness) which is composed of spherical chondrocytes and randomly rented collagen fibers. The ECM composition is different when compared to zone one. Proteoglycan content is higher while collagen as well as water concentrations are lower.

Zone three, the deep, radial layer has fibers and in column aligned chondrons (clusters of ellipsoid cells surrounded by matrix) oriented perpendicular to the subchondral plate. Collagen fibrils extend into the calcified zone to reinforce the bond between cartilage and bone [Maroudas 1979].

Zone four is the calcified layer before the subchondral bone, separated from the deep layer with a distinct , basophilic tidemark. This layer consists of only few cells, low water and high proteoglycan contents and an abundance of calcium salts, making it a place for growth of underlying bone tissue [Buckwalter 1998 p. 482-483 /2005 p. 469-471].

Besides this zonal differentiation, a distinction into three compartments or regions can also be seen: a pericellular regions, a territorial region and an interterritorial region [Buckwalter 1998 p. 483]. These three regions also show certain structural as well as biochemical differences and variations in the matrix composition.

5.5. Biomechanical Properties of Articular Cartilage

By design and function, articular cartilage is a tissue which gets exposed to large and complex stress fields during joint movement. The biomechanical structure of the tissue is composed to withstand this stress and adapt to it by reorganizing its structural composition [Laasanen 2003]. Immobilization of the joint or marked decrease in joint load alters chondrocyte activity so that degradation exceeds synthesis of at least the proteoglycan component of the matrix [Hinterwimmer 2004; Buckwalter 1995 p.33]. Under normal physiological conditions, in vivo loading can result in peak dynamic mechanical stresses on cartilage as high as 15 to 20MPa (150-200atm) during certain activities, such as stair climbing [Grodzinsky 2000 p. 692; Hogde 1986]. These peaks occur only short time and lead to small cartilage compressive strains of 1%-3%. If physiologic stresses of 3.5MPa (approximately 150% of bodyweight) are applied to knee joints for up to 210 minutes, compressive strains of the knee cartilage of 35% up to 45% can result with an estimated loss of cell height of chondrocytes resulting from physiological loading to be approximately 20% [Guilak 1995]. Within the first minute of static compression, approximately 7% of the final compression is reached, reaching 25% after eight minutes [Herberhold 1999 p. 1291-1292; Armstrong 1979 p. 750]. These findings however depended on the compartment of the joint, showing on average a higher overall deformation in the patella compared to the femoral condyles. Measurements have also revealed that the equilibrium compressive modulus of adult articular cartilage is in the order of approximately 0.5 to 1MPa, the shear modulus about 0.25 MPa and the tensile modulus about 10 to 50MPa [Buckwalter 2005 p.472].

5.6. Topographical Differences in the Knee Joint

The knee joint can be generally separated into weight-bearing and non weight-bearing regions, forming three different compartments with six articulating surfaces.

Scientific work has previously reported on regional differences across the knee joint. Articular cartilage composition and thickness vary from joint to joint and topographically in a joint as a function of age, load and among species [Athanasiou 1991 p. 336]. These differences have been described to be initialized during the early development of the cartilage anlagen [Brommer 2005].

Throughout life, these distinct areas of cartilage are exposed to different loading patterns resulting in biomechanically adapted tissue to meet the physical needs [Athanasiou 1991; Akizuki 1986; Cameron 1975]. The ability of the tissue to adapt to functional demands is essential. The imbalance between mechanical loads and tissue properties may initiate or worsen cartilage degeneration. In general the relation between the stress applied and the mechanical properties and restructuring mechanisms are highly complex [Arokoski 1993]. Cartilage areas regularly subjected to high levels of compressive stress show higher content of proteoglycans [Manicourt 1988; Kiviranta 1987; Slowmann 1986; Bjelle 1975] and may result in greater stiffness [Swann 1993; Kempson 1971]. Cartilage shows the highest tensile stiffness at the periphery of highly loaded areas where large tensile hoop stresses most likely exist [Akizuki 1986]. Besides these varying glycosaminoglycan contents, differing cellular density, cell shape and matrix morphology as well as collagen contents have been described [Darling 2004; Hunziker 2002]. Jurvelin et al. studied canine articular cartilage from five different locations within the knee joint [Jurvelin 2000 p. 671] regarding their Possion´s ratio and values for shear and aggregate moduli. Differences in stiffness found were also compared to biochemical analysis. This comparison revealed a negative correlation between the Possion´s ratio and collagen to proteoglycan content, indicating that the degree of lateral expansion in different tissue zones may be related to the individual collagen to proteoglycan ratio of that zone [Jurvelin 2000 p. 674].

Cartilage thickness, contributing fundamentally to tissue biomechanical function, varies considerably throughout the human knee joint [Franz 2001; Shepherd 1999]. Varying thickness thus not only compensates for joint incongruence but is also due to local differences in the articular mechanics.

Short term thickness changes in response to joint loading are rapid and site specific [Eckstein 1999; Waterton 2000]. Long term tissue response to alter loading conditions is only limited under increased loads [Shepherd 1999; Eckstein 2002] but reveals a dramatic thinning under decreased load [Vanwanseele 2002 p. 2075].

Seven topographically different locations were biopsied in human knee joints of diseased patients by Quinn et al, and analyzed for cell, matrix and tissue morphology searching for full thickness as well as zonal differences. Cell surface area per unit volume in the superficial zone was found to be greater in the medial femoral condyle than in the trochlea and tibial plateau. The transitional zone of the meniscus-covered lateral tibia showed highest chondrocyte densities compared to the other regions and the radial zone seemed to show a highly uniform morphology [Quinn 2005 p. 675].

Esquisatto et al. analyzed five different regions of articular cartilage in the knee joint of cattle [Esquisatto 1997 p. 434] for GAG content per gram tissue and the amount of soluble proteoglycans obtained for each region. The total amount of GAG/g of fresh tissue was significantly different, with

the trochlea as well as the femoral condyles presenting with the highest and the the tibial plateau with the lowest contents [Esquisatto 1997 p. 435].

Within the cartilage layer, the zonal structure also exhibits variations in matrix biochemical composition, cell morphology and cell-matrix structural organization as a function of depth [Hunziker 2002; Poole 1982]. These distinct tissue zones exhibit different biochemical properties [Chen 2001] as well as cell metabolic activities [Wong 1996]. These findings suggest differential adaptions to distinct biomechanical roles.

Interestingly, Bentley et al. reportet in a study comparing ACI with mosaicplasty that none of the five patients who underwent a mosaicplasty of the patella had good arthroscopic results. They reasoned that this is almost certainly due to the differing thicknesses of donor and recipient articular cartilage which can make healing and incorporation of the patellar articular cartilage difficult. Moreover, the structural orientation of the articular cartilage matrix in the donor and the recipient is different, and might not be adapted or able to adapt for the same stress resistance [Bentley 2003 p. 228].

How adaptable is articular cartilage or the chondrocytes themselves really when put into new mechanical surroundings?

Cellurarity in human cartilage is reduced sevenfold during the time of maturation from birth to adult life [Stockwell 1967 p. 759]. It is commonly believed that the cell density declines during adult life, mostly in the superficial zone [Stockwell 1978 p. 11]. In hip and knee joints, cell density in the first zone might fall by 30% to 50% [Stockwell 1967 p. 760] and cell distribution is not uniform within the adult tissue [Schuurmann 2009; Stockwell 1978 p. 10]. The maximum cell density found in the superficial zone of articular cartilage must be attributed to proximity to synovial fluid and its nutrient and other contents. Since in the young bovine knee it has been stated that a zonal organization is not yet achieved and only a superficial layer differentiation of cells is found [Darling 2005 p. 426] it is very interesting that nevertheless in our data, the yield per gram of cartilage biopsied between the distinct topographical regions differed significantly. Especially the femoral condyles contained the highest cellular density, followed by the tibia plateau, also the pure surface of the tibia plateau that was biopsied exceeded the condyles by far (as the biopsy pictures in the material and method section support). This was identically published from Stenhamre et al. who found highest cell numbers also in the femural condyles, followed by the tibia plateau and agreed with Quinn et al who found similar relationships [Quinn 2005 p. 675]. The patello-femoral joint seemed to have less cells, comparable to the NWB femoral notch, one of the traditional biopsy sites in ACT. Least cells were isolated from the NWB proximo-medial femur condyle which has also been proclaimed a reasonable biopsy site

[Brittberg 1994]. This finding was contrary to Stenhamre et al. who found most cells in the NWB proximo-lateral, followed by the proximo-medial femur condyle [Stenhamre 2008 p. 3].

Cellular density is connected to cartilage thickness (the overall cellularity of the tissue is inversely related to the common factor cartilage thickness [Stockwell 1978 p. 11, 1972 p. 424]) and mechanical forces, which have been reported to vary among distinct anatomical compartments within the knee joint [Cohen 1999]. The patello-femoral joint is experiencing the highest mechanical load during locomotion which results in a thicker matrix related to an increased extracellular proteoglycan deposition in dependency to the mechanical stimulation [Eckstein 2006]. Another theory proclaims, that thickness might not be related to the compressive stress but, however might be a sign of incongruent joint surfaces. Thus, thicker cartilage is needed to distribute high local stresses in incongruent joints [Athanasiou 1991 p. 337; Simon 1970, 1971, 1973]. As suspected in both theories we discovered fewer cells per gram among our initial biopsies from the patella and trochlea compared to the femoral condyles or tibia plateau which are more congruent in the surface as well as less mechanically stressed. The depth of cartilage biopsy in the bovine knee joint can nicely be estimated when comparing the biopsy pictures in the material and method part Image 5**: Biopsy Locations**. Obviously the femoral condyles as well as the tibia plateau show thinner cartilage than the patello-femoral joint and the non weight-bearing areas.

5.7. Autologous Chondrocyte Implantation

To estimate the amount of cartilage needed to be harvested for ACT, biopsies from 1000 patients were studied. The mean value of biopsy weight was 280mg (4-1700mg). The mean value for cells/mg studied on 500 patients was 2600 cells/mg [Brittberg 2003 p. 109-115]. Stenhamre et al. isolated an average of 1483 cells/mg cartilage [Stenhamre 2008 p. 3] from different locations of healthy human knee joints. This is 19-10 times less than obtained in the young bovine knee, where we were able to harvest between 27-50 million cells per gram biopsy. Since young cartilage is known to have a higher cellularity, this is not surprising [Stockwell 1967 p. 759].

Brittberg et al. calculated the average harvest per patient for ACT is 728.000 cells [Brittberg 2003]. They proclaimed that at least $2.6\text{-}5*10^6$ cells per 0,1ml are needed, thus monolayer expansion is indispensable when using the traditional NWB biopsy sites [Brittberg 1994 p. 890].

5.8. Monolayer Culture

Monolayer culture is a basic approach for chondrocyte propagation. It is economical and technically simple and allows the culture of large numbers of cells. Since mature cartilage tissue has a low cell density, expanding the harvested cells will be required before obtaining sufficient numbers for therapeutic treatments. However, chondrocytes have been shown to lose their phenotype in monolayer culture after being passaged. The cells become more fibroblast-like, losing their ability to secrete proteoglycans and changing collagen synthesis from type II to type I [Cheng 2012; Lin 2008; Darling 2005; Benya 1982]. Already in the late 1960ties observations were published that chondrocytes, when isolated and put into monolayer culture, lost their round appearance with passaging, flattened and no longer accumulated metachromatic matrix. They were said to have dedifferentiated and were described as fibroblastic like cells [Chacko 1969]. This description however is only to emphasize the absence of differentiated functions, not to imply a regression to an earlier bipotent or multipotent cell type.

Image 21: Isolated Chondrocytes in Monolayer Culture

Isolated chondrocytes in 2-D Monolayer Culture with round and flattening cell shape. Chondrocytes stretch out to establish cell to cell contact. Zeiss Microscop with Camera (Kappa), Axio Vision; Axiovert 25

Mayne et al. investigated these observations further, culturing clones of chick chondrocytes until senescence and characterizing collagen synthesis [Mayne 1976]. Besides cell shape changes towards a flattened amoebic appearance in culture they also found a pronounced drop in the α1:α2 chain

ratio of collagen synthesis. For the initial population of chondrocytes the synthesis of only α1 chains could be detected dropping to a ratio of 2:0 of α1: α2 until the fourth subculture [Mayne 1976 p. 1675]. This change in collagen synthesis towards type I collagen and type I trimers was analyzed to be due to the cellular senescence and dedifferentiation of the chondrocytes in 2-D culture [Mayne 1976 p. 1767]. Similar changes in collagen biosynthesis have also been observed for all other articular chondrocytes cultured in monolayer culture [von der Mark 1980; Benya 1977].

Darling et al. described the rapid phenotypic changes in passaged articular chondrocytes from 8-month old goats with changes in Collagen type I and II, SZP and Aggrecan mRNA expression levels. Collagen type II expression decreased 10 fold over culture time, while Collagen I increased 20 fold. SZP showed a dramatic drop of 600 fold different expression levels and was hardly detectable after only two passages while Aggrecan expression did not significantly change but showed an overall decreasing tendency [Darling 2005 p. 428].

Lin et al. investigated the expression profile of 27 chondrocyte-associated genes from passaged human articular chondrocytes by rtPCR and compared these finding to osteoarthritic cartilage. Besides the observation that the healthy passaged chondrocytes differed only very little from osteoarthritic cultured chondrocytes, indicating that the monolayer conditions have a higher impact on the chondrocytes than the disease itself, they found that gene expression profiles revealed a dramatic shift once the cells entered monolayer culture [Lin 2008 p. 1235]. Only chondrogenic transcription factors, such as Sox9 remained unchanged.

This dedifferentiation of chondrocytes in monolayer culture towards a more fibroblastic like cell type is still an unsolved problem. It was shown in numerous studies, that the in vitro expansion affects the response of chondrocytes to mechanical stimulation. This might lead to cellular failure and repair tissue of inferior quality [Das 2008].

Up to date, expansion cultures are still crucial to gain sufficient cell numbers to re-implant into defect sites, like when performing ACI. Thus, a lot of effort is made to redifferentiate the cells after monolayer expansion. For this purpose various approaches like media supplements, genetic transfection, low oxygen tension as well as 3-D culture and co-culture systems with and without mechanical load are more and less successfully tested [Lin 2014; Markway 2013; Babur 2013; Enochson 2012; Caron 2012; Darling 2009; Stoddart 2009; Crawford 2009; Grad 2003].

Schuurman et al. found no differences in population doubling in equine chondrocytes from different cartilage zones [Schuurman 2009]. In our work the population doubling did not differ significantly between the regionally distinct subpopulations for the entire time of culture (until P7, = 42 days) especially during the first passage P1 the proliferation capacity among the eight groups significantly

differed. We did detect a trend towards varying population doubling for the entire period, ranking the tibial groups highest, followed by the femoral condyles, then the NWB areas with overlapping counts towards the patello-femoral joint. This behavior was not true for the first passage were the difference was statistically significant. Interestingly the NWB biopsy sites X and Y showed the highest population doubling activity, followed by the groups as ranked above, a clear anatomic grouping was possible. This finding is contrary to Stenhamre et al. who observed a good attachment of cells isolated from the femur with good proliferation rates while the tibia biopsies had low attachment and proliferation behavior, and a slightly lower proliferation index for the NWB proximal femur condyles compared to the WB femur groups [Stenhamre 2008 p. 4]. If these observations are statistically relevant was not stated or graphically shown by the authors.

After the initial passage and a lag phase, like published by Wiseman et al. [Wiseman 2004 p. 288] the growing curves started to synchronize which might be due to the dedifferentiation effect of passage on chondrocytes which has been widely described before. Chick clonal chondrocytes could only be passaged on average 4 times with a total life span of 7.9 weeks after initiation [Mayne 1976 p. 1765]. They undergo between 15–35 population doublings [Mayne 1976 p. 1678]. For our bovine chondrocytes we found on average 24 PDs in 42 days with the growth curve already declining. Thus this typical growth behavior already observed for chick chondrocytes seems to be true for bovine ones as well. Human articular chondrocytes can be successfully grown from donors over a wide age range. The time to primary confluence is however directly correlated to donor age [Harrison 2000]. The effect of passage number has been extensively [Mandl 2004; Schnabel 2002; Mark 1977] shown before to have a significant impact on the phenotypic appearance of chondrocytes [Darling 2005] and thus on the overall ACI outcome and quality of tissue engineered constructs [Niemeyer 2012; Das 2008; Kang 2007; Veilleux 2004, Wiseman 2004; Homicz 2002]. Keeping this in mind, one might want to pick a topographical subpopulation of chondrocytes which have high cell numbers per gram cartilage and show the ability to adapt quickly to the 2-D environment for serial expansion since the number of population doublings and passages as well as the time for expansion culture should be as low as possible to avoid dedifferentiation. When put together for the patello-femoral group P and T we found not only the lowest number of cells per gram cartilage biopsied but cells also showed a distinct lower population growth when compared to other groups. This observation is not in favor for the patello-trochlear joint as a biopsy site.

Typical changes in the chondrocyte specific mRNA expression levels during dedifferentiation in monolayer culture are a downregulation of Col II, COMP and PRG-4 and an upregulation of Col I [Darling 2005; Benya 1978, 1982; von der Mark 1977 p. 532]. Already after one passage, significant changes in the chondrocytic phenotype can be observed, Darling et al. stated. Col II mRNA expression

dropped two times, SZP 2.4 times, and Col I increased 2.6 times for P1 and these phenotypic changes are alarming for only one expansion [Darling 2005 p. 428]. Over four passages they observed a 20 fold increase for Col I, a 10 fold drop for Col II and no SZP expression at all after two passages. Interestingly their Aggrecan levels did not change significantly over the course of the experiment [Darling 2005 p. 428]. Although the group of Darling et al. worked with goat chondrocytes and a different housekeeping gene when compared to our mRNA levels for Col I, II, SZP and Aggrecan we found partly a similar behavior. Col I displayed a striking increase between native cartilage and P1 as well (a little less between P0 and P1, since freshly isolated P0 cells showed an initial down-regulation of Col I), which were significantly distinct between the regional groups. Highest levels were reached for the femoral condyle groups with an upregulation up to 260-380 times. After P1 the changes were not significantly anymore between the passages of each subpopulation but LF. The NWB areas displayed by far the lowest Col I mRNA levels and differed significantly from the patello-femoral joint as well as the femoral condyles. For Col II the drop in mRNA expression levels over the time of culture was pronounced. Initially the level was even upregulated between native cartilage and P0 cells, but overall when compared to the levels detected in native cartilage, they dropped already 25 times in P1 and about 5600 times at P7. While for the Col I expression a plateau was reached after P1, Col II expression further declined with statistically significant differences for each subpopulation between all passages. For P1 the drop was most severe for the NWB biopsy sites X and Y. Interestingly those two groups had the most favorable Col I expression with the least increase though. Vice versa was observed for the femoral condyles. While MF and LF and the patello-femoral joint groups showed the highest levels of increase for Col I expression they also showed the least decrease for Col II together. Differences between groups were significant for the femur condyles and medial tibia plateau to the patello-trochlear joint as well as the proximo-medial femur condyle Y to the lateral femur condyle. Only the femoral notch (X) and the lateral tibia plateau had no statistical differences. When put into ratio Col I/Col II showed the most favorable ratio for the NWB areas over the entire time of culture. Thus the ranking was LF > MF > P > T > MT > LT > X > Y. These findings are contrary to Stenhamre et al. who found no topographic variation in Col I and II expression when they compared biopsies from NWB proximo-medial and -lateral femur condyles with the tibia plateau, femur condyles and the patellar groove in histology and immunohistochemistry [Stenhamre 2008 p. 3]. But on might argue that for one histology and immunohistochemistry are by far less sensible than rtPCR or biochemical analysis and also, that not all changes and differences in mRNA levels lead to different secretion or substrate production.

As in the work of Darling et al., we also did not observed big changes in the Aggrecan mRNA levels. The average change was only 1.6 fold, the overall drop between the native cartilage and P7 only 6,2 fold. The NWB areas X and Y as well as the patella P and lateral tibia plateau LT differed between

passages but there was no difference observed between the eight regional subpopulations for the monolayer culture for the Aggrecan mRNA levels. Darling et al. had published the same in concerns of the different subpopulations by zones in young cartilage [Darling 2004 p. 1185]. The same was true for the PRG-4 /SZP mRNA levels, although the tibial groups ranked lowest with 1,8-3,5 fold differences when compared to the other topographically difference groups. While Darling et al. were not able to detect SZP at all after 2 passages, which might be due to the method chosen we found a steady decline, overall about 800 fold with the most dramatic drop interestingly quite late, between P3 and P5 and not, as one might suspect earlier. The least decline in expression levels was found for the NWB proximo-medial femoral condyle Y, the trochlea T and the medial tibia plateau MT. We were not able to detect any anatomical grouping.

COMP has been known to be a sensitive marker of chondrocyte differentiation [Zaucke 2001 p. 358]. Between the eight regional groups, mRNA levels differed with the groups of the tibia plateau expressing the lowest level over the entire time of cultivation which was statistically significant 4-10 times lower. Also, all eight subpopulations displayed a significant dynamic in mRNA expression levels for COMP between all passages. Like in Col II the most dramatic decline of mRNA levels was found for the first passage (10.4 fold), being most pronounced for the lateral tibia, followed by the NWB biopsy areas and the medial tibia. When compared to the native cartilage, P7 mRNA levels were about 5200 times lower, as in Col II levels with a 5600 fold drop. COMP has been shown to be a ligand for chondrocytes equal to Col II [DiCesare 1994 p. 935].

Das et al also found a significant upregulation of Col I, as well as down regulation of Sox9, Col II, MMP-1,-3,-13 and an absent Col X expression [Das 2008 p. 386] when human chondrocytes were put into monolayer culture until P3, using redifferentiation medium and GAPDH as the house keeping gene for rtPCR. Changes for Aggrecan and PRG-4 were not significant. This again supports our findings. As does the upregulation for Col I and the down regulation for Col II as described earlier. For Sox9 we found, as for PRG-4 and Aggrecan no significant difference between the eight subpopulations until P7. And like in PRG-4 the biggest change in mRNA expression levels was between P3 and P5, fairly late within the expansion culture. But again, overall the mRNA levels for Sox9 at P7 were only 6 fold lower than native cartilage levels detected and thus the decline was only very modest and statistically not significant for most groups but for LT and the NWB biopsy sites suggesting a trend towards anatomical grouping. Our findings differed partly for the MMPs from Das et al. MMP-1 showed only an overall 1.5 fold increase in the expression between P1-P7 which was statistically not significant compared to Das et al. [Das 2008 p. 388]. Between the eight subpopulations distinct variations could be found though. Variation in MMP-1 mRNA level was 29 fold distinct between LT and Y. Cells of the NWB proximo-medial femoral condyle showed a

significant lower MMP-1 expression than all other groups but its anatomical partner X. It should be pointed out that MMP-1 was not detectable in experiment two and three for all eight subpopulations in the native cartilage. MMP-1 is mainly expressed in the superficial cartilage layer, while MMP-13 is chiefly expressed in the deep zone, where different deformation is experienced by the chondrocytes [Rannou 2006]. Since it was stated earlier that young bovine cartilage (as in goat) [Darling 2006] might mostly consist of superficial zone differentiated chondrocytes because maturation is not completed, we wonder why we were not able to detect MMP-1 but had no troubles detecting MMP-13. This finding is contrary to the opinion stated by other authors.

MMP-3 expression was very similar to MMP-1. Again, the cells of the NWB proximo-medial femoral condyle showed the lowest mRNA expression levels, distinct from all groups but the patella (including X this time). Overall as for Das et al. a down regulation was detected but only 5.7 fold compared to the native cartilage an only statistically significant for some subpopulations. The biggest change was again detected between P3 and P5 as for PRG-4 and Sox9. For MMP-3 a clear anatomical grouping was possible for P1 cells showing the highest expression for the tibia plateau, followed by the patello-femoral joint, the femoral condyle to the highest down regulation for the NWB areas. For MMP-13 a 30 fold drop in mRNA expression levels was found which was again like in MMP-3 statistically partly significant for all passages and also partly distinct between the subpopulation. An anatomical grouping was possible for the tibia plateau and the femoral condyles but distinct variations were detected within the patello-femoral joint as well as the NWB areas. The biggest drop was again between P3 and P5. It has to be noted though, that mRNA level declines between P3-5 which were significant for PRG-4, Sox9, MMP-3 and -13 are not statistically relevant when taken into account that there was a passage 4 in between.

For MMP-13 the NWB proximo-medial femoral condyle did not keep its distinct expression pattern compared to the other groups. Lin et al. found a decline for healthy human chondrocytes in MMP-1,-3,-13 over P2-4 but only for MMP-13 mRNA expression levels differed significant. Sox9 showed no drop but stayed within same range which is consistent with our data where only some subpopulations showed a distinct variation [Lin 2008 p. 1234]. Interestingly Malpeli et al. were able to detect Sox9 in chondrocytes cultured in serum free medium for up to 6 passages, whereas chondrocytes in serum medium lacked Sox9 expression [Malpeli 2004]. Lin et al. extensively investigated the gene expression profiles of cultured chondrocytes as well as osteoarthritic cartilage from patients undergoing joint replacement. They also showed that although dedifferentiation occurred during monolayer culture, for example that MMP -1, -3,-13, Aggrecan, Col II and X showed a drop in OA chondrocytes in culture compared to native tissue [Lin 2008 p. 1232], surprisingly gene expression profiles between OA and normal monolayer cultured chondrocytes were not significantly

different for most genes [Lin 2008 p. 1233] and gene expression levels of transcription factors necessary for chondrogenesis remain unchanged (Sox-9, c-fos, or c-jun). Interestingly our findings concerning the statistically significant behavior of Col II expression and Sox9 expression between some of the subpopulations can be put into relation. As Lefebvre et al. published in 1997 Sox9 plays a role in chondrogenesis, harboring a DNA-binding domain, acting as a transcriptional activator and taking part in controlling the Col II expression in chondrocytes [Lefebvre 1997 p. 2338]. Thus, high expression of Sox9 was found to correlate with the expression of Col II and when put into monolayer culture, chondrocytes' mRNA expression of Sox9 as well as Col II declined in the process of dedifferentiation [Lefebvre 1997 p. 2343]. This supports our finding as well. We did find partially significant differences for the regional behavior with a down regulation of the NWB areas X and Y as well as the lateral tibia plateau LT which proved to be significant. When compared to our Col II levels, the most severe drop in mRNA levels was found in P1 for the NWB areas, followed by the tibia plateau.

For PTHrp which has been far less addressed in the literature when chondrocytes and their behavior in monolayer culture are discussed we found no significant differences between the subpopulations and an anatomical grouping was not possible. Overall we showed a 3.15 fold decreased in the expression level until P7 with the biggest loss of mRNA expression in the first passage (6.1 fold). Again, some groups showed significant dynamics in mRNA expression levels over the 42 days of culture like the NWB femoral X and the patella P with higher levels when compared to MF and MT which showed overall lower levels. PTHrp is known to inhibit chondrocyte hypertrophy near the articular ends of the developing bone and is needed to maintain a pool of proliferating cells [Kobayashi 2002 p. 2985]. When we try to correlate this with the expression of Col X, a classic marker for chondrogenic hypertrophy we are able to detect an inverse relationship only when comparing the native cartilage mRNA levels with the freshly isolated P0 cells. Here we found a clear upregulation for Col X and a down-regulation of PTHrp. This difference could not be sustained over the course of monolayer culture. The Col X expression dropped 155 fold until P7 with the biggest drop between P1 and P2. Cells for the femoral condyle and the patella showed an initial increase of P1 when compared to the native cartilage but all other groups dropped their mRNA expression levels from the beginning. The patella showed the least drop in the Col X level (2.7 fold compared to cartilage) but also the highest PTHrp expression. We did find significant expression dynamics between the passages for all subpopulations except for LT and Y, but no group differences besides the NWB areas which differed significantly from each other. Since MMP-13 has been shown to be expressed by terminal hypertrophic chondrocytes [Ortega 2004; Inada 1999] our decreased levels perfectly fit to the decreased expression of the hypertrophic marker gene Col X and might even fit to a decreased PTHrp level [Mengshol 2001].

5.9. 3-D Culture Systems and mechanical Stimulation

To redifferentiate chondrocytes after monolayer expansion culture or to prevent dedifferentiation of freshly isolated chondrocytes, 3-D culture systems have been extensively investigated in the last years. Already in 1982 Benya et al. showed that in monolayer culture expanded chondrocytes were able to regain their chondrocyte specific characteristics after a several week culture period in a form giving alginate gel. Proteoglycan as well as collagen synthesize were shown to strongly increase [Benya 1982 p. 216], but depending on the time of previous monolayer culture period [Benya 1982 p. 220].

The use of scaffolds in tissue engineering as matrices for cells has paved the way for the use of functional tissue substitutes in the treatment of cartilage defects. In order to serve as a functional substitute to fill a cartilage defect, scaffolds must follow some basic principles. They must be biocompatible, structurally and mechanically stable and they must support the loading of an appropriate cell sources. Next to a successful infiltration and attachment of the cells they should also allow the use of bioactive molecules in order to promote differentiation, proliferation and maturation of cells.

The ideal material to provide a 3-D matrix to chondrocyte has not been proclaimed yet. There are two approaches in general. Research is carried out using biological as well as synthetical tissue replacement. Scaffolds composed of biological materials like hyaluronan- or collagen based have shown promising results [Niemeyer 2010; Mukaida 2005 p. 527]. Pavesio et al. published preliminary clinical trials using a hyaluronan-based scaffold known as Hyalograft C^{TM} in 2003. Early clinical findings showed results of 96,7 % of repair tissue biologically comparable with hyaline-like cartilage formation [Pavesio 2003 p. 215]. However, natural scaffolds may face problems of immunogenic compatibility, resulting in a faster degradation or batch inconsistency. Synthetic scaffolds have also shown promising results in numerous in vitro [Grad 2003] and in vivo experiments [van Bomhard 2013]. Poly-a-hydroxy ester substrate based scaffolds have been observed to enhance the promotion of proteoglycans, chondrocyte proliferation, differentiation and maturation in comparison to collagen based scaffolds [Grande1997 p. 214-215].

For this experimental set-up polyurethane-scaffolds were fabricated to serve as 3-D matrices for articular chondrocytes to be seeded on.

At the end of the experiment wet weight between loaded and non-loaded scaffolds was compared. There was no statistically significant difference between the wet weights of loaded and static free swell control scaffolds when compared after 21 days of 3-D culture. In a pairwise comparison within the static group one difference was found with MF weighing significantly more than the scaffolds of Y. For the mechanically loaded constructs a trend could be detected towards a heavier outcome for the trochlea group T.

When biochemically compared the difference for cell content (DNA), overall GAG content and GAG/DNA was strikingly higher between constructs of mechanically induced versus static conditions even though the difference was apparently not displayed in the wet weight of the scaffolds. Wiseman et al. found the same behavior for cells that had been passaged for up to two times, suggesting that the mechanotransduction pathways were not altered by monolayer culture [Wiseman 2004 p. 296]. Heyland et al. showed also an increase in GAG/DNA through load by intermittent hydrostatic pressure in porcine knee chondrocytes [Heyland 2006 Fig.2&6]. Since the group T tended towards the highest scaffold weight it is no surprise that we found the highest GAG/DNA content in this anatomical group. This finding was also supported by the histological analysis were it was clearly shown that mechanically loaded scaffolds with cells of the trochlear groove produced the thickest and most pronounced layer of ECM. Lowest values for GAG/DNA were found at both NWB areas, about 1/4th of the value of the patella, which was the highest group followed by the trochlea. In the static culture, highest GAG/DNA values were found for the medial femur condyle, lowest were produced by the medial tibia plateau, although they were statistically not relevant. When the WB areas were consequently compared within, the femur groups tended to have higher GAG/DNA values than the tibia groups. The same finding was published by Stenhamre et al. although they analyzed only GAG /pellet, not GAG /DNA [Stenhamre 2008 p. 4]. Additionally as a contrary finding they found the highest proteoglycan content per cell for the NWB areas of the proximo-medial and -lateral femur condyles. Interestingly in our static culture although statistically not significant we found a trend towards a higher GAG/DNA level for the femoral notch (X) which had the second highest overall content, even slightly more than the patellar group. When compared to the GAG content per mg tissue in the bovine knee joint similar findings are true. Biopsies of the trochlea and the femur condyle showed significant higher GAG/mg levels than the tibia plateau and the NWB proximo-medial condyle [Esquisatto 1997 p. 435]. Thus we can summarize that we did not only find differences between loaded and static culture in GAG/DNA content and saw histologically supported differences in ECM production and disposure, but also saw differences and trends within the topographically distinct groups.

One might wonder how cells of regionally different locations that were seeded after 3 passages, after a clear dedifferentiation as shown in our monolayer rtPCR values were able to restitute themselves to show similar behavior and thus regional differences as seen in the natural bovine knee joint?

Furthermore it demonstrates that biopsy regions that are classically chosen for ACT like X and Y might not be capable of manufacturing the amount of extracellular GAG required to withstand the common loads, for example of the patello-femoral joint. As earlier mentioned, same was questioned by Bentley at al. after discovering an increased failure rate in mosaicplasty of the patella defects

[Bentley 2003]. When compared to these groups it becomes obvious that they seem to respond much differently to mechanotransduction with a significant upregulation of GAG production. Since this difference became clear through the loaded conditions and was not obvious during static culture, it becomes also obvious that static in vitro culture is not a good predictor for in vivo behavior of chondrocytes when they are put into a mechanically stressed system. Although these findings are the result of 21 days of 3-D culture and will thus only display short-term results they might throw a light on the early weeks post transplantation behavior of NWB cells when weight bearing is initiated and ECM is being primed. Since novel rehabilitation procedures after matrix-assisted ACT follow an accelerated load bearing protocol this has to be kept in mind [Ebert 2008].

The mRNA gene expression levels analyzed were statistically significantly different between the topographically varying regions for all analyzed parameters except for PTHrp and Aggrecan for loaded and static constructs, as well as Sox9 for loaded scaffolds and PRG-4 in the static control. As shown before, Sox9 as well as PTHrp have significant roles in chondrogenesis and maturation. De Crombrugghe et al. published a hypothetical model in prehypertrophic chondrocytes describing the role of PTHrp triggering the phosphorylation of Sox9 to help prevent the chondrocytes from becoming hypertrophic [de Crombrugghe 2000 p. 393]. We did see differences between load and no load in these genes but almost no regional distinct ones. In the static constructs PTHrp was up regulated while it showed more or less the same values as the P0 cells in the loaded group and Sox9 was down regulated compared to P0 cells for both, load and control as it has also been for monolayer culture with a trend towards a more distinct down regulation for the NWB groups compared to the femoral WB groups (especially in static culture and also for monolayer culture). One might argue that mechanical transduction has an impact on chondrocytes from different regions in concerns to ECM production as shown in our results but not as distinct in concerns of basic chondrogenesis and maturation.

Collagen X mRNA expression levels seemed not to be affected by load, contrary to PTHrp which was down regulated, and an anatomical grouping was not as easily observed as for Col I and II. But, again contrary to PTHrp topographical differences were found. The NWB femoral notch displayed the highest Col X mRNA levels for loaded and non loaded scaffolds and was significantly up regulated compared to the weight bearing femur biopsy sites and even to the anatomical partner, the NWB proximo-medial femur condyle.

In the static control there was also no distinct difference for the expression of PRG-4/SZP but a very distinct one for the loaded scaffolds. This finding by rtPCR was nicely supported by the immunohistochemical staining of exemplary scaffolds from the anatomic regions. Interestingly in this staining we discovered that not only the surface seemed to produce the SZP but it was detected in

deeper layers as well, especially for the group of the NWB femoral notch. This might be due to the fact that because of the porous structure of the scaffolds and the more light ECM, cells behaved more like in the superficial layer and less like in the deep zone where structural differences in ECM composition and chondrocyte behavior have been observed before. Darling et al. showed for example in young cartilage that the superficial zone populations produced 3 fold higher SZP mRNA levels than the growth zone population (while the second had 8 fold higher Collagen II levels) but did not reveal any significant differences between the layers of different topographies (medial and lateral condyle and trochlea groove) [Darling 2004 p. 1185]. SZP production is enhanced by loading as published before, especially by oscillating movement as used in this protocol [Grad 2006 p. 3176; Grad 2006 p. 264] but also by stretch, even in dedifferentiated chondrocytes [Das 2008 p. 387]. P3 cells seemed to respond even more than primary cells to the mechanical stress. Thus, to find different PRG-4 mRNA levels for load vs. static control was expected and a clear upregulation compared to P0 cells was found for mechanically stressed constructs - but to find different levels within the eight regional subpopulations is very interesting since it again supports our hypothesis that cells from different regions within the knee joint might, because of their distinct origin from a WB or a NWB area respond differently when being subjected to load. The mechanotransduction pathways might not lead to the same result depending on the cells origin.

Aggrecan mRNA levels did not show regional differences for the mechanically loaded constructs as well as the static control. Darling et al. also detected no differences between different zones (superficial and growth zones) and between WB regions like the femur condyles and the trochlea [Darling 2004 p. 1185]. This supports our biochemical findings. Although the GAG content varied between the mechanically induced scaffolds when put into relation GAG/DNA was statistically not significant between the regions although histology showed striking differences. The trend that was detected for both load and no load was that the cells of the patella had a pronounced upregulation in mRNA while the NWB femoral notch cells had lower levels. One might argue that it is a question of distribution within the scaffold and that, in the short time of culture (21 days) the varying subpopulations distributed their ECM and cell content differently in response to the load in the scaffolds, with i.e. the trochlea displaying much more ECM at the border where as the NWB femoral notch showed a very light but evenly distributed ECM. Slight upregulation of Aggrecan by load was not reflected in GAG synthesis [Grad 2006 p.3177; Grad 2006 p. 264] but a significant upregulation of Aggrecan by load as in our data did reflect in the significantly different GAG production. The contrary has been hypothesized by Darling et al. [Darling 2004 p. 1186]. Thus, trends between different subpopulations in mRNA levels might not be statistically relevant due to the complex statistical model used but trends detected might still be valuable. Similar findings were true for the COMP mRNA expression level. As suspected [Grad 2006 p. 3176; Grad 2006 p. 264; Giannoni 2003] mRNA

levels were significantly upregulated by mechanical stress but as for Aggrecan a clear anatomical grouping was not possible. For loaded and non-loaded scaffolds, the tibia groups showed the least response in the COMP mRNA levels while highest levels were reached by the patella again for the loaded group and in the static group the NWB proximo-medial femur condyle showed by far the highest levels. Thus, the chondrocytes from the patella-femoral joint seemed to respond to mechanical load by a higher upregulation of mRNA for Aggrecan and COMP and a higher deposition of GAG/DNA biochemically and histologically although a statistically relevant p-value could not always be reached due to the complex analysis based on the inter-group dependencies and multiple variants. Areas of high levels of compression stress show a higher content of proteoglycans which is in accordance with our data [Brama 2000 p. 25; Jurnvelin 2000 p. 674;Kiviranta 1987 p. 275, p. 807; Bjelle 1975] since the patello-femoral joint has been defined as such [Simon 1970].

When comparing load versus no load, Collagen I, X and MMP-1 and -3 did not respond significantly to mechanical stress although trend could be detected here. But mRNA levels for Collagen II, Aggrecan, COMP, Sox9, PRG-4, PTHrp and MMP-13 differed significantly. To mechanical stress all of these genes showed an upregulation but PTHrp and MMP-13 , which seemed down regulated by load.

Collagen I expression has been previously shown to be down regulated by mechanical stress [Das 2008 p. 387; Salzmann 2009 p. 2513] which was not true when loaded groups were compared with static cultured groups in our experiment. The WB femoral groups of the condyle and the patello-femoral joint showed higher Col I mRNA levels than the NWB areas or the tibia plateau. This was already true for the monolayer expansion culture and was again detected in 3-D culture, for mechanically stressed and static scaffolds. Thus, cells of different topographical origin kept their different mRNA expression behavior although they had been dedifferentiated in monolayer culture and redifferentiated in 3-D culture plus subjected to load. While Collagen I expression seemed to be not responsive to mechanical stress, Collagen II was. But it was not only responsive to load but showed group differences within the eight topographical distinct cell populations in the static as well as in the loaded constructs. Darling et al. had previously published that although different mRNA levels for Col II were found for distinct zones, they could not detect topographically different values for the femoral condyles and the trochlea [Darling 2004 p. 1185]. But Stenhamre et al. found differences between the WB femur groups and the tibia plateau. Tibia groups had less PG, Col II and more Col I than femur groups in histological stains [Stenhamre 2008 p. 4]. Again, a distinct variation could be seen between the patella and the NWB proximo-medial femoral condyle, with highest values for the patello-femoral joint in our data. This was true for both major groups, load and no load. As for the monolayer culture, lowest and thus least favorable values were found for the NWB areas while the highest Col II mRNA expression was detected for the patello-femoral joint. This was

also the group of highest GAG/DNA values and seemed to show the highest cell metabolism and ECM production activity of all groups.

For the MMPs all topographical subpopulations showed distinct mRNA levels in the loaded as well as static group, but only MMP-13 levels seemed to be affected by the mechanical stress. Apparently MMP-1 and -3 expression is not sensitive to mechanic load, as also shown by Grad et al. [Grad 2006 p. 3174; Grad 2006 p. 264; Das 2008 p. 387]. MMP-1 showed distinct regional differences with the least upregulation for the NWB proximo-medial femoral condyle compared to some of the WB groups. This finding fits to the observation of Collagen II expression were the NWB proximo-medial femur condyle also showed lower levels than WB areas. Highest levels of expression were found for the patella group for load and control. MMP-3 showed an overall down regulation when compared to P0 cells (versus MMP-1 that was upregulated) but again the NWB area Y showed the highest downregulation which was distinct from the patello-femoral joint groups which showed again the highest expression of mRNA. Although it is discussed controversial in the current literature, MMP-3 expression has been hypothesized to be beneficial in chondrogenic differentiation by Pelttrari et al. [Pelttari 2008 p. 467] which would support our data. The patello-femoral joint, especially the patella has shown most favorable values in concerns of redifferentiation, mechanosensitivity and ECM production. MMP-13 was, as shown by Grad et al. [Grad 2006 p. 3174] down regulated in response to mechanical load as a sign of redifferentiation and had the overall lowest mRNA levels detected for the NWB proximo-medial femoral condyle again as for MMP-1 and -3. Furthermore, Y seemed to show the most pronounced response to mechanical load. Interestingly the NWB proximo-medial femur condyle Y showed not only this distinct difference in MMP expression levels for 3-D culture but also for the monolayer expansion culture. Thus, one might argue again that the cell population of Y kept a certain characteristic all through monolayer and 3-D culture which is distinct from other subpopulations, as has been shown by our data for the patello-femoral group.

Chondrocytes may comprehend a specific regional topology shown by the regionally individual biochemical and gene expression findings in monolayer culture, throughout dedifferentiation as well as in 3-D culture, even when mechanically stressed. Thus, the cartilage anlagen may not be totally lost during culture, which is supported by the work from Williams et al., who revealed expression of Notch 1, Delta 1, Jagged 1 and Jagged 2 in all limb mesenchymal cells at the early stages of cartilage anlagen development and reported that Notch 1 expression is needed and not lost to maintain the distinct zonal architecture by regulating cartilage differentiation and maturation [Williams 2009 p. 159; Brommer 2005 p. 148]. Stenhamre et al. also showed that differences between anatomical regions of the knee joint exist in concern of matrix components as GAG and collagen and that these differences are not abolished through de- and redifferentiation [Stenhamre 2008 p. 5]. And Bevill et

al. described gene expression differences among central and peripheral articular cartilage explants of porcine tibial groups in response to in vitro mechanical load [Bevill 2009]. The same observation is true for our data. It has been already published at the end of the 1990's that each type of zonal subpopulation of chondrocytes may respond differently to mechanical stimuli, and the cell products secreted may be specific to that zone´s function [Li 2007; Flannery 1999; Lorenzo 1998 p. 23468], thus one might hypothesize if the topographically distinct behavior is due to different fractions of zonal chondrocytes? Or are these differences based on different stages of maturation in the same population of cells as Archer et al. hypothesized already 1990 when they observed different behavior of chondroctes from different zones in agarose gel culture? [Archer 1990 p. 369]

Darling et al. published that the phenotypes of the two independent populations (superficial and growth zone in 8 month old goats) appeared to converge over the several passages, indicating a homogenization of the zonal chondrocytes to a phenotype that is not zone specific [Darling 2005 p. 430]. The same was true for pig chondrocytes from different zones in concern of gene expression and mechanical properties [Darling 2009 p. 7]. It was also published by Archer et al. that deep and superficial zone human chondrocytes did not show any differences after 14 days of monolayer culture [Archer 1990 p. 364]. Thus, we hypothesize that not zonal differences are responsible for the topology of chondrocytes in monolayer as well as 3-D culture but that different progenitor-cells or different cartilage anlagen have to exist to explain our distinct findings.

On the on hand this means that natural site specificity may be partially retained or regained on the other hand this means that chondrocytes from one region might not equal a chondrocyte from another region and that when put in a new environment, especially in concerns of mechanical load, needs may not be met. Sharma et al. published for example a study where the repair tissue of ACI (following the standard protocol by Brittberg) was biopsied after one year and compared to healthy cartilage of the same region. Differences in the GAG composition were significant and are one of the reasons why the repair tissue does not meet its requirements [Sharma 2007 p. 6].

Salter et al. published that NMDA receptor expression has a role in human articular chondrocyte mechanotransduction [Salter 2004]. So, maybe this behavior is due to different NMDA receptor types or mechanotransduction pathways in distinct cell types. Maybe there is some kind of mechano-memory of distinct chondrocytes [Grad 2009]? Or different locations inert different numbers or differently programmed progenitor cells which will behave diveresly in monolayer, 3-D culture and to mechanic force. Plumb et al. found for example, that contrary to healthy bovine cartilage, cyclic compressive loading was not stimulatory in cartilage biopsies from human femoral heads [Plumb 2005]. Is this a prove of the mechanical memory of cells? A progenitor cell population of the surface of articular cartilage has already been proclaimed 2004 by Dowthwaite et al. They were able to

isolate a progenitor cell from the surface zone of articular cartilage with certain characteristics. They suggested the Notch 1 expression in the surface zone to be a possible marker for progenitor cells in the bovine model [Dowthwaite 2004 p. 894-895] being in charge of maintaining a proliferative state [Dowthwaite 2004 p. 896]. Ito et al. also suggested a chondrocyte precursor cell residing in the jucta-fibrious portion of the cambium layer of the periosteum [Ito 2001]. Tallheden et al. published on the other hand that culture-expanded human articular chondrocytes presented similar characteristics to that of progenitor however they were not able to identify a specific progenitor cell population [Tallheden 2003].

5.10. Limitations

One limitation of this study includes the general application of a quadruped animal model which complicates data transfer to the clinical situation [Frisbie 2006 p. 142] as stated by some authors.

The strengths to this study however include suppression of an inter-individual variation which has been shown to inhibit uniformity to clinical studies in humans [Jakobson 2005] since cells were used from the same breed of animals of same age, sex, and surrounding conditions like food and activity.

Bioreactor conditions allow an optimal analysis and comparison of articular chondrocytes since standardized protocol can be applied. Thus, it is an artificial but very native stimulus avoiding individual differences. This standardized condition can never be found in any animal model, challenging in vivo studies until this point. Furthermore, any ethical challenges are being avoided.

The bovine stifle joints have been described to be similar to the human knee joint in concern of cartilage thickness, cell density per mm cartilage and anatomical regions [Stockwell 1971 p. 413]. Although when compared with quadrupeds human cartilage seems to be thicker at first than it would be predicted for man's body weight (cartilage thickness is related to body weight in a simple allometric manner stated by Simon in 1970), if adjusted to the fact of more limps like in quadrupedal animals, cartilage thickness is brought into closer conformity with large animal models [Stockwell 1971 p. 417] Following the August Krogh Principle " for a large number of problems there will be some animal of choice, or a few such animals, in which it can be most conveniently studied" [Krogh 1929] the bovine model was thus chosen because of the similarities to the human joint in cartilage thickness, anatomical regions, cell density and of course availability and numerous previous works establishing the bovine model for cartilage research [Williamson 2003; Kim 2003; Athanasiou 1991]. Another animal possibly used but harder to obtain cadaver specimens from would have been the horse [Frisbie 2006]. Femoral condylar cartilage shows a 25 fold difference between small species

(mice) which have very cellular cartilage and large species such as cow or man [Stockwell 1971 p. 413]. Cows show an average cell density of $2.0*10^4$ cells/mm^3 compared to human ($1.4*10^4$ cells/mm^3). When compared with smaller animals used in research such as mice $33.4*10^4$ cells/mm^3 or rat $26.5*10^4$ cells/mm^3 the cow seems to be most comparable to the human condition. More comparable seem even the number of cells to 1 mm^2 articular surface. In cows, the surface presents with $3.3*10^4$ cells /mm^3 whereas humans show only a little less on average ($3.1*10^4$ cells /mm^3).

Current articular cartilage engineering studies use homogenous cell mixtures from immature animals (1-6 weeks old) that include chondrocytes from all zones. This cell source while excellent at producing large amounts of extracellular matrix, is not clinically relevant or even practical. Furthermore, fetal cartilage does not exhibit the zonal structure present in mature articular cartilage [Darling 2005 p.426]. The zones develop incrementally, with the superficial zone forming first, followed by the middle and deep zones. In 8 month old goats for example, the superficial zone has been formed while the remaining depth of cartilage is not fully differentiated. This zone is termed the “growth zone” and will eventually develop into the middle and deep zone of mature articular cartilage [Darling 2004].

One might argue that only young, healthy bovine joint cartilage was used and that, when put into a model of diseased or aged cartilage as in treated patients cells will behave differently as shown in works of other authors [Acosta 2006; Williamson 2003]. We used a healthy cartilage model to carry out this basic research to investigate basic cellular behavior of chondrocytes of different topographical origin, thus diminishing further bias or variations in results due to a zonal variation of chondrocyte origin, or a diseased or aged state of the chondrocytes.

Many authors use GAPDH as the housekeeping gene [Darling 2005; Das 2008; Lin 2008] instead of 18S ribosomal RNA as in our experimental set up [Grad 2005 p. 251; Salzmann 2009]. Unfortunately this makes a direct comparison of mRNA levels between different authors impossible.

The medium used to cultivate the chondrocytes was used to make results comparable to previous works of other members under similar conditions and contained FCS. One might argue that a serum free medium might have had advantages as well. Serum free medium culture conditions have been described to redifferentiate chondrocytes, because the presence of serum is thought to impede chondrocyte expansion and differentiation at higher concentrations [Matmati 2013]. Chondrocytes cultured in serum-free medium supplemented with an insulin-containing serum substitute show more active expression of type II collagen and aggrecan than chondrocytes in standard monolayer culture [Blunk 2002; Goldring 1994]. Serum-free cultured chondrocytes also exhibit better redifferentiation capacity when they are subsequently cultivated in 3-D systems [Mandl 2004].

Besides the conditions of cell and construct culture, cell seeding densities for the 3-D cultures are also a topic of discussion. A key aspect of cell seeding is the initial number of cells that one should use per scaffold or per defect volume. Despite its importance, only few systematic studies have been performed to examine the effects of the initial cell density on cartilage formation and determine the optimal cell number [Concaro 2008]. Puelacher and colleagues published a study in 1994 [Puelacher 1994 p. 52] in which scaffolds were seeded at a density ranging from $20*10^6$ up to $100*10^6$ cells/ml. Higher seeding densities resulted in increased cartilaginous formation. High density is also another critical requirement for chondrocyte phenotype maintenance. Studies have shown that dedifferentiated chondrocytes regain their phenotype and re-express chondrocyte–specific type II collagen and aggrecan when they reach condensation [Deng 2002; Schulze-Tanzil 2002].

We used $4*10^6$ with 150µl fibrin-thrombin solution in a 4x8mm cylindrical scaffold in our experimental set up. Grad et al. seeded scaffolds of the same dimension for example with an initial density of $25\text{-}30*10^6$ [Grad 2005 p. 250; Wimmer 2004 p. 1441]. But it has been reported that chondrocytes can already stabilize their phenotype when cultured at a lower density of $4*10^5$ cells/cm^2 [Ruggiero 1993 p. 873].

It has to be kept in mind, that in the clinical application, such high numbers can often not be achieved. While in an animal model the number of chondrocytes might not be an issue, the biopsy amount in patients is limited due to its site and due to the fact that the new defect site should be held as small as possible to avoid new biomechanical problems. It has been shown by a study of Simonian et al., that none of the harvest sites for articular cartilage biopsies, declared to be non weight bearing, were truly non weight bearing [Simonian 1998]. Thus, keeping this in mind, it seems to be best to keep the biopsy size and extend as small as possible to prevent donor site morbidity.

Cell expansion during monolayer culture should be limited too, due to the problem of dedifferentiation [Kang 2007; Darling 2005; Wiseman 2004; von der Mark 1977; Mayne 1976] in monolayer culture into a more fibroblastic cell type and the occurrence of senescence and thus proliferation stop. In the original protocol of Brittberg published in 1994 $2.6\text{-}5*10^6$ cells were used in a suspension of 50 - 100µl [Brittberg 1994 p. 890], Henderson et al. obtained about $3*10^6$ cells/cm^2 [Henderson 2006 p. 254]. We chose comparable cell seeding and density conditions based on previous work of our group [Salzmann 2009 p. 215] and to keep the experiment close to the clinical reality. This is also the reason why we chose to take P3 cells that had previously been passaged and cultivated in monolayer expansion culture than taking P0, freshly isolated cells. If we had chosen larger seeding densities or had taken P0 cells [Grad 2005 p.250], the results might have been more striking especially the histological sections but would have been far from the clinical reality of cell harvest and culture and thus probably too positive and enthusiastic.

5.11. Future Directions

There are two major problems in cartilage repair that need to be addressed: The first is to fill the defect with a tissue that has the same mechanical properties as the articular cartilage it has to replace. The second is to promote a successful integration of this repair tissue into the native cartilage.

So far these two major problems have not yet been solved. There has not been found a satisfying method to fully redifferentiate chondrocytes after several monolayer passages - a method still needed to gain sufficient cell numbers. To further complicate this, we showed that there is a topology to de - and redifferentiation of chondrocytes in the knee joint. The composition and structural arrangement of ECM of a tissue engineered construct is never identical to the cartilage in the joint. And we furthermore added to this that cells from different topographical regions respond distinctly to mechanical force.

It has been shown before that distinct scaffolds of different material such as polyglycolic acid or collagen enhance distinct ECM components. The first enhanced proteoglycan synthesis whereas the second promoted collagen production [Grande 1997 p. 215]. Thus, when put into context with the findings that different regions are composed of different proportions of ECM components due to the static or intermittent compressive load and shear stress, as early studies carried out on dogs by Kiviranta et al. showed [Kiviranta 1987], one might reason that the defect has to be treated accordingly to its anatomical compartment. This means, since the topographical differences within the knee joint have been widely described they should not be ignored when restoration of function is discussed. The same material might not be the best matrix used for chondrocytes depending on the defect site since it has to meet different criteria.

We state that the same might be true for the cell source chosen. It has been shown before that chondrocytes from different cartilage zones behave differently in monolayer culture and when exposed to mechanical force [Li 2007], although these differences seemed to diminish after a few passages [Darling 2010]. This supports our finding that cells from different topographical regions behaved differently with the distinction that cells did behave differently partly over a long time in monolayer and did again behave differently when transferred back into a redifferentiating surrounding with a 3-D matrix and mechanical stress. Thus we conclude that the topological differences are not due to distinct fractions or distribution of zonal chondrocytes.

Benya et al. were able to redifferentiate chondrocytes in an alginate system after dedifferentiating them in monolayer culture back to the initial cell type [Benya 1982]. This has never been achieved again afterwards. Maybe because laboratory methods improved and analysis became more detailed and exact, maybe because the group had used 8 week old New Zealand rabbits with young, potent

chondrocytes. Darling et al. stated that contrary to their expectation, alginate encapsulation proved inadequate as a means to fully redifferentiate the expanded chondrocytes. The gene expression for all cells at all passage points never returned to initial levels. But they showed a more phenotypically correct gene expression when passaged less than 3 times. When compared to initial levels, however, all passage groups showed major phenotypic changes in gene expression [Darling 2005 p. 428].

To support redifferentiation after monolayer culture, a redifferentiation medium (high glucose DMEM, ITS (insulin transferring selenium A supplement) with ascorbic acid and TGF-bβ2, insulin like growth factor IGF-1 has been introduced [Mandl 2004]. Das et al. showed re-expression after monolayer expansion of Col II and X indicating the return to a more chondrogenic phenotype after this redifferentiation medium was applied [Das 2008 p. 387], Blunk et al. found an increase of GAG and construct growth as well as collagen content with IGF-1, IL-4 and TGF-β [Blunk 2002]. But this chondrogenic environment did not significantly change the alterations in gene expression levels of matrix component by expanded chondrocytes associated with stretch [Das 2008 p. 388]. BMP-2 was able to decelerate the dedifferentiation and reinforce the maintenance of chondrocyte phenotype in monolayer culture [Ma 2013] and has been shown to improve chondrocyte performance in a transduction model [Salzmann 2009]. TGF-β2 applied to monolayer culture maintains the proliferation ability [de Haart 1999].

Culture systems inducing hypoxia [Babur 2013 p. 5; Markway 2013 p. 6; Foldager 2011 p. 237] or establishing co-cultures [Taylor 2012 p. 580], medium supplements [Enochson 2012 p. 308], viral transduction [Salzmann 2009] to induce growth factor production and varying systems of bioreactors [Concaro 2009; Marolt 2006 p. 3; Pörtner 2005 p. 237; Darling 2003 p.15-23], all methods trying to enhance chondrocyte redifferentiation and ECM production are a wide field of intense research [Giannoni 2006]. It would be interesting to further investigate if for example cells of different topographical origin behave differently, not only under monolayer conditions and when exposed to mechanical stress but also when cultivated with redifferentiating medium supplements.

How could the ACI procedure be further adapted to these findings? One could be the right cell source for a certain defect location, others can be certain matrix materials and specific redifferentiating culture systems matching specific topographical needs as well as a mechanical training to induce certain matrix compositions and arrangements before putting the engineered tissue back into a patient. A specific donor site could be chosen depending on the requirements for the defect it has to meet. Cells would be expanded in monolayer in specific culture medium and put into customized scaffolds of certain materials further promoting certain cellular behavior and production of certain components. These constructs could then be trained ex vivo with distinct training protocols varying stress and shear to establish a defect-matched ECM meeting certain criteria to fill the defect not only visibly but also in terms of the mechanical integrity.

Forming an adequate tissue substitute for joint cartilage repair is still an unsolved problem with many variables to be worked on [Steinert 2007; Redman 2005]. ACI is still a cost intensive procedure requiring two surgeries for the patient. It is an individualized medicine but since expenses in our health system are growing (especially in the aging society) the question is, if these costs are justifiable. A single step procedure would be a huge advantage and cost reduction.

Gigante et al. published a case report with suggesting a new protocol combining the single-step procedure of microfracture with a bone marrow concentrate from the iliac crest and a carrier scaffold [Gigante 2012]. A one year follow-up seemed successful - but if this approach is not a step back from ACI, leading to more fibrous cartilage repair result, has to be reevaluated in the long run.

If the right supplements and factors to promote the formation of hyaline cartilage would be discovered or combined , a scenario could be to i.e. harvest elastic, auricular cartilage, mince it and install it with a carrier gel or scaffold into a joint cartilage defect with the right supplements to promote cartilage conversion in vivo - this could be done as a single step procedure without additional laboratory cost.

To safe one surgical procedure other cell sources could be used. Mesenchymal stem cells, known to be able to differentiate into articular chondrocytes [Olderoy 2014; Knight 2013; Montoya 2013], bone marrow aspirate [Matsuda 2005] or even umbilical cord blood-derived unrestricted somatic stem cells [Soleimani 2014] could be harvested. They could be differentiated ex vivo with growth factors, or viral transfection to promote chondrocytic differentiation, maybe even completely predesigned in a bioreactor setting [Cashion 2014] before implantation. Although this would require an ex vivo laboratory part, it would still safe the patient one surgery.

This approach could also be combined with a minimal invasive technique combining precultured, differentiated mesenchymal stem cells with a carrier gel such as long-term stabile hypoallergenic hyaluronic acid like Amalian© (S&V Skin Technologies; Henningsdorf) or Surgiderm© (Allergan, Ettlingen) or a hydroxyappatit hydrogel like Radiesse© (Merz, Frankfurt) to produce an injectible cell-based gel - maybe even further enriched with encapsulated supplements that, at certain time points release messenger substances to enhance tissue integration and maturation. This might be even done under radiological control in local anesthesia - saving the patient two surgeries and reducing the costs.

Cartilage research is a prospering, busy, promising and ever growing field of intense research. It will be interesting to follow and challenging to take part in the developments of the upcoming years. As Franz Werfel (1890-1945) statet: Die Welt fängt im Menschen an.

6. Summary

By design and function, articular cartilage is a tissue which gets exposed to large and complex stress fields during joint movement. The biomechanical structure is composed to withstand this stress and adapt to it by reorganizing its structural composition. But when defects occur the self healing and intrinsic repair abilities are low.

The autologous chondrocyte implantation (ACI) is one way to repair cartilage defects, mostly used in the knee joint. Generally, cartilage biopsies can be taken from several non weight-bearing (NWB) areas of the knee joint, a gold standard as not been implemented yet. After monolayer expansion culture the chondrocytes are then implanted into a load-bearing defect site with multiple techniques available.

ACI has been applied for the treatment of cartilage defects of all six joint surfaces in the knee joint for almost 20 years now. Just recently the DGOU, German Society of Orthopaedic Surgery and Traumatology published a review with recommendations for the indications for ACI installing it as a gold standard for large diameter defects.

Still, results and outcomes, especially long term developments of joint function and patient satisfaction/ quality of life are not uniform. They vary from region to region, from method to method and author to author. The highest frequency of repair is found at the distal femur and to a lesser extent at the patello-femoral joint. It has - been stated in several studies that defect repair of the patello-femoral joint has a higher rate of failure compared to other regions of the knee joint. These heterogeneous outcomes might be due to the topographical and zonal variations of the knee joint, which include diverseness at the cellular, biochemic-compositional and structural level reported. These differences might influence the integration of repair tissue, mechanical response, matrix composition and structure - even on a cellular level.

We ask whether topographic differences observed in the native cartilage of the knee joint can be found not only on a compositional level but on a cellular one. How durable is this diverseness? Can it still be observed after the isolation of chondrocytes, a monolayer expansion phase with dedifferentiation of cells and maybe even after reentering into a redifferentiating 3-D culture system? Moreover, do these topographically distinct subpopulations of chondrocytes show distinct responses to a biomechanical stimulation to simulate in vivo condition of the joint, using a bioreactor system?

The quickly emerging field of tissue engineering holds great promise for the generation of functional tissue as substitutes for various tissues from head to toe. But a matrix formed by mixing appropriate concentrations of water and cartilage macromolecules such as collagens, proteoglycans and non-

collagenous proteins will never achieve the properties of articular cartilage. To produce a tissue that could duplicate the normal function of the synovial joint, the chondrocytes must first synthesize the right types and amounts of molecules for the extracellular matrix and then assemble, organize and structure them into a highly ordered framework. Scientific investigations have shown that chondrocyte activity is stimulated by the mechanical demands made by the environment and extracellular matrix is assembled accordingly.
Bioreactors for tissue engineering have hence been used in a diversity of experimental settings to promote chondrocyte redifferentiation after monolayer culture, tissue growth and maturation.

To compare different donor sites for healthy cartilage biopsies in ACI, biopsies where taken at eight topographically distinct locations in freshly slaughtered stifle joints. Cells were expanded in a conventional monolayer expansion culture since in the clinical protocol of ACI, this step is still crucial to gain sufficient cell numbers for implantation. During this monolayer culture (42 days until Passage 7) the different subpopulation were analyzed for their proliferation and dedifferentiation behavior.
At passage 3 (P3) the cells were seeded into biodegradable polyurethane-scaffolds. These scaffolds were either cultured as free swelling control groups or mechanically loaded to simulate in vivo conditions. Biomechanical stress was induced with a customized bioreactor following a special loading protocol, simulating in vivo joint movement with specific compressive load and shear stress forces. After 21 days of 3-D culture the constructs were analyzed biochemically as well as for mRNA expression levels for 11 chondrocyte specific genes and histologically examined.
Biochemically, DNA and Glycosaminoglycan (GAG) content were analyzed in the 3-D constructs. DNA content was evaluated to gain information about proliferation activity during 3-D culture and to put into perspective the GAG content to cell number and thus examine the differences in cellular productivity.
Chondrocyte characterizing gene expression levels were evaluated using rtPCR to gain information about mRNA expression levels. For this purpose the following markers were chosen: Collagen (Col) I, II, X, cartilage oligomeric protein (COMP), Aggrecan, PRG-4 (Superficial Zone Protein), Sox9, Matrix Metalloproteinase (MMP) -1,-3,-13 and Parathyroid hormone related peptide (PTHrp).

The initial number of cells that was enzymatically isolated from the articular cartilage biopsies showed significant regional differences. Peak cellular densities were found at the femoral condyles and the medial tibia plateau, followed by the lateral tibia plateau. The lowest cell density was found at the proximo-medial femoral condyle.

During monolayer culture only for the first passage a striking difference between regional sub-populations in population doubling was seen**.** The cells of the non weight-bearing area biopsy sites, showed the highest cell proliferation activity, together with the tibial groups, followed by the condyle

groups. The chondrocytes from the patello-femoral contact showed the poorest cell dividing activity. These initial differences seemed to diminish over passage time.

Significant diverseness in the gene expression levels between the eight topographically different populations during monolayer culture was found depending on the gene and the passage number.

Overall significant differences for the eight subpopulations could be found for Col I, II, X, COMP, MMP-1 and MMP-3 and partly MMP-13. No topographically distinct differences could be detected for the entire time of culture for Aggrecan, PRG-4 and Sox9 and PTHrp. Partly this significant diverseness was clearly anatomically grouped, sometimes even distinct between weight-bearing and non weight-bearing areas. The Col I/II level for example showed a clear trend and grouping depending on the anatomical compartment of origin over the entire cultivation period. Starting with the first passage, the ratio between Col I/II was lowest for the cells of non weight-bearing origin, followed by the tibia plateau, the patello-femoral joint and the femoral condyles, showing the highest, and thus least favorable ratio.

After 3-D culture there was no statistically significant difference between loaded and non-loaded, free swell control groups in concerns of the wet weight of scaffolds. But when comparing mean overall DNA, mean overall GAG and GAG/DNA among all scaffolds that were cultured under static conditions with constructs that were cultured within the bioreactor, the difference was significant for all parameters. Total mean DNA content was higher for the loaded groups as well as average GAG production, thus the ratio of GAG/DNA was significantly higher for the bioreactor groups as well. Mean overall DNA content was significantly different between the regional subpopulations that were cultured under static conditions, while this diverseness was not significant between regions that were cultured within the bioreactor. There was no significant difference between the subpopulations in the mean overall GAG production among constructs that were cultured under static conditions, while mean overall GAG production among the distinct regions subjected to load was significantly different for the distinct regions, especially between the patella region and the non weight-bearing proximo-medial femoral condyle. This finding was nicely supported with histological staining clearly showing the difference in extracellular matrix deposition between these anatomically distinct regions.

The mRNA gene expression levels detected were significantly different between the topographically varying regions in 3-D culture for all analyzed parameters except for the expression of Aggrecan and PTHrp for loaded constructs as well as the static control scaffolds. For PRG-4 in loaded scaffolds this finding could be supported by immunohistochemical stains. No distinct regional variations were also found for PRG-4 for the static control constructs as well as for Sox9 for the mechanically loaded

scaffolds. When expression levels were compared between load and no load in general, all mRNA expression levels seemed to respond significantly to mechanical stress but Collagen I, X, MMP-1 and -3, although trends could be detected here. Thus, this comparison was significant for the remaining genes Collagen II, Aggrecan, COMP, Sox9, PRG-4, PTHrp and MMP-13. Among those, the expression of genes was constantly higher in response to the mechanical load except for the expression of PTHrp and MMP-13, which was more strongly expressed among static controls and seemed to be down regulated in response to mechanical stress.

In the static, free swell 3-D culture Collagen I, MMP-1 and PTHrp were upregulated, while expression of Collagen II and X, Aggrecan, Sox9, COMP and MMP-3 were down-regulated when compared to freshly isolated chondrocytes, P0. Levels for PRG-4 and MMP-13 showed no significant differences when compared to P0 cells.

In the mechanically stressed 3-D cultures Collagen I, PRG-4, MMP-1 were upregulated while expression for Collagen II and X, COMP, Sox9, and MMP-3 were downregulated when compared to freshly isolated chondrocytes, P0. Aggrecan, MMP-13 and PTHrp displayed more or less the same values as P0 cell.

In our experimental set-up we were able to show that topographical variances in the knee joint seem to originate from different cellular behavior. Persistent regional differences were observed in dedifferentiating monolayer culture, as well as in redifferentiating 3-D culture with and without mechanical stimulation on an intracellular level (rtPCR), biochemically (GAG production) as well as microscopically (histology).

On the one hand this means that natural site specificity of chondrocytes may be partially retained or regained. On the other hand this means, that chondrocytes from one region might not equal chondrocytes from another region and that, when put in a new environment, especially in concerns of mechanical load, needs may not be met. Thus, when put into context with the findings that different regions are composed of different proportions of extracellular matrix components due to the static or intermittent compressive load and shear stress we add to this that they also comprise differently behaving cells. We thus reason that the defect has to be treated accordingly to its anatomical compartment and biomechanical needs. This means, since the topographical differences within the knee joint have been widely described they should not be ignored when restoration of function is discussed. We hypothesize that not zonal differences are responsible for the topology of chondrocytes in monolayer as well as 3-D culture. Maybe different progenitor-cells or different cartilage anlagen exist to explain our distinct findings.

7. Literature

Acosta, C. A.; Izal, I.; Ripalda, P.; Douglas-Price, A. L.; Forriol, F.
Gene expression and proliferation analysis in young, aged, and osteoarthritic sheep chondrocytes effect of growth factor treatment
J Orthop Res 2006, 24: 2087–2094

Akizuki, S.; Mow, V. C.; Muller, F.; Pita, J. C.; Howell, D. S.; Manicourt, D. H.
Tensile properties of human knee joint cartilage: I. Influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus
J Orthop Res 1986, 4: 379–392

Archer, C. W.; McDowell, J.; Bayliss, M. T.; Stephens, M. D.; Bentley, G.
Phenotypic modulation in sub-populations of human articular chondrocytes in vitro
J Cell Sci 1990, 97: 361–371

Archer, C. W.; Francis-West, P.
The chondrocyte
Int J Biochem Cell Biol 2003, 35: 401–404

Aristotle.
Arts of Animals.
Vol. XII, Cambridge, MA:Harvard University Press. 1918. Trans. By E.S. Forster, p. 167-169

Armstrong, C. G.; Bahrani, A. S.; Gardner, D. L.
In vitro measurement of articular cartilage deformations in the intact human hip joint under load
J Bone Joint Surg Am. 1979, 61: 744–755

Arokoski, J.; Kiviranta, I.; Jurvelin, J.; Tammi, M.; Helminen, H. J.
Long-distance running causes site-dependent decrease of cartilage glycosaminoglycan content in the knee joints of beagle dogs
Arthritis Rheum 1993, 36: 1451–1459

Athanasiou, K. A.; Rosenwasser, M. P.; Buckwalter, J. A.; Malinin, T. I.; Mow, V. C.
Interspecies comparisons of in situ intrinsic mechanical properties of distal femoral cartilage
J Orthop Res 1991, 9: 330–340

Athanasiou, K. A.; Shah, A. R.; Hernandez, R. J.; LeBaron, R. G.
Basic science of articular cartilage repair
Clin Sports Med 2001, 20: 223–247

Avicenna.
The Canon of Medicine.
New York: A.M. Kelley, 1970. Trans. By O.C. Gruner, Chap. 16, p.94

Aydelotte, M. B.; Kuettner, K. E.
Differences between sub-populations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production
Connect Tissue Res 1988, 18: 205–222

Babur, B. K.; Ghanavi, P.; Levett, P.; Lott, W. B.; Klein, T.; Cooper-White, J. ; Crawford, R.; Doran, M. R.
The interplay between chondrocyte redifferentiation pellet size and oxygen concentration
PLoS One 2013, 8: e58865

Bachrach, N. M.; Valhmu, W. B.; Stazzone, E.; Ratcliffe, A.; Lai, W. M.; Mow, V. C.
Changes in proteoglycan synthesis of chondrocytes in articular cartilage are associated with the time-dependent changes in their mechanical environment
J Biomech 1995, 28: 1561–1569

Behrens, P.; Bitter, T.; Kurz, B.; Russlies, M.
Matrix-associated autologous chondrocyte transplantation/implantation (MACT/MACI)-5-year follow-up
Knee 2006, 13: 194–202

Benedek, T. G.
A history of the understanding of cartilage
Osteoarthritis Cartilage 2006, 14: 203–209

Bentley, G.; Biant, L. C.; Carrington, R W J; Akmal, M.; Goldberg, A.; Williams, A. M.; Skinner, J. A.; Pringle, J.
A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee
J Bone Joint Surg Br 2003, 85: 223–230

Benya, P. D.; Padilla, S. R.; Nimni, M. E.
Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture
Cell 1978, 15: 1313–1321

Benya, P. D.; Padilla, S. R.; Nimni, M. E.
The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis
Biochemistry 1977, 16: 865–872

Benya, P. D.; Shaffer, J. D.
Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels
Cell 1982, 30: 215–224

Bevill, S. L.; Briant, P. L.; Levenston, M. E.; Andriacchi, T. P.
Central and peripheral region tibial plateau chondrocytes respond differently to in vitro dynamic compression
Osteoarthritis Cartilage 2009, 17: 980–987

Bjelle, A.
Content and composition of glycosaminoglycans in human knee joint cartilage. Variation with site and age in adults
Connect Tissue Res 1975, 3: 141–147

Blunk, T.; Sieminski, A. L.; Gooch, K. J.; Courter, D. L.; Hollander, A. P.; Nahir, A. M.; Langer, R.; Vunjak-Novakovic, G.; Freed, L. E.
Differential effects of growth factors on tissue-engineered cartilage
Tissue Eng 2002, 8: 73–84

Bonassar, L. J.; Grodzinsky, A. J.; Frank, E. H.; Davila, S. G.; Bhaktav, N. R.; Trippel, S. B.
The effect of dynamic compression on the response of articular cartilage to insulin-like growth factorI
J Orthop Res 2001, 19: 11–17

Braham, R.; Dawson, B.; Goodman, C.
The effect of glucosamine supplementation on people experiencing regular knee pain
Br J Sports Med 2003, 37: 45-9

Brama, P. A.; Tekoppele, J. M.; Bank, R. A.; Karssenberg, D.; Barneveld, A.; van Weeren, P R
Topographical mapping of biochemical properties of articular cartilage in the equine fetlock joint
Equine Vet J 2000, 32: 19–26

Brittberg M.
Autologous chondrocyte transplantation.
Clin Orthop Relat Res. 1999 Oct;(367 Suppl):S147-55

Brittberg, M.; Peterson, L.; Sjögren-Jansson, E.; Tallheden, T.; Lindahl, A.
Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments.
J Bone Joint Surg Am. 2003;85-A Suppl 3:109-15

Brittberg, M.; Lindahl, A.; Nilsson, A.; Ohlsson, C.; Isaksson, O.; Peterson, L.
Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation
N Engl J Med 1994, 331: 889–895

Brittberg, M.; Sjögren-Jansson, E.; Thornemo, M.; Faber, B.; Tarkowski, A.; Peterson, L.; Lindahl, A.
Clonal growth of human articular cartilage and the functional role of the periosteum in chondrogenesis
Osteoarthr Cartil 2005, 13: 146–153

Brittberg, M.
Autologous chondrocyte implantation--technique and long-term follow-up
Injury 2008, 39: 40-9

Brommer, H.; Brama, P A J; Laasanen, M. S.; Helminen, H. J.; van Weeren, P R; Jurvelin, J. S.
Functional adaptation of articular cartilage from birth to maturity under the influence of loading: a biomechanical analysis
Equine Vet J 2005, 37: 148–154

Buckwalter, J. A.
Articular cartilage: injuries and potential for healing
J Orthop Sports Phys Ther 1998, 28: 192–202

Buckwalter, J. A.
Activity vs. rest in the treatment of bone, soft tissue and joint injuries
Iowa Orthop J 1995, 15: 29–42

Buckwalter, J. A.; Mankin, H. J.
Articular cartilage: degeneration and osteoarthritis, repair, regeneration, and transplantation
Instr Course Lect 1998, 47: 487–504

Buckwalter, J. A.; Martin, J.; Mankin, H. J.
Synovial joint degeneration and the syndrome of osteoarthritis
Instr Course Lect 2000, 49: 481–489

Buckwalter, J. A.; Mankin, H. J.; Grodzinsky, A. J.
Articular cartilage and osteoarthritis
Instr Course Lect 2005, 54: 465–480

Bueno, E. M.; Bilgen, B.; Carrier, R. L.; Barabino, G. A.
Increased rate of chondrocyte aggregation in a wavy-walled bioreactor
Biotechnol Bioeng 2004, 88: 767–777

Bujia, J.; Sittinger, M.; Minuth, W. W.; Hammer, C.; Burmester, G.; Kastenbauer, E.
Engineering of cartilage tissue using bioresorbable polymer fleeces and perfusion culture
Acta Otolaryngol 1995, 115: 307–310

Buschmann, M. D.; Gluzband, Y. A.; Grodzinsky, A. J.; Hunziker, E. B.
Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture
J Cell Sci 1995, 108: 1497–1508

Cameron, H. U.; Pillar, R. M.; Macnab, I.
The microhardness of articular cartilage
Clin Orthop Relat Res 1975, 275–278

Caron, M. M. J.; Emans, P. J.; Coolsen, M. M. E.; Voss, L.; Surtel, D. A. M.; Cremers, A.; van Rhijn, L. W.; Welting, T. J. M.
Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures
Osteoarthritis Cartilage 2012, 20: 1170–1178

Cashion, A. T.; Caballero, M.; Halevi, A.; Pappa, A.; Dennis, R. G.; van Aalst, J. A.
Programmable mechanobioreactor for exploration of the effects of periodic vibratory stimulus on mesenchymal stem cell differentiation.
Biores Open Access. 2014 Feb 1;3: 19-28

Chacko, S.; Abbott, J.; Holtzer, S.; Holtzer, H.
The loss of phenotypic traits by differentiated cells. VI. Behavior of the progeny of a single chondrocyte
J Exp Med 1969, 130: 417–442

Chen, C. T.; Burton-Wurster, N.; Borden, C.; Hueffer, K.; Bloom, S. E.; Lust, G.
Chondrocyte necrosis and apoptosis in impact damaged articular cartilage
J Orthop Res 2001, 19: 703–711

Chen, H-C; Lee, H-P; Sung, M-L; Liao, C-J; Hu, Y-C
A novel rotating-shaft bioreactor for two-phase cultivation of tissue-engineered cartilage
Biotechnol Prog 2004, 20: 1802–1809

Cheng, T.; Maddox, N. C.; Wong, A. W.; Rahnama, R.; Kuo, A. C.
Comparison of gene expression patterns in articular cartilage and dedifferentiated articular chondrocytes
J Orthop Res 2012, 30: 234–245

Cherubino, P.; Grassi, F. A.; Bulgheroni, P.; Ronga, M.
Autologous chondrocyte implantation using a bilayer collagen membrane: a preliminary report
J Orthop Surg 2003, 11: 10–15

Cohen, Z. A.; McCarthy, D. M.; Kwak, S. D.; Legrand, P.; Fogarasi, F.; Ciaccio, E. J.; Ateshian, G. A.
Knee cartilage topography, thickness, and contact areas from MRI: in-vitro calibration and in-vivo measurements
Osteoarthr Cartil 1999, 7: 95–109

Concaro, S.; Gustavson, F.; Gatenholm, P.
Bioreactors for tissue engineering of cartilage
Adv Biochem Eng Biotechnol 2009, 112: 125–143

Concaro, S.; Nicklasson, E.; Ellowsson, L.; Lindahl, A.; Brittberg, M.; Gatenholm, P.
Effect of cell seeding concentration on the quality of tissue engineered constructs loaded with adult human articular chondrocytes
J Tissue Eng Regen Med 2008, 2: 14–21

Correia, C.; Pereira, A. L.; Duarte, A. R C; Frias, A. M.; Pedro, A. J.; Oliveira, J. T.; Sousa, R. A.; Reis, R. L.
Dynamic culturing of cartilage tissue: the significance of hydrostatic pressure
Tissue Eng Part A 2012, 18: 1979–1991

Crawford D. C., DeBerardino T. M., Williams R. J. 3rd.
NeoCart, an autologous cartilage tissue implant, compared with microfracture for treatment of distal femoral cartilage lesions: an FDA phase-II prospective, randomized clinical trial after two years.
J Bone Joint Surg Am. 2012 Jun 6;94:979-89.

Crawford D. C., Heveran C. M., Cannon W. D. Jr, Foo L. F., Potter H. G.
An autologous cartilage tissue implant NeoCart for treatment of grade III chondral injury to the distal femur: prospective clinical safety trial at 2 years.
Am J Sports Med. 2009 Jul;37(7):1334-43.

Crombrugghe, B. de; Lefebvre, V.; Behringer, R. R.; Bi, W.; Murakami, S.; Huang, W.
Transcriptional mechanisms of chondrocyte differentiation
Matrix Biol 2000, 19: 389–394

Curl, W. W.; Krome, J.; Gordon, E. S.; Rushing, J.; Smith, B. P.; Poehling, G. G.
Cartilage injuries: a review of 31,516 knee arthroscopies
Arthroscopy 1997, 13: 456–460

Darling, E. M.; Athanasiou, K. A.
Rapid phenotypic changes in passaged articular chondrocyte subpopulations
J Orthop Res 2005, 23: 425–432

Darling, E. M.; Athanasiou, K. A.
Articular cartilage bioreactors and bioprocesses
Tissue Eng 2003, 9: 9–26

Darling, E. M.; Hu, J. C. Y.; Athanasiou, K. A.
Zonal and topographical differences in articular cartilage gene expression
J Orthop Res 2004, 22: 1182–1187

Darling, E. M.; Pritchett, P. E.; Evans, B. A.; Superfine, R.; Zauscher, S.; Guilak, F.
Mechanical properties and gene expression of chondrocytes on micropatterned substrates following dedifferentiation in monolayer
Cell Mol Bioeng 2009, 2: 395–404

Das, R. H. J.; Jahr, H.; Verhaar, J. A. N.; van der Linden, J. C.; van Osch, G. J. V. M.; Weinans, H.
In vitro expansion affects the response of chondrocytes to mechanical stimulation
Osteoarthr Cartil 2008, 16: 385–391

Davisson, T.; Sah, R. L.; Ratcliffe, A.
Perfusion increases cell content and matrix synthesis in chondrocyte three-dimensional cultures
Tissue Eng 2002, 8: 807–816

Deng, Y.; Zhao, K.; Zhang, X-F.; Hu, P.; Chen, G-Q.
Study on the three-dimensional proliferation of rabbit articular cartilage-derived chondrocytes on polyhydroxyalkanoate scaffolds
Biomaterials 2002, 23: 4049–4056

Deutsches Institut für Normung. Wear; Terms; System Analysis of Wear Processes; Classification of Wear Phenomena. DIN 50320-1. Berlin: Beuth-Verlag, 1979;
Deutsches Institut für Normung. Tribology: Terms. DIN 50323-2. Berlin: Beuth- Verlag, 1995

DiCesare, P. E.; Morgelin, M.; Mann, K.; Paulsson, M.
Cartilage oligomeric matrix protein and thrombospondin 1. Purification from articular cartilage, electron microscopic structure, and chondrocyte binding
Eur J Biochem 1994, 223: 927–937

Dowthwaite, G. P.; Bishop, J. C.; Redman, S. N.; Khan, I. M.; Rooney, P.; Evans, D. J. R.; Haughton, L.; Bayram, Z.; Boyer, S.; Thomson, B.; Wolfe, M. S.; Archer, C. W.
The surface of articular cartilage contains a progenitor cell population
J Cell Sci 2004, 117: 889–897

Dozin, B.; Malpeli, M.; Cancedda,R.; Bruzzi,P.; Calcagno,S.; Molfetta,L.; Priano,F.; Kon,E.; Marcacci, M.
Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial
Clin J Sport Med 2005, 15: 220–226

Driesang, I. M.; Hunziker, E. B.
Delamination rates of tissue flaps used in articular cartilage repair
J Orthop Res 2000, 18: 909–911

Ebert, J. R.; Robertson, W. B.; Lloyd, D. G.; Zheng, M. H.; Wood, D. J.; Ackland, T.
Traditional vs accelerated approaches to post-operative rehabilitation following matrix-induced autologous chondrocyte implantation (MACI): comparison of clinical, biomechanical and radiographic outcomes
Osteoarthritis Cartilage 2008, 16: 1131–1140

Eckstein, F.; Faber, S.; Mühlbauer, R.; Hohe, J.; Englmeier, K-H; Reiser, M.; Putz, R.
Functional adaptation of human joints to mechanical stimuli
Osteoarthr Cartil 2002, 10: 44–50

Eckstein, F.; Hudelmaier, M.; Putz, R.
The effects of exercise on human articular cartilage
J Anat 2006, 208: 491–512

Eckstein, F.; Tieschky, M.; Faber, S.; Englmeier, K. H.; Reiser, M.
Functional analysis of articular cartilage deformation, recovery, and fluid flow following dynamic exercise in vivo
Anat Embryol 1999, 200: 419–424

Edlich, M.; Yellowley, C. E.; Jacobs, C. R.; Donahue, H. J.
Oscillating fluid flow regulates cytosolic calcium concentration in bovine articular chondrocytes
J Biomech 2001, 34: 59–65

Ehrlich, M. G.; Mankin, H. J.; Jones, H.; Wright, R.; Crispen, C.; Vigliani, G.
Collagenase and collagenase inhibitors in osteoarthritic and normal cartilage
J Clin Invest 1977, 59: 226–233

Enochson, L.; Brittberg, M.; Lindahl, A.
Optimization of a chondrogenic medium through the use of factorial design of experiments
Biores Open Access 2012, 1: 306–313

Erggelet, C.; Sittinger, M.; Lahm, A.
The arthroscopic implantation of autologous chondrocytes for the treatment of full-thickness cartilage defects of the knee joint
Arthroscopy 2003, 19: 108–110

Esquisatto, M. A.; Pimentel, E. R.; Gomes, L.
Extracellular matrix composition of different regions of the knee joint cartilage in cattle
Ann Anat 1997, 179: 433–437

Falsafi, S.; Koch, R. J.
Growth of tissue-engineered human nasoseptal cartilage in simulated microgravity
Arch Otolaryngol Head Neck Surg 2000, 126: 759–765

Flannery, C.R.; Hughes, C.E.; Schumacher, B.L.; Tudor, D.; Aydelotte, M.B.; Kuettner, K.E.; Caterson, B.
Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and Is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism
Biochem Biophys Res Commun 1999, 254: 535–541

Foldager, C. B.; Nielsen, A. B.; Munir, S.; Ulrich-Vinther, M.; Soballe, K.; Bunger, C.; Lind, M.
Combined 3D and hypoxic culture improves cartilage-specific gene expression in human chondrocytes
Acta Orthop 2011, 82: 234–240

Franz, T.; Hasler, E. M.; Hagg, R.; Weiler, C.; Jakob, R. P.; Mainil-Varlet, P.
In situ compressive stiffness, biochemical composition, and structural integrity of articular cartilage of the human knee joint
Osteoarthritis Cartilage 2001, 9: 582–592

Freed, L. E.; Marquis, J. C.; Nohria, A.; Emmanual, J.; Mikos, A. G.; Langer, R.
Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers
J Biomed Mater Res 1993, 27: 11–23

Freed, L. E.; Vunjak-Novakovic, G.
Microgravity tissue engineering
In Vitro Cell Dev Biol Anim 1997, 33: 381–385

Frisbie, D. D.; Cross, M. W.; McIlwraith, C. W.
A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee
Vet Comp Orthop Traumatol 2006, 19: 142–146

Galen.
On the Usefulness of Various Parts of the Body.
Othaca, NY: Cornell University Press, 1968. Trans. By M.T. May, Book 16, ii (1:683)

Giannoni, P.; Cancedda, R.
Articular chondrocyte culturing for cell-based cartilage repair: needs and perspectives
Cells Tissues Organs 2006, 184: 1–15

Giannoni, P.; Siegrist, M.; Hunziker, E. B.; Wong, M.
The mechanosensitivity of cartilage oligomeric matrix protein (COMP)
Biorheology 2003, 40: 101–109

Gigante, A.; Cecconi, S.; Calcagno, S.; Busilacchi, A.; Enea, D.
Arthroscopic knee cartilage repair with covered microfracture and bone marrow concentrate. Arthrosc Tech. 2012 Sep 14;1:e175-80

Goldring M. B., Birkhead J. R., Suen L. F., Yamin R., Mizuno S., Glowacki J., Arbiser J. L., Apperley J. F.
Interleukin-1 beta-modulated gene expression in immortalized human chondrocytes
J Clin Invest. 1994, 94(6): 2307-16.

Goldring, M. B.
Update on the biology of the chondrocyte and new approaches to treating cartilage diseases
Best Prac Res Clin Rheumatol 2006, 20: 1003–1025

Goldring, M. B.; Tsuchimochi, K.; Ijiri, K.
The control of chondrogenesis
J Cell Biochem 2006, 97: 33–44

Gorna, K.; Gogolewski, S.
Biodegradable polyurethanes for implants. II. In vitro degradation and calcification of materials from poly(epsilon-caprolactone)-poly(ethylene oxide) diols and various chain extenders
J Biomed Mater Res 2002, 60: 592–606

Grad, S.; Salzmann, G. M.
Chondrocytes - one cell type, different subpopulations
Orthopade 2009, 38: 1038-44

Grad, S.; Gogolewski, S.; Alini, M.; Wimmer, M. A.
Effects of simple and complex motion patterns on gene expression of chondrocytes seeded in 3D scaffolds
Tissue Eng 2006, 12: 3171–3179

Grad, S.; Kupcsik, L.; Gorna, K.; Gogolewski, S.; Alini, M.
The use of biodegradable polyurethane scaffolds for cartilage tissue engineering: potential and limitations
Biomaterials 2003, 24: 5163–5171

Grad, S.; Lee, C. R.; Gorna, K.; Gogolewski, S.; Wimmer, M. A.; Alini, M.
Surface motion upregulates superficial zone protein and hyaluronan production in chondrocyte-seeded three-dimensional scaffolds
Tissue Eng 2005, 11: 249–256

Grad, S.; Lee, C. R.; Wimmer, M. A.; Alini, M.
Chondrocyte gene expression under applied surface motion
Biorheology 2006, 43: 259–269

Grande, D. A.; Halberstadt, C.; Naughton, G.; Schwartz, R.; Manji, R.
Evaluation of matrix scaffolds for tissue engineering of articular cartilage grafts
J Biomed Mater Res 1997, 34: 211–220

Grodzinsky A. J., Levenston M. E., Jin M., Frank E. H.
Cartilage tissue remodeling in response to mechanical forces.
Annu Rev Biomed Eng. 2000;2:691-713

Guilak, F.; Ratcliffe, A.; Mow, V. C.
Chondrocyte deformation and local tissue strain in articular cartilage: a confocal microscopy study
J Orthop Res 1995, 13: 410–421

Haart, M. de; Marijnissen, W. J.; van Osch, G J; Verhaar, J. A.
Optimization of chondrocyte expansion in culture. Effect of TGF beta-2, bFGF and L-ascorbic acid on bovine articular chondrocytes
Acta Orthop Scand 1999, 70: 55–61

Haddo, O.; Mahroof, S.; Higgs, D.; David, L.; Pringle, J.; Bayliss, M.; Cannon, S. R.; Briggs, T W R
The use of chondrogide membrane in autologous chondrocyte implantation
Knee 2004, 11: 51–55

Hall, B. K.
A role for epithelial-mesenchymal interactions in tail growth/morphogenesis and chondrogenesis in embryonic mice
Cells Tissues Organs 2000, 166: 6–14

Harrison, P. E.; Ashton, I. K.; Johnson, W. E.; Turner, S. L.; Richardson, J. B.; Ashton, B. A.
The in vitro growth of human chondrocytes
Cell Tissue Bank 2000, 1: 255–260

Hayes, D W Jr; Brower, R. L.; John, K. J.
Articular cartilage. Anatomy, injury, and repair
Clin Podiatr Med Surg 2001, 18: 35–53

Hedbom, E.; Antonsson, P.; Hjerpe, A.; Aeschlimann, D.; Paulsson, M.; Rosa-Pimentel, E.; Sommarin, Y.; Wendel, M.; Oldberg, A.; Heinegard, D.
Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage
J Biol Chem 1992, 267: 6132–6136

Henderson, I.; Lavigne, P.; Valenzuela, H.; Oakes, B.
Autologous chondrocyte implantation: superior biologic properties of hyaline cartilage repairs
Clin Orthop Relat Res 2007, 455: 253–261

Hendrickson, D. A.; Nixon, A. J.; Erb, H. N.; Lust, G.
Phenotype and biological activity of neonatal equine chondrocytes cultured in a three-dimensional fibrin matrix
Am J Vet Res 1994, 55: 410–414

Herberhold, C.; Faber, S.; Stammberger, T.; Steinlechner, M.; Putz, R.; Englmeier, K. H.; Reiser, M.; Eckstein, F.
In situ measurement of articular cartilage deformation in intact femoropatellar joints under static loading
J Biomech 1999, 32: 1287–1295

Heyland, J.; Wiegandt, K.; Goepfert, C.; Nagel-Heyer, S.; Ilinich, E.; Schumacher, U.; Portner, R.
Redifferentiation of chondrocytes and cartilage formation under intermittent hydrostatic pressure
Biotechnol Lett 2006, 28: 1641–1648

Hinterwimmer, S.; Krammer, M.; Krötz, M.; Glaser, C.; Baumgart, R.; Reiser, M.; Eckstein, F.
Cartilage atrophy in the knees of patients after seven weeks of partial load bearing
Arthritis Rheum. 2004, 50: 2516–2520

Hjelle, K.; Solheim, E.; Strand, T.; Muri, R.; Brittberg, M.
Articular cartilage defects in 1,000 knee arthroscopies
Arthroscopy 2002, 18: 730–734

Hodge, W. A.; Fijan, R. S.; Carlson, K. L.; Burgess, R. G.; Harris, W. H.; Mann, R. W.
Contact pressures in the human hip joint measured in vivo
Proc Natl Acad Sci U S A 1986, 83: 2879–2883

Homicz, M. R.; Schumacher, B. L.; Sah, R. L.; Watson, D.
Effects of serial expansion of septal chondrocytes on tissue-engineered neocartilage composition
Otolaryngol Head Neck Surg 2002, 127: 398–408

Horas, U.; Pelinkovic, D.; Herr, G.; Aigner, T.; Schnettler, R.
Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial
J Bone Joint Surg Am 2003, 85: 185–192

Hunter W.
Of the structure and diseases of articular cartilage.
Philos Trans R Soc Lond 1743; 42: 514-521

Hunziker E. B., Rosenberg L. C.
Repair of partial-thickness defects in articular
cartilage: cell recruitment from the synovial membrane.
J Bone Joint Surg Am. 1996, 78: 721-33

Hunziker, E. B.
Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects
Osteoarthr Cartil 2002, 10: 432–463

Hunziker, E. B.; Driesang, I. M.; Morris, E. A.
Chondrogenesis in cartilage repair is induced by members of the transforming growth factor-beta superfamily
Clin Orthop Relat Res 2001, 171-81

Hunziker, E. B.; Driesang, I. M.; Saager, C.
Structural barrier principle for growth factor-based articular cartilage repair
Clin Orthop Relat Res 2001: 182-9

Hunziker, E. B.; Quinn, T. M.; Häuselmann, H-J
Quantitative structural organization of normal adult human articular cartilage
Osteoarthr Cartil 2002, 10: 564–572

Inada, M.; Yasui, T.; Nomura, S.; Miyake, S.; Deguchi, K.; Himeno, M.; Sato, M.; Yamagiwa, H.; Kimura, T.; Yasui, N.; Ochi, T.; Endo, N.; Kitamura, Y.; Kishimoto, T.; Komori, T.
Maturational disturbance of chondrocytes in Cbfa1-deficient mice
Dev Dyn 1999, 214: 279–290

Ito, Y.; Fitzsimmons, J. S.; Sanyal, A.; Mello, M. A.; Mukherjee, N.; O'Driscoll, S. W.
Localization of chondrocyte precursors in periosteum
Osteoarthritis Cartilage 2001, 9: 215–223

Jakobsen, R. B.; Engebretsen, L.; Slauterbeck, J. R.
An analysis of the quality of cartilage repair studies
J Bone Joint Surg Am. 2005, 87: 2232–2239

Jeffery, A. K.; Blunn, G. W.; Archer, C. W.; Bentley, G.
Three-dimensional collagen architecture in bovine articular cartilage
J Bone Joint Surg Br 1991, 73: 795–801

Jin, M.; Frank, E. H.; Quinn, T. M.; Hunziker, E. B.; Grodzinsky, A. J.
Tissue shear deformation stimulates proteoglycan and protein biosynthesis in bovine cartilage explants
Arch Biochem Biophys 2001, 395: 41–48

Jurvelin, J. S.; Arokoski, J. P.; Hunziker, E. B.; Helminen, H. J.
Topographical variation of the elastic properties of articular cartilage in the canine knee
J Biomech 2000, 33: 669–675

Kang, S-W.; Yoo, S. P.; Kim, B-S.
Effect of chondrocyte passage number on histological aspects of tissue-engineered cartilage
Biomed Mater Eng 2007, 17: 269–276

Kempson, G. E.; Spivey, C. J.; Swanson, S. A.; Freeman, M. A.
Patterns of cartilage stiffness on normal and degenerate human femoral heads
J Biomech 1971, 4: 597–609

Kim, T-K; Sharma, B.; Williams, C. G.; Ruffner, M. A.; Malik, A.; McFarland, E. G.; Elisseeff, J. H.
Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage
Osteoarthritis Cartilage 2003, 11: 653–664

Kiviranta, I.; Jurvelin, J.; Tammi, M.; Säämänen, A. M.; Helminen, H. J.
Weight bearing controls glycosaminoglycan concentration and articular cartilage thickness in the knee joints of young beagle dogs
Arthritis Rheum 1987, 30: 801–809

Kiviranta, I.; Tammi, M.; Jurvelin, J.; Helminen, H. J.
Topographical variation of glycosaminoglycan content and cartilage thickness in canine knee (stifle) joint cartilage. Application of the microspectrophotometric method
J Anat 1987, 150: 265–276

Knight, M. N.; Hankenson, K. D.
Mesenchymal Stem Cells in Bone Regeneration.
AdvWound Care (New Rochelle). 2013 Jul;2:306-316.

Knutsen, G.; Engebretsen, L.; Ludvigsen, T. C.; Drogset, J. O.; Grontvedt, T.; Solheim, E.; Strand, T.; Roberts, S.; Isaksen, V.; Johansen, O.
Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial
J Bone Joint Surg Am 2004, 86: 455–464

Kobayashi, T.Soegiarto, D. W.; Yang, Y.; Lanske, B.; Schipani, E.; McMahon, A. P.; Kronenberg, H. M.
Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP
J Clin Invest 2005, 115: 1734–1742

Kon, E.; Gobbi, A.; Filardo, G.; Delcogliano, M.; Zaffagnini, S.; Marcacci, M.
Arthroscopic second-generation autologous chondrocyte implantation compared with microfracture for chondral lesions of the knee: prospective nonrandomized study at 5 years
Am J Sports Med 2009, 37: 33–41

Kreuz P. C., Steinwachs M. R., Erggelet C., Krause S. J., Konrad G., Uhl M., Südkamp N.
Results after microfracture of full-thickness chondral defects in different compartments in the knee.
Osteoarthritis Cartilage. 2006 Nov;14:1119-25

Krogh, A.
Progress of Physiology
Am J Physiol 1929, 89: 243-51

Laasanen, M. S.; Töyräs, J.; Korhonen, R. K.; Rieppo, J.; Saarakkala, S.; Nieminen, M. T.; Hirvonen, J.; Jurvelin, J. S.
Biomechanical properties of knee articular cartilage
Biorheology 2003, 40: 133–140

Lanske, B.; Karaplis, A. C.; Lee, K.; Luz, A.; Vortkamp, A.; Pirro, A.; Karperien, M.; Defize, L. H.; Ho, C.; Mulligan, R. C.; Abou-Samra, A. B.; Juppner, H.; Segre, G. V.; Kronenberg, H. M.
PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth
Science 1996, 273: 663–666

LaPrade R. F., Bursch L. S., Olson E. J., Havlas V., Carlson C. S.
Histologic and immunohistochemical characteristics of failed articular cartilage resurfacing procedures for osteochondritis of the knee: a case series.
Am J Sports Med. 2008 Feb;36:360-8.

Lefebvre, V.; Huang, W.; Harley, V. R.; Goodfellow, P. N.; Crombrugghe, B. de
SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene
Mol Cell Biol 1997, 17: 2336–2346

Li, Z.; Yao, S.; Alini, M.; Grad, S.
Different response of articular chondrocyte subpopulations to surface motion
Osteoarthr Cartil 2007, 15: 1034–1041

Lin, L.; Shen, Q.; Xue, T.; Duan, X.; Fu, X.; Yu, C.
Sonic hedgehog improves redifferentiation of dedifferentiated chondrocytes for articular cartilage repair
PLoS One 2014, 9: e88550

Lin, Z.; Fitzgerald, J. B.; Xu, J.; Willers, C.; Wood, D.; Grodzinsky, A. J.; Zheng, M. H.
Gene expression profiles of human chondrocytes during passaged monolayer cultivation
J Orthop Res 2008, 26: 1230–1237

Lin, Z.; Willers, C.; Xu, J,; Zheng, M-H.
The chondrocyte: biology and clinical application
Tissue Eng 2006, 12: 1971–1984

Lohmander, L. S.; Saxne, T.; Heinegard, D. K.
Release of cartilage oligomeric matrix protein (COMP) into joint fluid after knee injury and in osteoarthritis
Ann Rheum Dis 1994, 53: 8–13

Lorenzo, P.; Bayliss, M. T.; Heinegard, D.
A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age
J Biol Chem 1998, 273: 23463–23468

Ma, B.; Leijten, J C H; Wu, L.; Kip, M.; van Blitterswijk, C A; Post, J. N.; Karperien, M.
Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture
Osteoarthritis Cartilage 2013, 21: 599–603

Malpeli, M.; Randazzo, N.; Cancedda, R.; Dozin, B.
Serum-free growth medium sustains commitment of human articular chondrocyte through maintenance of Sox9 expression.
Tissue Eng. 2004, 10: 145-55

Mandl, E. W.; van der Veen, S. W.; Verhaar, J. A. N.; van Osch, G. J. V. M.
Multiplication of human chondrocytes with low seeding densities accelerates cell yield without losing redifferentiation capacity
Tissue Eng 2004, 10: 109–118

Manicourt, D. H.; Pita, J. C.
Quantification and characterization of hyaluronic acid in different topographical areas of normal articular cartilage from dogs
Coll Relat Res 1988, 8: 39–47

Mankin, H. J.
The response of articular cartilage to mechanical injury
J Bone Joint Surg Am 1982, 64: 460–466

Mansour, J. M.; Mow, V. C.
The permeability of articular cartilage under compressive strain and at high pressures
J Bone Joint Surg Am 1976, 58: 509–516

Marcacci, M.; Kon, E.; Zaffagnini, S.; Vascellari, A.; Neri, M. P.; Iacono, F.
New cell-based technologies in bone and cartilage tissue engineering. II. Cartilage regeneration
Chir Organi Mov 2003, 88: 42–47

Marcacci, M.; Berruto, M.; Brocchetta, D.; Delcogliano, A.; Ghinelli, D.; Gobbi, A.; Kon, E.; Pederzini, L.; Rosa, D.; Sacchetti, G. L.; Stefani, G.; Zanasi, S.
Articular cartilage engineering with Hyalograft C: 3-year clinical results.
Clin Orthop Relat Res. 2005, 435: 96-105

Marijnissen, A. C. A.; Lafeber, F. P. J. G.
Re: E. B. Hunziker.
Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. Osteoarthr. Cartil. 2002, 10: 432-63
Osteoarthr Cartil 2003, 11: 300-1; author reply 302-4

Mark, K. von der; Gauss, V.; Mark, H. von der; Müller, P.
Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture
Nature 1977, 267: 531–532

Markway, B. D.; Cho, H.; Johnstone, B.
Hypoxia promotes redifferentiation and suppresses markers of hypertrophy and degeneration in both healthy and osteoarthritic chondrocytes
Arthritis Res Ther 2013, 15: 92

Marlovits, S.; Tichy, B.; Truppe, M.; Gruber, D.; Schlegel, W.
Collagen expression in tissue engineered cartilage of aged human articular chondrocytes in a rotating bioreactor
Int J Artif Organs 2003, 26: 319–330

Marlovits, S.; Hombauer, M.; Tamandl, D.; Vècsei, V.; Schlegel, W.
Quantitative analysis of gene expression in human articular chondrocytes in monolayer culture
Int J Mol Med 2004, 13: 281–287

Marlovits, S.; Tichy, B.; Truppe, M.; Gruber, D.; Vécsei, V.
Chondrogenesis of aged human articular cartilage in a scaffold-free bioreactor
Tissue Eng 2003, 9: 1215–1226

Marlovits, S.; Zeller, P.; Singer, P.; Resinger, C.; Vécsei, V.
Cartilage repair: generations of autologous chondrocyte transplantation
Eu J Radiol 2006, 57: 24–31

Marolt, D.; Augst, A.; Freed, L. E.; Vepari, C.; Fajardo, R.; Patel, N.; Gray, M.; Farley, M.; Kaplan, D.; Vunjak-Novakovic, G.
Bone and cartilage tissue constructs grown using human bone marrow stromal cells, silk scaffolds and rotating bioreactors.
Biomaterials. 2006, 27: 6138-49

Martinek, V.
Anatomy and Pathophysiology of hyaline Cartilage
Dt. Zeitschrift für Sportmedizin 2003, 54: 166-170

Matmati, M.; Ng, T. F.; Rosenzweig, D. H.; Quinn, T. M.
Protection of bovine chondrocyte phenotype by heat inactivation of allogeneic serum in monolayer expansion cultures
Ann Biomed Eng 2013, 41: 894–903

Matsuda, C., Takagi, M., Hattori, T., Wakitani, S., Yoshida, T.
Differentiation of Human Bone Marrow Mesenchymal Stem Cells to Chondrocytes for Construction of Three-dimensional Cartilage
Tissue. Cytotechnology. 2005 Jan;47:11-7

Mau, E.; Whetstone, H.; Yu, C.; Hopyan, S.; Wunder, J. S.; Alman, B. A.
PTHrP regulates growth plate chondrocyte differentiation and proliferation in a Gli3 dependent manner utilizing hedgehog ligand dependent and independent mechanisms
Dev Biol 2007, 305: 28–39

Mayne, R.; Vail, M. S.; Mayne, P. M.; Miller, E. J.
Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity
Proc Natl Acad Sci U.S.A. 1976, 73: 1674–1678

Mengshol, J. A.; Vincenti, M. P.; Brinckerhoff, C. E.
IL-1 induces collagenase-3 (MMP-13) promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation by p38 MAPK and JNK pathways
Nucleic Acids Res 2001, 29: 4361–4372

Micheli, L. J.; Browne, J. E.; Erggelet, C.; Fu, F.; Mandelbaum, B.; Moseley, J. B.; Zurakowski, D.
Autologous chondrocyte implantation of the knee: multicenter experience and minimum 3-year follow-up
Clin J Sport Med 2001, 11: 223–228

Minas, T.; Bryant, T.
The role of autologous chondrocyte implantation in the patellofemoral joint
Clin Orthop Relat Res 2005, 30–39

Montoya, F.; Martínez, F.; García-Robles, M.; Balmaceda-Aguilera, C.; Koch, X.; Rodríguez, F.; Silva-Álvarez, C.; Salazar, K.; Ulloa, V.; Nualart, F.
Clinical and experimental approaches to knee cartilage lesion repair and mesenchymal stem cell chondrocyte differentiation.
Biol Res. 2013;46:441-51

Morgagni, J.B.
The Seats and Causes of Diseases Investegated by Anatomy.
London: Millar and Cadell, 1769; Vol. 3. Trans. by B. Alexander (original 1761), p. 321

Mow, V. C.; Kuei, S. C.; Lai, W. M.; Armstrong, C. G.
Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments
J Biomech Eng 1980, 102: 73–84

Mukaida, T.; Urabe, K.; Naruse, K.; Aikawa, J.; Katano, M.; Hyon, S-H.; Itoman, M.
Influence of three-dimensional culture in a type II collagen sponge on primary cultured and dedifferentiated chondrocytes
J Orthop Sci 2005, 10: 521–528

Niemeyer, P.; Porichis, S.; Steinwachs, M.; Erggelet, C.; Kreuz, P. C.; Schmal, H.; Uhl, M.; Ghanem, N.; Südkamp, N. P.; Salzmann, G.
Long-term outcomes after first-generation autologous chondrocyte implantation for cartilage defects of the knee.
Am J Sports Med. 2014 Jan;42(1):150-7.

Niemeyer, P.; Andereya, S.; Angele, P.; Ateschrang, A.; Aurich, M.; Baumann, M.; Behrens, P.; Bosch, U.; Erggelet, C.; Fickert, S.; Fritz, J.; Gebhard, H.; Gelse, K.; Günther, D.; Hoburg, A.; Kasten, P.; Kolombe, T.; Madry, H.; Marlovits, S.; Meenen, N. M.; Müller, P.E.; Nöth, U.; Petersen, J. P.; Pietschmann, M.; Richter, W.; Rolauffs, B.; Rhunau, K.; Schewe, B.; Steinert, A.; Steinwachs, M. R.; Welsch G. H.; Zinser, W.; Albrecht, D.
Autologous chondrocyte implantation (ACI) for cartilage defects of the knee: a guideline by the working group "Tissue Regeneration" of the German Society of Orthopaedic Surgery and Traumatology (DGOU).
Z Orthop Unfall. 2013 Feb;151:38-47

Niemeyer, P.; Pestka, J. M.; Kreuz, P. C.; Salzmann, G. M.; Köstler, W.; Südkamp, N. P.; Steinwachs, M.
Standardized cartilage biopsies from the intercondylar notch for autologous chondrocyte implantation (ACI).
Knee Surg Sports Traumatol Arthrosc 2010 Aug;18:1122-7

Niemeyer, P.; Pestka, J. M.; Kreuz, P. C.; Erggelet, C.; Schmal, H.; Suedkamp, N. P.; Steinwachs, M.
Characteristic complications after autologous chondrocyte implantation for cartilage defects of the knee joint.
Am J Sports Med. 2008 Nov;36:2091-9.

Niemeyer, P.; Lenz P.; Kreuz, P.C.; Salzmann, G. M.; Sudkamp, N. P.; Schmal, H.; Steinwachs, M.
Chondrocyte-seeded type I/III collagen membrane for autologous chondrocyte transplantation: prospective 2-year results in patients with cartilage defects of the knee joint
Arthroscopy 2010, 26: 1074–1082

Niemeyer, P.; Steinwachs, M.; Erggelet, C.; Kreuz, P. C.; Kraft, N.; Köstler, W.; Mehlhorn, A.; Südkamp, N. P.
Autologous chondrocyte implantation for the treatment of retropatellar cartilage defects: clinical results referred to defect localisation
Arch Orthop Trauma Surg 2008, 128: 1223–1231

Niemeyer, P.; Pestka, J. M.; Salzmann, G. M.; Südkamp, N. P.; Schmal, H.
Influence of cell quality on clinical outcome after autologous chondrocyte implantation. Am J Sports Med. 2012 Mar;40:556-61.

Nöthen, M.; Böhm K.
Themenheft 48: Krankheitskosten; Gesundheitsberichterstattung des Bundes
Robert Koch Institut; Statistisches Bundesamt, 2009

Nugent, G. E.; Aneloski, N. M.; Schmidt, T. A.; Schumacher, B. L.; Voegtline, M. S.; Sah, R. L.
Dynamic shear stimulation of bovine cartilage biosynthesis of proteoglycan 4
Arthritis Rheum 2006, 54: 1888–1896

Olderøy, M. Ø.; Lilledahl, M.B.;Beckwith, M. S.; Melvik, J. E.; Reinholt, F.; Sikorski, P.;Brinchmann, J. E.
Biochemical and structural characterization of neocartilage formed by mesenchymal stem cells in alginate hydrogels.
PLoS One. 2014 Mar 13;9:e91662

Ortega, N.; Behonick, D. J.; Werb, Z.
Matrix remodeling during endochondral ossification
Trends Cell Biol 2004, 14: 86–93

Ossendorf, C.; Kaps, C.; Kreuz, P. C.; Burmester, G. R.; Sittinger, M.; Erggelet, C.
Treatment of posttraumatic and focal osteoarthritic cartilage defects of the knee with autologous polymer-based three-dimensional chondrocyte grafts: 2-year clinical results
Arthritis Res Ther 2007, 9: 41

Parkkinen, J. J.; Lammi, M. J.; Karjalainen, S.; Laakkonen, J.; Hyvarinen, E.; Tiihonen, A.; Helminen, H. J.; Tammi, M.
A mechanical apparatus with microprocessor controlled stress profile for cyclic compression of cultured articular cartilage explants
J Biomech 1989, 22: 1285–1291

Pavesio, A.; Abatangelo, G.; Borrione, A.; Brocchetta, D.; Hollander, A. P.; Kon, E.; Torasso, F.; Zanasi, S.; Marcacci, M.
Hyaluronan-based scaffolds (Hyalograft C) in the treatment of knee cartilage defects: preliminary clinical findings
Novartis Found Symp 2003, 249: 203-17; discussion 229-33, 234-8, 239-41

Pelttari, K.; Lorenz, H.; Boeuf,S.; Templin, M.F.; Bischel, O.;Goetzke, K.; Hsu,H-Y.; Steck, E.; Richter, W.
Secretion of matrix metalloproteinase 3 by expanded articular chondrocytes as a predictor of ectopic cartilage formation capacity in vivo
Arthritis Rheum 2008, 58: 467–474

Peterson, L.; Minas, T.; Brittberg, M.; Nilsson, A.; Sjögren-Jansson, E.; Lindahl, A.
Two- to 9-year outcome after autologous chondrocyte transplantation of the knee
Clin Orthop Relat Res 2000, 212–234

Peterson, L.; Brittberg, M.; Kiviranta, I.; Akerlund, E. L.; Lindahl, A.
Autologous chondrocyte transplantation. Biomechanics and long-term durability
Am J Sports Med 2002, 30: 2–12

Peterson L.; Vasiliadis H.S.; Brittberg M.; Lindahl A.
Autologous chondrocyte implantation: a long-term follow-up.
Am J Sports Med. 2010 Jun;38(6):1117-24

Plumb, M. S.; Aspden, R. M.
The response of elderly human articular cartilage to mechanical stimuli in vitro
Osteoarthritis Cartilage 2005, 13: 1084–1091

Poole, A. R.; Pidoux, I.; Reiner, A.; Rosenberg, L.
An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage
J Cell Biol 1982, 93: 921–937

Portner, R.; Nagel-Heyer, S.; Goepfert, C.; Adamietz, P.; Meenen, N. M.
Bioreactor design for tissue engineering
J Biosci Bioeng 2005, 100: 235–245

Puelacher, W. C.; Kim, S. W.; Vacanti, J. P.; Schloo, B.; Mooney, D.; Vacanti, C. A.
Tissue-engineered growth of cartilage: the effect of varying the concentration of chondrocytes seeded onto synthetic polymer matrices
Int J Oral Maxillofac Surg 1994, 23: 49–53

Quinn, T. M.; Grodzinsky, A. J.; Buschmann, M. D.; Kim, Y. J.; Hunziker, E. B.
Mechanical compression alters proteoglycan deposition and matrix deformation around individual cells in cartilage explants
J Cell Sci 1998, 111: 573–583

Quinn, T. M.; Hunziker, E. B.; Häuselmann, H-J.
Variation of cell and matrix morphologies in articular cartilage among locations in the adult human knee
Osteoarthr Cartil 2005, 13: 672–678

Rannou, F.; Francois, M.; Corvol, M-T.; Berenbaum, F.
Cartilage breakdown in rheumatoid arthritis
Joint Bone Spine 2006, 73: 29–36

Reboul, P.; Pelletier, J. P.; Tardif, G.; Cloutier, J. M.; Martel-Pelletier, J.
The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis
J Clin Invest 1996, 97: 2011–2019

Redman, S. N.; Oldfield, S. F.; Archer, C. W.
Current strategies for articular cartilage repair
Eu Cell Mater 2005, 9: 23-32; discussion 23-32

Reinholz, G. G.; Lu, L.; Saris, D. B. F.; Yaszemski, M. J.; O'Driscoll, S. W.
Animal models for cartilage reconstruction
Biomaterials 2004, 25: 1511–1521

Roth, V.; Mow, V. C.
The intrinsic tensile behavior of the matrix of bovine articular cartilage and its variation with age
J Bone Joint Surg Am 1980, 62: 1102–1117

Ruggiero, F.; Petit, B.; Ronziere, M. C.; Farjanel, J.; Hartmann, D. J.; Herbage, D.
Composition and organization of the collagen network produced by fetal bovine chondrocytes cultured at high density
J Histochem Cytochem 1993, 41: 867–875

Saini, S.; Wick, T. M.
Concentric cylinder bioreactor for production of tissue engineered cartilage: effect of seeding density and hydrodynamic loading on construct development
Biotechnol Prog 2003, 19: 510–521

Salter, D. M.; Wright, M. O.; Millward-Sadler, S. J.
NMDA receptor expression and roles in human articular chondrocyte mechanotransduction
Biorheology 2004, 41: 273–281

Salzmann, G. M.; Nuernberger, B.; Schmitz, P.; Anton, M.; Stoddart, M. J.; Grad, S.; Milz, S.; Tischer, T.; Vogt, S.; Gansbacher, B.; Imhoff, A. B.; Alini, M.
Physicobiochemical synergism through gene therapy and functional tissue engineering for in vitro chondrogenesis
Tissue Eng 2009, 15: 2513–2524

Saris, D. B. F.; Vanlauwe, J.; Victor, J.; Haspl, M.; Bohnsack, M.; Fortems, Y.; Vandekerckhove, B.; Almqvist, K. F.; Claes, T.; Handelberg, F.; Lagae, K.; van der Bauwhede, J.; Vandenneucker, H.; Yang, K. G. A.; Jelic, M.; Verdonk, R.; Veulemans, N.; Bellemans, J.; Luyten, F. P.
Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture
Am J Sports Med 2008, 36: 235–246

Saris, D. B.; Vanlauwe, J.; Victor, J.; Almqvist, K. F.; Verdonk, R.; Bellemans J.; Luyten, F.P.; TIG/ACT/01/2000&EXT Study Group.
Treatment of symptomatic cartilage defects of the knee: characterized chondrocyte implantation results in better clinical outcome at 36 months in a randomized trial compared to microfracture.
Am J Sports Med. 2009, 37: Suppl 1:10S-19S

Sarzi-Puttini, P.; Cimmino, M. A.; Scarpa, R.; Caporali, R.;Parazzini, F.;Zaninelli, A.; Atzeni, F.; Canesi, B.
Osteoarthritis: an overview of the disease and its treatment strategies
Semin Arthritis Rheum 2005, 35: 1–10

Schnabel, M.; Marlovits, S.; Eckhoff, G.; Fichtel, I.; Gotzen, L.; Vécsei, V.; Schlegel, J.
Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture
Osteoarthr Cartil 2002, 10: 62–70

Schulze-Tanzil, G.; de Souza, P.; Villegas Castrejon, H.; John, T.; Merker, H. J.; Scheid, A.; Shakibaei, M.
Redifferentiation of dedifferentiated human chondrocytes in high-density cultures
Cell Tissue Res. 2002, 308: 371-9

Schuurman, W.; Gawlitta, D.; Klein, T. J.; ten Hoope, W.; van Rijen, M. H.; Dhert, W. J.; van Weeren, P. R.; Malda, J.
Zonal chondrocyte subpopulations reacquire zone-specific characteristics during in vitro redifferentiation.
Am J Sports Med. 2009 Nov;37 Suppl 1:97S-104

Setton, L. A.; Mow, V. C.; Muller, F. J.; Pita, J. C.; Howell, D. S.
Mechanical behavior and biochemical composition of canine knee cartilage following periods of joint disuse and disuse with remobilization
Osteoarthritis Cartilage 1997, 5: 1–16

Shapiro, F.; Koide, S.; Glimcher, M. J.
Cell origin and differentiation in the repair of full-thickness defects of articular cartilage
J Bone Joint Surg Am 1993, 75: 532–553

Sharma, A.; Wood, L. D.; Richardson, J. B.; Roberts, S.; Kuiper, N. J.
Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage
Arthritis Res Ther 2007, 9: 79

Shepherd, D. E.; Seedhom, B. B.
Thickness of human articular cartilage in joints of the lower limb
Ann Rheum Dis 1999, 58: 27–34

Shum, L.; Nuckolls, G.
The life cycle of chondrocytes in the developing skeleton
Arthritis Res. 2002, 4: 94–106

Simon, W. H.
Scale effects in animal joints. I. Articular cartilage thickness and compressive stress
Arthritis Rheum 1970, 13: 244–256

Simon, W. H.; Friedenberg, S.; Richardson, S.
Joint congruence. A correlation of joint congruence and thickness of articular cartilage in dogs
J Bone Joint Surg Am 1973, 55: 1614–1620

Simon, W. H.; Green, W T Jr
Experimental production of cartilage necrosis by cold injury: failure to cause degenerative joint disease
Am J Pathol 1971, 64: 145–154

Simonian, P. T.; Sussmann, P. S.; Wickiewicz, T. L.; Paletta, G. A.; Warren, R. F.
Contact pressures at osteochondral donor sites in the knee
Am J Sports Med 1998, 26: 491–494

Sittinger, M.; Bujia, J.; Minuth, W. W.; Hammer, C.; Burmester, G. R.
Engineering of cartilage tissue using bioresorbable polymer carriers in perfusion culture
Biomaterials 1994, 15: 451–456

Slowman, S. D.; Brandt, K. D.
Composition and glycosaminoglycan metabolism of articular cartilage from habitually loaded and habitually unloaded sites
Arthritis Rheum 1986, 29: 88–94

Smith, R. L.; Donlon, B. S.; Gupta, M. K.; Mohtai, M.; Das, P.; Carter, D. R.; Cooke, J.; Gibbons, G.; Hutchinson, N.; Schurman, D. J.
Effects of fluid-induced shear on articular chondrocyte morphology and metabolism in vitro
J Orthop Res 1995, 13: 824–831

Soleimani M., Khorsandi L., Atashi A., Nejaddehbashi F.
Chondrogenic Differentiation of Human Umbilical Cord Blood-Derived Unrestricted Somatic Stem Cells on A 3D Beta-Tricalcium Phosphate-Alginate-Gelatin Scaffold.
Cell J. 2014 Feb 3;16:43-52

Steinert, A. F.; Ghivizzani, S. C.; Rethwilm, A.; Tuan, R. S.; Evans, C. H.; Noth, U.
Major biological obstacles for persistent cell-based regeneration of articular cartilage
Arthritis Res Ther 2007, 9: 213

Stenhamre, H.; Slynarski, K.; Petrén, C.; Tallheden, T.; Lindahl, A.
Topographic variation in redifferentiation capacity of chondrocytes in the adult human knee joint
Osteoarthr Cartil 2008, 16: 1356–1362

Stockwell, R. A.
Chondrocytes
J Clin Path 1978, 12: 7–13

Stockwell, R. A.
Inter-relationship of articular cartilage thickness and cellularity
Ann Rheum Dis 1972, 31: 424

Stockwell, R. A.
The interrelationship of cell density and cartilage thickness in mammalian articular cartilage
J Anat 1971, 109: 411–421

Stockwell, R. A.
The cell density of human articular and costal cartilage
J Anat 1967, 101: 753–763

Stoddart, M. J.; Grad, S.; Eglin, D.; Alini, M.
Cells and biomaterials in cartilage tissue engineering
Regen Med 2009, 4: 81–98

Swann, A. C.; Seedhom, B. B.
The stiffness of normal articular cartilage and the predominant acting stress levels: implications for the aetiology of osteoarthrosis
Br J Rheumatol 1993, 32: 16–25

Tallheden, T.; Dennis, J. E.; Lennon, D. P.; Sjogren-Jansson, E.; Caplan, A. I.; Lindahl, A.
Phenotypic plasticity of human articular chondrocytes
J Bone Joint Surg Am 2003, 85: 93–100

Taylor, D. W.; Ahmed, N.; Hayes, A. J.; Ferguson, P.; Gross, A. E.; Caterson, B.; Kandel, R. A.
Hyaline cartilage tissue is formed through the co-culture of passaged human chondrocytes and primary bovine chondrocytes
J Histochem Cytochem 2012, 60: 576–587

Temenoff, J. S.; Mikos, A. G.
Review: tissue engineering for regeneration of articular cartilage
Biomaterials 2000, 21: 431–440

Tuan, R. S.
A second-generation autologous chondrocyte implantation approach to the treatment of focal articular cartilage defects
Arthritis Res Ther 2007, 9: 109

Van Assche, D.; Staes, F.; Van Caspel, D.; Vanlauwe, J.; Bellemans, J.; Saris, D.B.; Luyten, F.P.
Autologous chondrocyte implantation versus microfracture for knee cartilage injury: a prospective randomized trial, with 2-year follow-up.
Knee Surg Sports Traumatol Arthrosc. 2010,;18:486-95.

van den Borne, M. P.; Raijmakers,. NJ.; Vanlauwe, J.; Victor, J.; de Jong, S. N.; Bellemans, J.; Saris, D. B.; International Cartilage Repair Society.
International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture.
Osteoarthritis Cartilage. 2007, 15(12):1397-402.

Vanlauwe, J.; Saris, D. B.; Victor, J.; Almqvist, K. F.; Bellemans, J.; Luyten, F. P.; TIG/ACT/01/2000&EXT Study Group.
Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters.
Am J Sports Med. 2011, 39(12):2566-74.

van Saase, J. L.; van Romunde, L. K.; Cats, A.; Vandenbroucke, J. P.; Valkenburg, H. A.
Epidemiology of osteoarthritis: Zoetermeer survey. Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations
Ann Rheum Dis 1989, 48: 271–280

van Susante, J. L.; Buma, P.; van Osch, G. J.; Versleyen, D.; van der Kraan, P. M.; van der Berg, W. B.; Homminga, G. N.
Culture of chondrocytes in alginate and collagen carrier gels
Acta Orthop Scand 1995, 66: 549–556

Vanwanseele, B.; Eckstein, F.; Knecht, H.; Stussi, E.; Spaepen, A.
Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement
Arthritis Rheum 2002, 46: 2073–2078

Vasiliadis, H.S.; Wasiak, J.; Salanti, G.
Autologous chondrocyte implantation for the treatment of cartilage lesions of the knee: a systematic review of randomized studies.
Knee Surg Sports Traumatol Arthrosc. 2010 Dec;18:1645-55

Veilleux, N. H.; Yannas, I. V.; Spector, M.
Effect of passage number and collagen type on the proliferative, biosynthetic, and contractile activity of adult canine articular chondrocytes in type I and II collagen-glycosaminoglycan matrices in vitro
Tissue Eng 2004, 10: 119–127

Vesalius A.
On the Fabric of the Human Body. Cartilage, Its Nature, Function and Differentiation.
San Francisco: Norman Publishing 1998; Volume 1. Trans. By W.F. Richardson and J.B. Carman, Chap. 2 pp. 269-293

van Bomhard, A.; Veit, J.; Bermueller, C.; Rotter, N.; Staudenmaier, R.; Storck, K.; The H. N.
Prefabrication of 3D cartilage contructs: towards a tissue engineered auricle--a model tested in rabbits.
PLoS One. 2013 Aug 9;8:e71667

von der Mark, K.
Immunological studies on collagen type transition in chondrogenesis
Curr Top Dev Biol 1980, 14: 199–225

von der Mark, K; Gauss, V.; von der Mark, H; Muller, P.
Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture
Nature 1977, 267: 531–532

Vortkamp, A.; Lee, K.; Lanske, B.; Segre, G. V.; Kronenberg, H. M.; Tabin, C. J.
Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein
Science 1996, 273: 613–622

Waterton, J. C.; Solloway, S.; Foster, J. E.; Keen, M. C.; Gandy, S.; Middleton, B. J.; Maciewicz, R. A.; Watt, I.; Dieppe, P. A.; Taylor, C. J.
Diurnal variation in the femoral articular cartilage of the knee in young adult humans
Magn Reson Med 2000, 43: 126–132

Wheatley, S.; Wright, E.; Jeske, Y.; MCCormack, A.; Bowles, J.; Koopman, P.
Aetiology of the skeletal dysmorphology syndrome campomelic dysplasia: expression of the Sox9 gene during chondrogenesis in mouse embryos
Ann N Y Acad Sci 1996, 785: 350–352

Widuchowski, W.; Widuchowski, J.; Trzaska, T.
Articular cartilage defects: study of 25,124 knee arthroscopies
Knee 2007, 14: 177–182

Williams, R.; Nelson, L.; Dowthwaite, G. P.; Evans, D. J R; Archer, C. W.
Notch receptor and Notch ligand expression in developing avian cartilage
J Anat 2009, 215: 159–169

Williamson, A. K.; Chen, A. C.; Masuda, K.; Thonar, E. J-M A.; Sah, R. L.
Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components
J Orthop Res 2003, 21: 872–880

Wimmer, M. A.; Grad, S.; Kaup, T.; Hänni, M.; Schneider, E.; Gogolewski, S.; Alini, M.
Tribology approach to the engineering and study of articular cartilage
Tissue Eng 2004, 10: 1436–1445

Wiseman, M.; Bader, D. L.; Reisler, T.; Lee, D. A.
Passage in monolayer influences the response of chondrocytes to dynamic compression
Biorheology 2004, 41: 283–298

Whitman W.;
Leaves of grass (song of myself), in Kaplan J: Walt Whitman: Complete Poetry and Collected Prose. New York, NY, Library of America, 1982

Wong, M.; Wuethrich, P.; Eggli, P.; Hunziker, E.
Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic stereology and quantitative autoradiography
J Orthop Res 1996, 14: 424–432

Zaucke, F.; Dinser, R.; Maurer, P.; Paulsson, M.
Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes
Biochem J 2001, 358: 17–24

8. Images

9. Acknowledgment

My greatest gratitude to my thesis supervisor Prof. Dr. A. Imhoff for giving me the opportunity to complete my thesis in his department and to Priv. Doz. Dr. G. Salzmann, my supervising tutor, for the project, mentoring, patronage and review.

It was an honor and pleasure to conduct the research work at the AO Research Institute under the supervision of Dr. M. Alini, Dr. M. Stoddart and Dr. S. Grad. They always shared their knowledge and thoughts and it is a researchers dream to work at an institute like that with best technical equipment and continuous research exchange and feedback. Technical assistance of R. Peters was always very much appreciated.

I also thank my family for their love, support and encouragement - my parents for giving me the priceless chance of freedom to pursue whatever dream and plan I had and my husband for his endless support and backup.