Advances in Autologous Chondrocyte Implantation and Related Techniques for Cartilage Repair

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PREFACE

Centuries ago, long before mapping of the genome, discovery of stem cells, and proposing of tissue engineering principles, it was acknowledged that damage to the articular cartilage would cause doctors problems. In his work from 1743, William Hunter illustrates the issue in his famous quote: "If we consult the standard Chirurgical Writers from Hippocrates down to the present Age, we shall find, that an ulcerated Cartilage is universally allowed to be a very troublesome Disease; that it admits of a Cure with more Difficulty than carious Bone; and that, when destroyed, it is not recovered"[1]. I feel confident in saying that he was not trying to predict the future but rather making a statement on the current status of the time. However, despite all the major discoveries of modern science the former has remained true – at least until very recently. During the past two decades, several new modalities for cartilage repair have been introduced aiming at restoring the articular cartilage rather than replacing it with an artificial joint prosthesis, and one may once again ask: Does Hunter's statement remain true?

The present thesis focuses on advances of an existing treatment for repair of articular cartilage after injury – the autologous chondrocyte implantation. It is based on projects carried out at the Orthopaedic Research Laboratory at Aarhus University Hospital, Institute for Clinical Medicine and MR-Research Center at Skejby Sygehus in Denmark, and Department of Orthopaedics at Brigham and Women's Hospital, Harvard Medical School, Boston, MA, and VA Medical Center, Boston, MA during my enrollment as a PhD-student at the Faculty of Health Sciences at Aarhus University.

The thesis consists of five papers and the present summery, which contain an overall background as well as discussions on the methods used in the papers and additional discussions on the results and their implications. The thesis may be read as desired, but because the five papers all contain substantial information related to the discussions in the summary, they may preferably be read prior to those chapters.

LIST OF PAPERS

(In chronological order)

PAPER 1

Foldager CB, Munir S, Ulrik-Vinther M, Soballe K, Bunger C, Lind M; Validation of Suitable Housekeeping Genes for Hypoxia-cultured Human Chondrocytes. BMC Mol Bio, 2009, Oct 9; 10 (1):94

PAPER 2

Foldager CB, Nielsen AB, Munir S, Ulrich-Vinther M, Søballe K, Bünger C, Lind M; Combined 3D- and Hypoxic Culturing Improve Cartilage-Specific Gene Expression in Human Chondrocytes. Acta Orthop, 2011 Apr;82(2):234-40

PAPER 3

Foldager CB, Ringgard S, Pedersen M, Bünger C, Lind M; Chondrocyte Gene Expression Is Affected by VSOP-Labeling in Long-Term In Vitro MRI Tracking. J Magn Reson Imaging, 2011 (33): 724-730

PAPER 4

Foldager CB, Gomoll AH, Lind M, Spector M; Cell Seeding Densities in Chondrocyte Transplantation **Techniques. Cartilage** 2012 April; 3(2): 108-117.

PAPER 5

Foldager CB, Nielsen AB, Munir S, Bünger C, Everland H, Lind M; Dermatan Sulfate in MPEG-PLGA Scaffolds Upregulates Fibronectin Gene Expression but has no Effect on in vivo Osteochochondral Repair. Int Orthop, 2012 July (36): 1507-1513.

BACKGROUND

Although maintenance of the viable human body is a result of continuous regeneration and renewal of organs and tissues throughout life, few of these are capable of full regeneration after an injury. Articular cartilage has very low intrinsic regenerative capacity, which might be due to the nature of the tissue itself, being avascular, aneural, and alymfatic, or a result of the harsh external environment, where it is exposed to a combination of shear and compression forces and restricted access to oxygen and nutrition [2-5]. Diagnosing cartilage injuries is challenging because

it cannot be determined by physical examination alone and because the clinical symptoms and findings such swelling, activity-related pain, limping, catching, locking, and feeling of instability are all non-specific. Hence, the diagnosis requires other modalities such as arthroscopy and/or magnetic resonance imaging (MRI).[6, 7] After focal cartilage damages a major concern is the increase in risk of early development of osteoarthritis [8, 9].
Articular cartilage has its own unique and complex structure. Based on the morphological appearance on a cross-section it can be divided into four layers with different arrangements of the fibers in the extracellular matrix (ECM) and the cells in these different layers have been shown to express distinct characteristics (Fig. 1) [10].

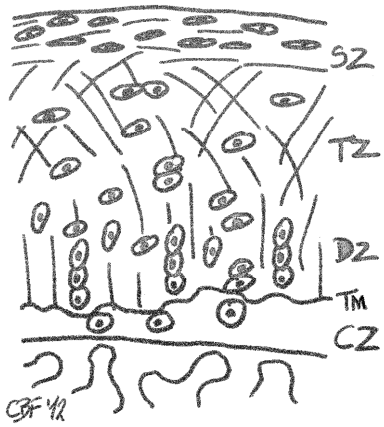

Figure 1: Schematic illustration of different zones in a cross-section of articular cartilage. In the superficial zone (SZ) the cells are flattened and the cellularity is relatively high compared to the other layers and the collagen fibers are horizontally aligned. The transitional zone (TZ) is the zone between the superficial and deep zone where the cells become more rounded and the collagen fibers change orientation from horizontal to more vertical alignment. In the deep zone (DZ) cells are aligned in columns and the collagen fibers are generally vertically aligned. The tide-mark (TM) represents a line separating the hyaline from the calcified cartilage. In the calcified zone (CZ) the cells are larger in size (hypertrophic).

What separates it from other tissues is that the healthy articular cartilage contains no blood vessel, no lymfatic system, and no nerves and the cells are subsequently exposed to low oxygen and poor nutrition [11]. Nutrients and oxygen can reach the articular cartilage by two different routes the synovial fluid and the subchondral bone. Controversy between which route is more important still exists, but there seems to be a trend among authors supportive of the subchondral bone for supply of nutrients [12] and the synovial fluid for the supply of oxygen [13, 14]. To be able to survive under these conditions chondrocytes consume oxygen to stimulate glycolysis unlike most mammalian cell types in vascularized tissues that use oxygen for oxidative phosphorylation in the mitochondria [15]. The turnover rate of the extracellular matrix is very low [16]. While the cells are able to maintain an equilibrium between the anabolic and catabolic activity under normal conditions, thus providing a homeostatic environment, the ability to adjust to an increase in anabolic demand such as in changed biomechanics or under regenerative processes is very limited. This results in limited ability for spontaneous tissue regeneration after injury.

CARTILAGE MICROENVIRONMENT

The chondrocytes are mesenchymally derived and are the sole cell type in articular cartilage and although different subpopulations are identified chondrocytes are generally accepted as a homogenous group [17]. They synthesize the ECM of cartilage but they comprise less than 5 % of cartilage dependent on patient age and joint [18, 19]. The major component of the ECM is water, which contributes to approximately 70% of its weight [19]. The main parts of the dry weight are collagen type II (50-73%) that is responsible for strong tensile properties, and proteoclycans (PG) (15-30%) mainly aggrecan that contribute to swelling pressure and compressive modulus, in part due to the acidic charged groups in the attached glycosaminoglycan (GAG) side chains [20-23] (Fig. 2). Another naturally occurring proteoglycan in articular cartilage is dermatan sulfate (DS) as part of decorin, biclycan, and the α2(IX) chain of collagen type IX [21]. The proteoglycans are bound to hyaluronic acid (HA), which is synthesized without a core protein and is the only GAG that is exclusively found in non-sulfated form.

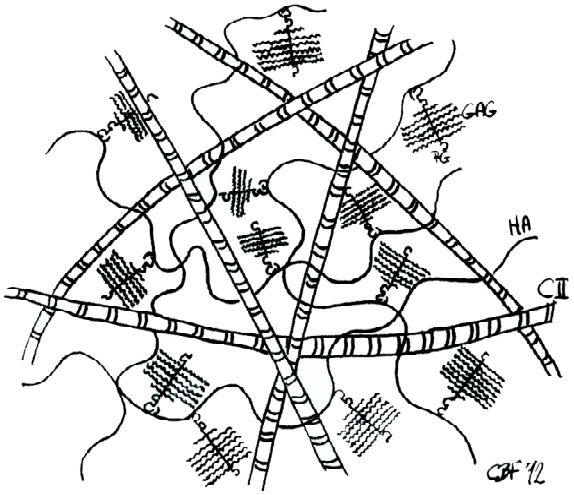

Figure 2: Schematic illustration of the cartilage ECM organization. The collagen type II (CII) constitutes a network in which proteoglycans (PG) with attached glycosaminoglycans (GAGs) are located in between attached to hyaluronic acid (HA). The negative charges on the GAGs bind water and contributes to the swelling and compressive modulus of the tissue.

The oxygen tension (partial pressure; pO_2) of ambient air progressively decreases from 21% (160 mmHg) after entering the lungs and travelling in the blood throughout the body. By the time it reaches organs and tissues, pO_2 levels have dropped to 2%–9% (14–65 mmHg) [24]. The term hypoxia being a relative measure may thus be misleading in some situations, as it is actually a physiological normoxia, and a physiological normoxia for one cell may be different from that of another cell [25]. The term hypoxia in this thesis is used as being "oxygen tension lower than expected-to-be" and in cell culture terminology hypoxia is then referred to as lower than 21%. Hence, even though the physiologic normoxia for chondrocytes is low it is termed hypoxia when

used for *in vitro* culture descriptions, as it is lower than expected-to-be for conventional cell culture. The majority of oxygen to the chondrocytes comes form the synovial fluid and the oxygen tension in synovial fluid was in 1970 estimated by the Dane Knud Lund-Olesen to be approximately 9% (65 mmHg) in patients with either osteoarthritis or rheumatoid arthritis [26]. It has later been shown that articular chondrocytes are exposed to oxygen tension between approximately 1 and 10% being highest at the superficial zone and lowest in the deep zone [14, 27].

FOCAL CARTILAGE INJURIES: ETIOLOGY AND INCIDENCE

Focal damages to the articular cartilage can be a result of traumatic compactions, shearing, or avulsion, or underlying pathologies such as in osteochondritis dissicans (OCD) [28, 29]. Chondral lesions are common and are reported in 57-66 % of patients undergoing arthroscopies [30-34]. Of these 28-67% were focal chondral lesions, 29-44% osteoarthritic lesions, and 0.7-2% OCD lesions. Curl *et al*. [30], performed a very large retrospective study including 31,516 patients undergoing arthroscopy, but they did not discriminate between osteoarthritic and focal cartilage lesions and instead divided the patients' injuries into OCD, articular fractures, and chondromalacia (CM). The latter was found in 98% of the patients with deep fissuring of the cartilage without exposed bone, which was the most frequent type of damage (41% of all patients with cartilage injury). The most common location of focal cartilage damage is the medial femoral condyle 32-58%,[32, 33] except in one study where defects in the patellar articular surface were slightly more prevalent (37.5% and 32.2%, respectively) among Outerbridge grade III and IV damages [34].

The International Cartilage Repair Society (ICRS) has recommended a classification system for focal damages where these are graded from I-IV based on both pre- and post-debridement size and depth of the defect. While the grade is based on the defect depth the exact dimensions of the defect is mapped separately [35]. The classification is largely based on the Outerbridge classification that was originally developed to assess chondromalacia of the patella at the time of meniscectomy [36]. Several other systems have also been proposed [37]. The repair of focal cartilage lesions can be managed by two different strategies: Either by transplantation of a cells or biomaterials or by stimulation of endogenous repair (e.g., by injections of growth factors etc.). The present thesis focuses mainly on the cell transplantation approach, which is a tissue engineering strategy for cartilage repair. Acknowledging that currently, no treatment is able to regenerate articular cartilage in a consistent and predictable fashion, the term cartilage repair has replaced the term regeneration.

ARTICULAR CARTILAGE REPAIR TREATMENTS

Focal cartilage damage can be managed surgically by a number of different treatments modalities. As the cartilage tissue is not spontaneously regenerated the treatment options comprise an array of possibilities to replace or restore the lost and damaged tissue. There are many different treatment strategies, but these can roughly be arranged based on defect size and whether the subchondral bone is damaged or intact. When the subchondral bone is damaged the treatment options for cartilage tissue replacement consists of transplantation of synthetic, autologous or allogenous osteochondral plugs. If the subchondral bone is intact the traditional treatment options are marrow stimulation techniques reaching the blood supply such as drilling, abrasion arthroplasty, and microfracture. The latter is an arthroscopic technique often used for defects <2cm^2 where the subchondral bone is penetrated by applying small holes allowing blood and cells from the bone marrow to form a blood cloth in the defect, which produces the regeneration tissue. For larger defects (>2cm^2) autologous chondrocyte implantation (or transplantation) can be used. This is a 2-step surgery with arthroscopic harvesting or a cartilage biopsy, isolation and culturing of the cells in vitro, and re-implantation of the cells in an open surgery procedure (Fig. 3). It should be noted that the expectations of each patient as well as other factors such as defect location and co-morbidities should be carefully addressed before selection of the appropriate treatment. More recent techniques include transplantation of particulated cartilage. These treatments are discussed in the general discusson.

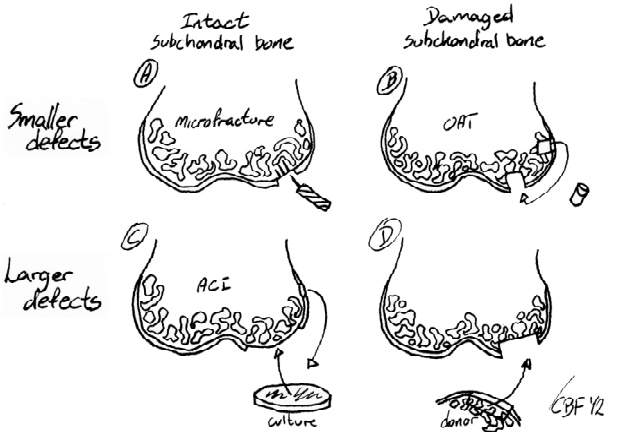

Figure 3: Overview of cartilage repair treatments. Cartilage injuries can roughly be categorized by size and whether the subchondral bone is intact or damaged. A) Microfracture with penetration of the subchondral bone. B) Osteochondral autograft transplants (OATs). An osteochodral plug is transferred from an non-weight bearing are to the defect. Alternatively, a synthetic scaffold is used. C) ACI. A biopsy is harvested in the 1st arthroscopic surgery. The cells are isolate and cultured before re-implantation in a 2nd surgery. D) Transplantation of osteochondral allografts from donors. It should be noted that this is a simplified illustration that cannot be used as an evidence-based algorithm for cartilage repair.

AUTOLOGOUS CHONDROCYTE IMPLANTATION

The present thesis focuses on optimization of the treatment modality named autologous chondrocyte implantation (ACI). In the 1970's Swedish doctor Lars Peterson was concerned with the absence of a good treatment for cartilage injuries in his athletic patients, which lead him to the idea of culturing autologous chondrocytes to expand the cell number and then re-implant them in a cartilage defect under a periosteal membrane. In 1982 Dr. Peterson was invited by professor Victor Frankel to the Hospital for Joint Disease at the Orthopaedic Institute in New York as visiting researcher. They began working on proving the hypothesis in a rabbit model and in 1984 Dr. Peterson presented the first evidence of cartilage regeneration by ACI at the Research Society meeting of the American Academy of Orthopaedic Surgeons in Atlanta, Georgia. These results were later published in 1987 by Grande *et al.* [38] Back in Sweden Dr. Peterson, Dr. Brittberg and Dr. Lindahl worked on the culture of autologous chondrocytes for

human use, and in October 1987 the first patient in the world received ACI. The outcome of the first 23 patients enrolled was later presented in 1994 by Brittberg, Peterson *et al.* [39] showing the efficacy of ACI for treatment of deep cartilage defects (personal correspondence with prof. Lars Peterson).

In brief, the ACI is two-step procedure where a cartilage biopsy is harvested arthroscopically from a non-weight bearing area in the affected joint. The cells from the biopsy are isolated and expanded *in vitro*. In the initial work of ACI the cells were cultured for 2-3 while the culture time in more recent commercial ACI-treatments is 4-6 weeks after which they are re-implanted in the defect in a second surgical procedure either under a cover of periosteum (1st generation, ACI-p); under a membrane, which is often made of collagen type I/III (2nd generation, ACI-c); or seeded onto a scaffold matrix (3rd generation, ACI-m). Third generation ACI, which is addressed in the projects in the present thesis, is often termed matrix-assisted chondrocyte implantation (MACI®) but this has been adopted as a trademark of Genzyme Biosurgery (Cambridge, MA). Thus, we use the term 3rd generation ACI or ACI-m for this treatment.

The four basic steps of the ACI treatment are depicted in Fig. 4 and each individual step is potential target for optimization and we have chosen to investigate the cell culture method [40, 41], the biomaterial used as cell carrier [42], the density of cells implanted [43] and the development of a method for tracking the cells after implantation [44] (Fig. 4).

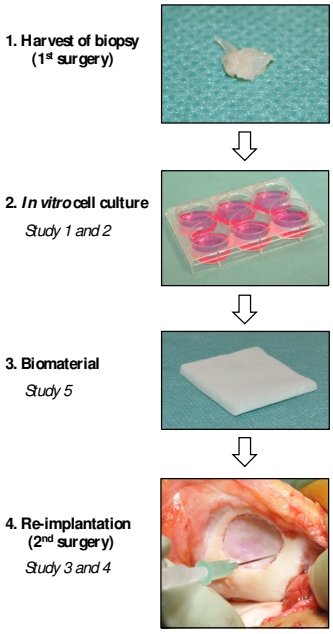

Figure 4: The four basic steps of autologous chondrocyte implantation (ACI). 1. A cartilage biopsy (2-300mg) is harvested at a non-weight bearing area in the joint in the first surgery, which is an arthroscopic procedure. 2. The cells are isolated and cultured *in vitro* to increase the number of the cells. This step usually takes 4-6 weeks. 3. In the traditional ACI-p a periosteum cover harvested from the tibia is used, but more recent ACI techniques utilize biomaterials as cover (ACI-c) or carrier/support of the cells (ACI-m). 4. Re-implantation of the cells into the defect in the second surgery where all the damaged cartilage has been surgically removed.

CLINICAL OUTCOMES IN ACI TECHNIQUES

In the original clinical work by Brittberg and Peterson using the ACI procedure with periosteal cover for treatment of full-thickness cartilage defects not involving the subchondral bone they found that 14 of 16 patients with femoral condyle lesions had good-to-excellent clinical outcomes at two years follow-up. Of the 7 patients included with patellar lesions only 2 had good-to-excellent outcomes [39]. In 2002, Peterson *et al.* [45] reported good or excellent outcomes in 50 of 61 patients 24 month after surgery and good or excellent outcomes in 51 of 61 patients after 5-11 years and showed that in 8 of 12 biopsies of the repair tissue showed hyaline characteristics. In 2003, Peterson *et al.* [46] reported the outcome of 58 patients with osteochondritis dissicans treated with ACI with 2 to 11 years follow-up in 2003. At 24 months follow-up 91% of the patients had a good (22/58) or excellent (31/58) outcomes. Other authors have confirmed the original findings, which are reviewed elsewhere [47].

To bypass the surgical step of harvesting periosteum biomaterials were investigated as substitutes for covering the defect. An experimental study in sheep comparing periosteum and collagen type I/III-covered ACI from 2005 found that periosteum stimulated osteochondral bone densification but that there were no difference in cartilage repair [48]. Similar observations on cartilage repair were reported in the first clinical study that compared 1st and 2nd generation ACI. They did not investigate the subchondral bone but they found no differences in complications and reoperations due to hypertrophy [49]. Other studies have reported significantly more patients with graft hypertrophy using periosteum cover compared to collagen type I/III membrane cover and higher reoperation rate (9%) [50, 51]. The safety and efficacy of collagen type I/III membranes have been reported in a number of prospective case series [52, 53].

In addition to using a membrane as a cover biomaterials have also been applied as a carriers for the cultured cells (*viz.*, 3rd generation ACI). Steinwachs *et al*., described the use of the collagen type I/III scaffold as cell carrier in 2009 [52], and the group later described the clinical findings with 2-years follow-up [54], while Vijayan *et al*. [55], presented 2-8 years follow-up using this method. Some treatments using 3rd generation ACI apply a membrane or porous scaffold that is only intended as a carrier for delivery of the cells while other treatments utilize biomaterials as a structural compartments for cartilage tissue engineering. All clinically approved ACI-associated treatments are addressed in paper 5, although the main focus of that paper was the density of the cells applied in the various treatments [43]. In brief, good or excellent clinical outcomes are found in approximately 75-80% of the patients [47], and the few studies using second-look arthroscopy and biopsies for histology show that no treatments have been able to generate hyaline cartilage in a consistent and predictable manner [56]. Other treatments methods for cartilage repair as well as the associated considerations are addressed in the general discussion.

SUMMARY OF THE PAPERS

(For additional figures please see the original papers)

AIM AND HYPOTHESIS

The overall aim of the present thesis was to address and investigate methods for optimizing the selected steps involved in the ACI treatments. We hypothesized that these areas were eligible for targeted optimization, which has been addressed in the five

papers constituting the work performed in the present thesis. The objectives of the separate studies and the related hypotheses are found below.

Study 1: Validation of hypoxia-suitable house-keeping genes for hypoxia-cultured human chondrocytes

We wanted to determine a set of reference genes (house-keeping genes, HKG) that were stable in hypoxic culture conditions. We hypothesized that low oxygen tension affected the expression levels of genes usually considered stable in normoxic conditions in human chondrocyte cultures. The stability was tested using two validated algorithms, geNorm [57] and Normfinder [58], by culturing human chondrocytes in 21%, 5%, and 1% oxygen and in both monolayer and on a 3D scaffold for up to 6 days. By testing nine potential reference genes for quantitative gene expression analysis we found that ribosomal protein L13a (*Rpl13A*), β_2-microglobulin (*B2m*), and human RNA polymerase II (*RpII*) were the most stable under hypoxic conditions, while traditional reference genes such as glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β-actin (*Actb*) were unstable. We also showed that the expression of these genes were stable when cultured on a 3D scaffold and that there was no benefit of using more than two reference genes for evaluating gene expression levels.

Study 2: Combined 3D- and Hypoxic Culturing Improve Cartilage-Specific Gene Expression in Human Chondrocytes

We aimed at improving traditional chondrocyte-culturing conditions by combining 3D and hypoxic culturing. We hypothesized that culturing in a native-like environment would favor expression of chondrogenic genes. Human articular chondrocytes from patients undergoing anterior cruciate ligament reconstruction was cultured in either monolayer or on a MPEG-PLGA scaffold and in 21%, 5%, and 1% oxygen for 1, 2, and 6 days. We were able to show that there was a combined positive effect on gene expression of chondrogenic markers by both 3D culture and hypoxic culture compared to culture in 2D and normoxia. This was illustrated by a gradual increase in gene expression of *Sox9*, *Agc* and *Col2a1* with lowering of oxygen tension, and that there was an additional increase in expression of *Col2a1* by addition of a 3D culturing surface. We also found that the positive effect of a 3D culturing surface was not present until 6 days of culture.

Study 3: Chondrocyte Gene Expression Is Affected by VSOP-Labeling in Long-Term In Vitro MRI Tracking

To be able to track implanted chondrocytes after implantation into a defect we aimed at determining the optimal chondrocyte labeling-concentration of human chondrocytes with very small iron oxide particles (VSOPs) in terms of gene expression and proliferation and to investigate the labeling efficacy in order to track alginate-embedded chondrocytes during a four-week period using clinically available MRI systems.

We hypothesized that VSOPs could be used for labeling of chondrocytes without affecting cell viability and that this will allow sequential tracking of these chondrocytes in clinical 1.5 T MRI system.

Intracellular labeling was obtained without the use of transfection agents simply by incubating the VSOPs with the cells for 90 minutes. We found that we were able to visualize the labeled cells in alginate beads for up to 4 weeks. However, the intracellular labeling affected the gene expression of the chondrogenic markers *Sox9*, *Col2a1* and *Agc* in an unpredictable manner. Based on these results we decided not to pursue this method as a valid technique for *in vivo* tracking of chondrocytes after implantation.

Study 4: Cell Seeding Densities in Chondrocyte Transplantation Techniques

We review of the clinical literature with the objective to investigate the cell seeding densities used in cell-based treatments currently available in the clinic for cartilage repair. We hypothesized that the chondrocyte seeding density in cell-based cartilage repair could affect the clinical outcome. In a literature study we reviewed chondrocyte seeding densities at the time of implantation in clinically approved ACI techniques. Data on chondrocyte seeding densities was obtained from the manuscripts in published clinical studies and from the company websites or representatives that process the cells. We found that relatively few authors reported chondrocyte seeding densities, and that there was little consensus of what density to use between authors of the studies that did report seeding densities. The preclinical evidence was additionally sparse. We found that in the included studies the densities used were between 0.5 and 14 million cells per square centimeter. There did not appear to be a correlation between the density of the implanted chondrocytes and clinical outcome, and although there was a tendency in favor of high seeding densities in the discussed pre-clinical studies there were no consistency in the results favoring either high- or low seeding densities.

Study 5: Dermatan Sulfate in MPEG-PLGA Scaffolds Upregulates Fibronectin Gene Expression but has no Effect on in vivo Osteochochondral Repair

In both *in vitro* and *in vivo* conditions we wanted to test the effect of dermatan sulfate (DS) addition to a clinically approved MPEG-PLGA scaffold for cartilage repair. We hypothesized that addition of DS to MPEG-PLGA scaffolds would improve chondrogenic gene expression in chondrocytes and cartilage repair in an osteochondral drill hole defect in a rabbit model. As a new biomaterial for 3rd generation ACI we tested MPEG-PLGA scaffolds with and without addition of DS *in vitro* and *in vivo*. Human chondrocytes were cultured *in vitro* for up to 14 days in 5% oxygen. We found that addition of DS increased the gene expression of *fibronectin*. This result along with encouraging unpublished results from other cell types by the research department at the company developing the scaffold led us to pursue *in vivo* experiments in an osteochondral rabbit model. Twenty New Zealand white rabbits received an osteochodral drill hole in the trochlear groove (*defects n=20*) with a diameter of 5 mm. When bleeding was observed an MPEG-PLGA scaffold with or without DS (Ø = 6 mm) was press-fitted into the defect and secured with fibrin glue. The animals were observed for 12 weeks before euthanization. The defects were evaluated by histology using hematoxylin and eosin and safranin-O stain, and immunohistochemistry using collagen type II antibodies. By the histological evaluation and scoring by the semi-quantitative O'Driscoll score we found that treatment with neither of the scaffolds resulted in regeneration of hyaline cartilage and that there was no benefit of adding DS to the scaffold.

METHODOLOGICAL DISCUSSION

In this chapter essential methods of the studies in the thesis will be addressed. The basic methodology or models will be described in order to explain the potential limitations of different models or methods and their applicability for valid interpretations and translations of the results.

GENE EXPRESSION ANALYSES: METHODOLOGY AND POTENTIAL SHORTCOMINGS

Genomic transcription and translation is the backbone of endogenous synthesis of proteins such as structural components and hormones. Through intracellular signaling pathways the transcription system is activated in the cell nucleus. Although the process is very complex it can be divided into an overall series of events from transcription to the final protein, and the general understanding of these events is essential for some of the limitations related to gene expression analyses and data interpretation. The process begins with uncoiling of DNA strands that are separated allowing the transcriptional apparatus consisting of RNA polymerase, a promoter (a DNA sequence that promotes transcription such as a TATA box), and transcription factors and various activators and suppressors to transcribe the code of the DNA strand into a complementary pre-messenger RNA strand (pre-mRNA) from the 5' end towards the 3' end of the RNA strand. The introns in the pre-mRNA are looped out in the splicosome and the exons are spliced creating mature mRNA consisting only of exons (posttranscriptional modification). This mRNA strand translocates from the nucleus to the cytosol where the translation takes place in the ribosome. Hence, the mRNA apart from being a messenger of the DNA sequence (i.e., the gene) to the ribosome, becomes an indirect measure of the expression of the specific gene [59].

Polymerase chain reaction (PCR) is a thermal cycling technique to amplify a few numbers of a specific DNA strands into a large numbers, which can be measured. Reverse-transcriptase RT-PCR is a variant of the conventional PCR method where a single RNA strand is reversed transcribed into its complementary DNA (cDNA) using the enzyme reverse transcriptase, which in turn is amplified similar to the conventional PCR method. This should not be confused with real-time PCR, also called quantitative PCR (qPCR), which is described below.

Real-time RT-PCR (RT-qPCR) is a method for quantification of mRNA and thus indirectly of the expression of a specific gene at a specific time point. In RT-qPCR techniques the probes contain a fluorescent signal (a flourophore), which is accumulated during sequence amplification of the gene sequence and detected by a RT-PCR machine. The machine measures the cyclic threshold (Ct) value, which is the number of cycles required for the amplification curve to cross the threshold line. The Ct value is thus inversely proportional to the amount of target sequence RNA. In the studies in the present thesis we have used commercially available TaqMan primers (Applied Biosystems). The TaqMan® Assay utilizes the 5' nuclease activity of Taq DNA polymerase to cleave a fluorescently labeled probe (FAM™-labeled MGB).

RT-qPCR is routinely used in gene expression analyses but there are limitations related to the method as well as the interpretation and translation of the results. One of the most important, and perhaps the method-related topic receiving the most attention, is the quality of the analyzed RNA. RNA is more unstable than DNA and is degraded thermologically and by endogenous RNAases. The stability is dependent on the 3' untranslated regions (UTR) and the 5' UTR. The down-stream consequence of low-quality RNA increases in situations where very little RNA is amplified. There is evidence that cDNA yield from sequences close to the 5' end of only partially degraded mRNA is significantly less than sequences close to the polyA tail in the 3' end [60]. However, others advocate that since amplicons used in RT-qPCR are rather small (usually <80 base pars (bp)), partially degraded RNA can safely be used (up to 250bp) as long as the expression is normalized to an internal reference gene [61]. Shorter amplicons are also amplified more efficiently than longer amplicons and are more tolerant of reaction conditions [59]. RNA quality encompasses both its purity and integrity. The quality is traditionally measured by estimation of the 28S:18S rRNA ratio using Northern analyses and/or the sample purity by the spectrometrical absorbance ratio A260/280 measuring protein contamination.[62] The theoretical values for both ratios are 2 for pure RNA samples. In the present thesis we applied the RNA Integrity Number (RIN) as a measure of RNA degradation. This method has been described by Schroeder *et al*., and although the superiority over conventional methods is debatable it has the advantage that the RNA quality measurement requires only very small samples [61, 63].

In RT-qPCR methods the magnitude of the expression of a specific gene is matched to a set of stable reference genes allowing for a relative measure of the expression. However, the stability or consistency of the expression of the reference genes needs be thoroughly addressed. This motivated paper 1 investigating the stability of hypoxia-stable reference genes [40]. The term housekeeping gene (HKG) are often used in place of "reference gene" as these often participate in general cell survival functions or as structural proteins. The term HKG may though insinuate that their expression is stable do to their function, which for a number of reasons is not the case [64]. The methods for determination of stable reference genes are described in paper 1 and in detail the papers validating the applied algorithms [40, 57, 58]. Another algorithm for evaluation of gene stability is "BestKeeper" where a graded index of up to 10 candidate reference genes can also be matched to up to 10 target genes to show whether these are differentially expressed [65]. The addition of target gene analysis separates it from the other methods for evaluation of gene expression stability. While the importance of the use of stable reference genes sometimes seems to be ignored, others have advocated that gene expression may be normalized to total RNA with accuracy of that of reference genes [59]. Using total RNA level as a reference may though be less accurate in comparing normal and tumor cells as the latter are highly proliferative and will have increased total RNA.

When using gene expression analyses it is important to keep in mind that it, for most practical applications, is the active protein and not the gene or the pre-protein that exhibit the functions. Post-translational modifications such as the addition of functional groups, phosphorylation, enzymatic removal of amino acids, and appropriate protein folding are important and determining the function of the protein. Direct use of mRNA levels as surrogate measures for protein synthesis and/or even protein function dictates that all these processes occur in a predictable manner, which is not always the case [66].

In study 1 we found that *Rpl13a*, *B2m* and *RplI* were the most stable under hypoxic conditions while traditional reference genes such as *Gapdh* and *Actb* were unstable. The expression of *Gapdh* was shown in study 2 to increase significantly with decrease in oxygen tension further emphasizing its inefficacy as a reference gene in hypoxia.[41] An issue, which has not been addressed in paper 1, is the discrepancy between the most stable genes identified using geNorm and Normfinder, respectively. GeNorm relies on the principle that in a pair of two candidate reference genes at least one of these genes is not constantly expressed. The advantage of pair-wise correlation of two reference genes limits the influence of technical variability due to different amouts of cDNA input, as this would affect both genes. However, the method makes it susceptible to bias if two or more of the analyzed genes are co-regulated as this will lead to an incorrect outcome of the most optimal normalizer pair [58]. This potential erroneous high ranking of a co-regulated candidate gene-pair is though inversely

related to the number of candidate genes analyzed. Genes coding for proteins belonging to the same protein complex are examples of genes that are often highly co-regulated. Expression patterns and possible co-regulation are difficult to foresee, and Normfinder was therefore also used for the evaluation of stable reference genes. It is an algorithm less susceptible to bias by covariance. Normfinder enables estimation of the intra- and intergroup variation, which is then combined into a stability value. Candidate control genes with the lowest intra- and intergroup variation will have the lowest stability value and will subsequently be top ranked.

HYPOXIA

Experimental hypoxia can be achieved either chemically or physical control of the oxygen tension in closed chambers [67, 68]. Working in hypoxia requires that all solutions and media used for cell cultures are buffered to achieve the oxygen tension used in the experimental setup before application. Otherwise, handling such as media change would lead to intermittent exposure of higher pO_2 and potentially confounding the results. In addition, all handling of the cells before lysing or fixation of the cells before evaluations (e.g., RT-qPCR) must be performed in hypoxia to limit confounding by changes in oxygen tension prior to the evaluation. The hypoxic culture in study 1 and 2 is performed in a closed precalibrated work chamber (Xvivo System, BioSpherix, NY), thus limiting the potential confounders described above.

In study 2 investigating the effect of combined hypoxic- and 3D culture of chondrocytes the hypoxic challenge on cellular level was validated by depicting a gradual increase in the expression of ankyrin repeat domain 37 (*Ankrd37*) with lowering of oxygen tension [41]. *Ankrd37* is a target for hypoxia inducible factor 1 (HIF-1) [69]. Hypoxia inducible factors (HIFs) are transcription factors that respond to changes in oxygen tension and they are commonly introduced in hypoxic experiments. HIF-1 plays a key role in developmental, physiological, and pathological conditions and its presence affect survival, cell-cycle progression, and metabolism [70, 71]. Although first identified as the main transcription factor activated under low oxygen tensions, HIF is a key transcription factor activated by cytokines, oncogenes, and reactive oxygen species under normal oxygen tensions [72]. HIFs are heterodimeric transcription factors consisting of an α- and a β-unit. In HIF-1 the β-unit is constitutively expressed while two oxygen dependent pathways regulate the α-unit: propyl-hydroxylase domain proteins (PHDs) and asparaginyl hydroxylases also termed factor inhibiting HIF-1 (FIH-1) [73]. The PHDs use oxygen as co-substrate for hydroxylation, which mediates binding of the von Hippel-Lindau (VHL) tumor suppressor gene, a E3 ubiquitin ligase that targets the complex for proteasome degradation. This is inhibited in hypoxia leading to an increase in HIF-1. We have not been able show an increase in *Hif-1α* expression in response to hypoxia in articular chondrocytes, while it is increased in mesenchymal stem cells in hypoxia compared to normoxia (unpublished data). Hence, HIFs were not used as controls of the hypoxic challenge. The roles and complexity of HIFs was further illustrated when two independent groups presented similar findings in the same issue of Nature Medicine of HIF-2α being an important cartilage catabolic factor in osteoarthritis [74, 75].

The oxygen tension in the micro-environment may differ depending on culture method. Culture in alginate beads and in pellets lead to oxygen gradients from the surface decreasing towards the center of the spheroid due to dependency of oxygen diffusion. This may potentially confound the data on hypoxia-related outcomes. In study 1 and 2 in the present thesis we used monolayer cultures and culture on porous scaffolds with theoretically much smaller diffusion distances, thus limiting this confounder.

THE TISSUE ENGINEERING APPROACH

Tissue engineering as described by Robert Langer and Joseph P. Vacanti is an interdisciplinary approach to produce whole organs or improve tissue or organ function by combining cells, biomaterials and stimulating factors [76]. In the past years, improving ACI-related methods applying the tissue engineering approach have mainly been focused on the biomaterials. These biomaterials are either scaffolds of natural [77] or synthetic [78] polymers or injectable materials such as gels [79]. Several factors are related to a successful development of a biomaterial for tissue engineering depending on the application. Initially the material should be biocompatible and often biodegradable. Some scaffolds serve as a delivery vehicle for exogenous cells; as a matrix facilitating migration, adhesion, proliferation, and differentiation; or provide structural support. In study 2 we used an MPEG-PLGA scaffold to simulate a 3D environment [41] and in study 5 we compared this MPEG-PLGA scaffold with an MPEG-PLGA scaffold with addition of DS, which is a naturally occurring proteoglycan in articular cartilage [21, 42]. The MPEG-PLGA is a synthetic polymer as opposed to natural polymers as the collagen type I/III scaffold often used for cartilage repair. The use of MPEG-PLGA scaffolds for cartilage repair have also been evaluated by our group in goats [80] and in rabbits [81].

The cells ability to bind to the scaffold is closely related to the cell attachment and migration. Cells attach to the ECM and scaffold materials by integrins that are heterodimeric transmembrane proteins consisting of an α- and a β-unit. In humans there are 24 combinations of α- and β-subunits, but additional variants of the α- and β-subunits are created by differential splicing [82, 83]. The biomaterials contain either ligands for integrins or bind ligands to the material. Important ligands for integrins are fibronectin, laminin, vibronectin and collagens [84]. We found that addition of dermatan sulfate (DS) lead to an increase in fibronectin expression, which we hypothesized, might facilitate early attachment and migration and subsequently cartilage repair. Fibronectin is present in from the early stages of chondrogenesis and in mature articular cartilage [85, 86]. A previous study showed that chondrocyte binding to fibronectin through α_5 containing integrins (i.e., $\alpha_5\beta_1$-integrin) provided a cell survival signal [87], while binding to fibronectin fragments by $\alpha_5\beta_1$-integrin lead to an increase in MMP13 synthesis [88]. A methodological limitation of study 5 was that we did not investigate the actual synthesis of fibronectin nor the cell migration and attachment after seeding, and the explanations for the results remain speculations. As highlighted above there are some potential benefits of the increase in fibronectin. However, there are also potential negative effects of both fibronectin and even the DS itself. DS has been shown to inhibit fibrillogenensis *in vitro* of both collagen type I and II [89], and the possible *in vivo* effect this posttranslational modification has not been addressed in our study since we used only gene expression analysis (as described earlier). Moreover, a recent study found that inhibition of fibronectin by siRNA lead to an increase in aggrecan expression and increase in aggrecan/vercican expression ratio in TGFβ1-induced chondrogenesis *in vitro* [90]. Versican is the other aggregating proteoglycan besides aggrecan but is not normally present in articular cartilage but rather in fibrous tissue. Fibronectin, which in our study

showed an increase in expression in chondrocytes *in vitro* by DS-addition to the MPEG-PLGA scaffolds, may play a role in cartilage maturation [91], but its increase have been linked to the development of osteoarthritis [92].
A major limitation of study 5 was the use of primary chondrocytes for *in vitro* analyses and an *in vivo* application that most evidently relied on chondrogenic differentiation of bone marrow-derived MSCs by implantation of a cell-free scaffold. Hence, the results *in vitro* may not be used to explain the *in vivo* effects. Because there are bidirectional effects of fibronectin and negative effect of fibronectin fragments we do not know whether the lack of improvement of cartilage repair *in vivo* was due to a the negative effects of fibronectin or whether the potential positive effects were overruled by other factors related to cartilage repair such as catabolic factors, biomechanical alterations etc. [93].
The cell density is another parameter in tissue engineering. Study 4 reviewing chondrocyte seeding densities used in the literature did not find any correlation between cell seeding density and the outcome although there was a tendency in the pre-clinical literature favoring high seeding densities. This may be a result of the multifactorial nature of cartilage repair, which in addition the type of tissue engineering approach is dependent on the post-operative rehabilitation regimen [94]. An unpublished Danish randomized controlled trial from the 90's showed no effect on clinical outcome of addition of *in vitro* cultures autologous chondrocytes in ACI-p.[95] Due to issues not related to the validity of the results, the study has never been published. The controversy of cell seeding densities still exists and although cell condensation and chondrogenesis of MSCs require high densities it is still reasonable to speculate how these processes are involved in regeneration in cartilage defects.

HISTOLOGICAL EVALUATION
Several histological scoring systems are available for semi-quantitative evaluation of cartilage regeneration and repair *in vitro* [96, 97] and *in vivo* [98-104] and an algorithm for selecting the most suitable evaluation score have been proposed [105]. Based on the proposed algorithm by Rutgers *et al*., we utilized the O'Driscoll score that were originally developed for cartilage repair in rabbits [106, 107]. The scoring systems contain of a number of subcategories and the score of each category is then summed to a total score. This potentially confounds the interpretation of the outcome since two histologically very different repair tissues might have the equal scores. For this reason we have developed an algorithm based on stereological methods for a truly unbiased and quantitative evaluation of cartilage repair that may prove an advantageous supplement to exiting semi-quantitative modalities in future studies [108]. The algorithm was developed after the completion of the studies comprising the present thesis, and is hence not applied in these.

ANIMAL MODELS: TRANSLATION OF RESULTS
Experimental animal models are commonly used for preclinical testing of drugs, devices, and surgical techniques. In study 5 we used New Zeeland White rabbits that received an osteochondral drill hole in the trochlear groove with a diameter of 5 mm (Fig. 5). The depth of the defect was approximately 2 mm. This model has been used in numerous studies although our defect diameter is larger than the average size used. There are limitations related to the model and the translation of the results to clinical practice. The anatomy of the rabbit knee is obviously different from the human knee and perhaps most significantly is the angulation of the joint leading to altered mechanical loading. In addition, the body weight of the rabbit is relatively low compared to the joint size [109]. The subchondral bone plate is relatively thick compared to the size of the joint and the cartilage layer is only <0.5 mm thick [110]. The thin cartilage layer limits the model for testing of clinical devices since most biomaterials are thicker and can thus not be implanted in a purely chondral defect. In our study the defect was made in the trochlear grove and hence in the patellofemoral compartment. Damages in the cartilage in the articulating surfaces of the patellofemoral joint have been shown to be more difficult to regenerate – at least in humans – which was demonstrated in the original Swedish trial of ACI-p [39].

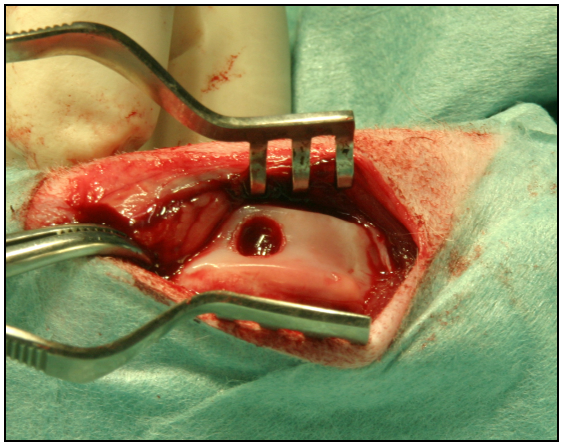

Figure 5: Perioperative image of the osteochondral defect in the trochlear groove in a rabbit knee before implantation of the scaffold. The access is made through an antero-medial incision. The penetration of the subchondral bone results in bleeding from the bottom of the defect.

Clinical cartilage repair is not performed immediately following the injury but rather 6 month or more after the acute phase. In the rabbit model used in our study, we performed repair of an acute injury and the environment may thus be different in terms of inflammation compared to the "chronic" injury.
The rabbit has the advantage of being a small animal, which reduces the costs and it is thus advantageous for screening purposes and as an initial model for *in vivo* validation. Larger animals such as goats, pigs, and horses, may be provide better evidence for clinical cartilage repair, but are limited mainly due to their cost, which could reduce the number of animals included in each group, thus subjecting the study results to the possibility of type II errors.
In our study the observation period was chosen to be 12 weeks, which other authors have found to be a sufficient observation period to see evidence of cartilage regeneration/repair. The length of the observation periods in all types of animal models for cartilage repair is an important topic as many argue that, at least in larger animals, the observation period should be at least a year to be able to show if the regenerated cartilage is able to integrate and remodel, or if the tissue deteriorates.
In our study we did not use present empty defects as negative controls. The age of the rabbits was approximately 6 month, which should ensure skeletal maturity [111]. It can be argued

whether these should have been included or not, but the defect size have previously shown critical and thus does not induce spontaneous regeneration as seen in young and adolescent animals [109]. Also, the aim was to show whether the addition of DS would improve cartilage repair, and no not to show that MPEG-PLGA scaffolds provided better cartilage repair than an untreated injury. Positive controls are difficult to produce since cartilage repair is challenging. However, comparisons to gold standard treatment could have been included, and in the present study we did in fact schedule for two groups with MPEG-PLGA scaffolds with and without DS with addition of autologous chondrocytes that was cultured for 4-6 weeks. Unfortunately, do to issues related to the handling and seeding of the cells (including 200-times differences in cell seeding densities) we were not able to include the results in our evaluations. Hence, we did in fact not analyze the impact of adding DS to MPEG-PLGA scaffolds in 3rd generation ACI, but for a type of scaffold-supported microfracture or autologous matrix-induced chondrogenesis (AMIC®) [112, 113].

NON-INVASIVE CHONDROCYTE TRACKING

The fate of the cells after implantation has important perspectives. Do the cells actually survive and to what extent? Do they settle in the defect or are they distributed throughout the joint? And finally, what are the temporal events in the defect in terms of cellular distribution and viability. Intracellular labeling for cell tracking is a potential strategy for investigation of these events. In study 3 we investigated the use of very small iron-oxide particles (VSOPs) for intracellular labeling and tracking of chondrocytes. These are citric acid coated particles with a diameter of 11 nm including the iron core of 5 nm. They produce a hypointense signal using spin echo (SE) T2-weighed magnetic resonance imaging (MRI). VSOPs are smaller than other more commonly used superparamagnetic iron oxide (SPIO) particles ultra-small SPIO and do not require transfection agents to augment the uptake in non-phagocytic cells but are taken up by endocytosis (non-specific, adsorptive pinocytosis) [114, 115]. Others authors have successfully used SPIO for intracellular labeling of MSCs and chondrocytes *in vitro [116, 117]* and recently labeling of chondrocytes *in vivo [118]*.

The utilization of a clinical 1.5 Tesla (T) MRI scanner allows clinical application and tracking of the labeled cells compared to high-field scanners, but has the disadvantage of lower resolution. Estimating cell viability and distribution *in vivo* requires a thorough *in vitro* investigation of the influence of the labeling agent. Cytotoxicity leading to decreased proliferation rate and increased cell death, interference with cell attachment, migration, and synthesis of ECM molecules are all factors that would seriously confound results obtained *in vivo* after transplantation of labeled chondrocytes.

Other methods for non-invasive labeling include optical and radioactive methods. Positron emission tomography (PET) utilizes specific radioactive tracers or tracers labeled with a radioactive isotopes. This method hold great promise for several areas including in orthopaedics [119], but due to the rapid decay of the tracer radioactivity this method is not suitable for long-term cell labeling and tracing. Most often the tracer is applied intravenously, which may limit the tracer availability in avascular cartilage tissue. Due to the similarity of the method of single-photon emission computed tomography (SPECT) this method shares the same limitations in cartilage imaging. Optical methods include fluorescent and bioluminescent methods, but both of these methods are limited by a relatively short distance of signal penetration [120].

GENERAL DISCUSSION

This chapter will provide a discussion of the results in addition to what is already found in the papers comprising the thesis. Hereafter a more general discussion of the aspects of ACI techniques for cartilage repair will be presented.

Gene expression in chondrogenesis: Interpretation of results

The master regulator of chondrogenesis of mesenchymal stem cells (MSCs) is Sry Related HMG-box 9 (SOX9), which works in conjunction with L-SOX5 and SOX6 (the "SOX trio") during chondrogenic differentiation [121]. SOX9 is a transcription factor that exerts many effects in both chondrocytes as well as in other cell types [122]. *Sox9* is expressed in chondroprogenitor and chondrocytes but the expression is decreased under chondrocyte hypertrophy [123, 124]. Transcriptional enhancers for collagen type II (*Col2a1*) and XI (*Col11a2*) have specific binding sites for SOX9 and collagen type II was originally found to be regulated directly by SOX9 [125-127]. Later it was shown that in osteoarthritic chondrocytes where *Sox9* expression is low, *Sox9* did not correlate with *col2a1* expression [128]. This finding, along with other results showing that high (compared to low) overexpression of *Sox9* led to a decrease in *Col2a1* expression and that overexpression of *Sox9* in general led to decreased *Col2a1* expression in dedifferentiated chondrocytes, highlighted a potential bifunctional effect of SOX9 on COL2A1 [129]. These discrepancies are likely to be related to expressions of *L-Sox5* and *Sox6*, which ensure the binding of SOX9 to *Col2a1* enhancers [130]. While we chose only *Sox9* for our evaluations, the importance of L-SOX5 and SOX6 was demonstrated in double mutant *Sox5*-/-;*Sox6*-/- mice that died in utero with severe generalized chondrodysplasia [131]. However, *Sox5* and *Sox6* are expressed downstream of *Sox9* and their expression is thus linked to the expression of *Sox9* adding to its properties as an appropriate marker for chondrogenesis and phenotypic evaluation of chondrocytes [123, 130].

Aggrecan (AGC) is the most abundant core protein of proteoglycans in articular cartilage and is encoded by a single gene [132]. It consists of three globular domains, G1-3, and G2 is unique to aggrecan compared to other proteoglycans. The interglobular domains containing chondroitin sulfate and keratan sulfate is located between G2 and G3 and it can contain as many as 130 GAG side chains (Fig. 6) [21]. On gene expression level *Sox9* enhances the expression of the *Agc* promotor/enhancer region [133], but this is dependent on co-expression of *L-Sox5* and *Sox6* to increase the potency of activation and ensuring the binding to the enhancer [134].

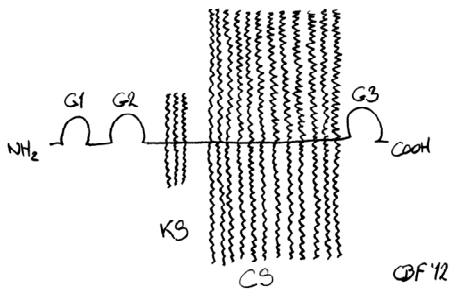

Figure 6: Aggrecan structure. It consists of three globular domains (G1-3). The interglobular domain containing keratan sulfate (KS) and chondroitin sulfate (CS) are located between G2 and G3.

Aggrecans exists in aggregates of up to 100 aggrecan molecules non-covalently bound to hyaluronic acid in the G1 region. The binding is stabilized by link protein (not shown).

Col2a1 and *Agc* gene expressions are often used as markers for chondrocyte differentiation and sometimes as surrogate markers for synthesis of the two most abundant extracellular matrix molecules in normal articular cartilage. The complex structure of these proteins requires sufficient posttranslational processing, which, as mentioned earlier, limits the assumption of proportional relationship between gene expression and presence of active protein. Also as earlier explained, SOX9 is not the sole regulator of collagen type II synthesis. In study 2 we found that the increase in *Sox9* and *Agc* gene expressions were only dependent on oxygen tension, while gene expression of *Col2a1* was additionally dependent on culture surface potentiating its expression in combined 3D and hypoxic culture. After six days of culture in hypoxia, the 3D surface did seem to facilitate the *Agc* expression compared to monolayer culture but it was not significant [41]. Whether these differences in expression are related to the co-expression patterns of *L-Sox5* and *Sox6* remain to be determined.

Collagen type I is the most abundant collagen in the human body. In articular cartilage and cartilage repair tissue it is expressed and synthesized by fibroblasts, fibrochondrocytes and chondrocytes in arthritic cartilage. Similar to collagen type II it contains three α-chains, and these are found in the combination $\alpha1(I)_2$ and α2(I) [135], and the adverse change in biomechanical properties in cartilage repair tissue compared to normal articular cartilage may in part be explained be abundance of collagen type I instead of collagen type II [136, 137]. Rather than using the expression of collagen type I as a marker for dedifferentiation of chondrocytes into a fibroblastic phenotype the ratio of collagen type II/I have been proposed [138]. In cell cultures there is a gradual increase in collagen type I and a decrease in collagen type II [139], but is uncertain what extent the individual cell express collagen type I and II simultaneously. Certainly, the initial studies advocated that the single cells must have an abrupt change in expression as only few cells showed simultaneous expression of the two collagen types [140]. Relative expression ratio was not used in our studies because of the combined information in the ratio, which means that a change in ratio can be do to either changes in the numerator or denominator.

Other important collagens participate in the fibrilliar cartilage network: Collagen IX decorates the surface for collagen type II [141] while collagen type XI functions as a template for lateral growth of the collagen type II/IX/XI heterofibrils [142], and the evaluation of the expressions of these is sometimes used in conjunction with collagen type II for matrix synthesis evaluation. Others have suggested the use of collagen type IX and Cartilage Oligomeric Matrix Protein (COMP) as markers for chondrocytes differentiation [143]. In study 2 we found that while collagen type I expression was highest in 21% oxygen, its expression in hypoxia (moderate or severe) was dependent on the culture surface. We did not investigate the possible influence of collagen type IX, XI or COMP expression in that study [41].

In prolonged *in vitro* culture chondrocytes loose their spherical shape and become elongated and begin to express collagen type I as described above [139, 140, 144]. It has been argued that the apparent dedifferentiation of chondrocytes observed during prolonged culture in monolayer is in fact not a true dedifferentiation and it has long been known that chondrocytes can re-express their differentiated phenotype when transferred to culture in more suitable environment [145, 146]. Dedifferentiation is a naturally occurring response in regeneration of certain tissues [147] and it is a mechanism in which terminally differentiated cells revert back to a less differentiated state within its own lineage to be able to proliferate before redifferentiate thus replacing the lost or damaged tissue [148]. The term phenotypic modulation may perhaps be more accurate characterization of the events observed in prolonged culture of chondrocytes because the changes in gene and protein expressions do not resemble that of chondrocyte dedifferentiation *per se* (see Fig. 7) but rather that of a fully differentiated fibroblast [149].

Robins *et al*. [150], were the first to describe an association between low oxygen and the expression of *Sox9*. Later others have found that hypoxia lead to an increase in cartilage matrix synthesis [151, 152] and to cause redifferentiation of dedifferentiated (phenotypically modulated) chondrocytes [146, 153].

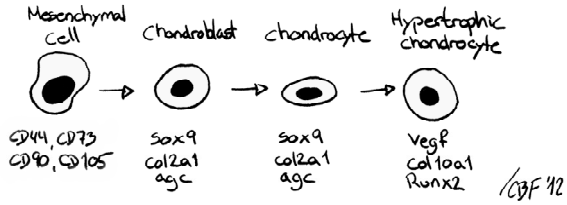

Figure 7: Chondrocyte differentiation. Expression of key markers is noted below each step.

The chondrogenic response seems to be mediated by both SOX9-dependent and SOX9-independent pathways.[154] Below the role of hypoxia will be discussed in relation to paper 1 and 2 [40, 41]. In study 2 we found that the gene expression of *Sox9* increased significantly with decrease in oxygen tension, and that this increase was not affected by culture time or culture surface. Since the expressions of *Agc* and *Col2a1* was dependent on both oxygen tension and culture surface this supports the findings that the chondrogenic response is mediated through both SOX9-dependent and SOX9-independent pathways as mentioned above. In study 2 we also found that there was a significant increase in the gene expression of *Gapdh* with decrease in oxygen tension and that during the culture on the MPEG-PLGA scaffolds there was a linear correlation between the hypoxic response and the (measured by gene expression of *Ankrd37*) and the gene expression of *Gapdh*. Hence, the use of *Gapdh* as reference gene would lead to bias since it was systematically regulated by hypoxia, which supports the finding in study 1 of its unsuitability as reference gene in hypoxia.

Cartilage homeostasis dictates a balance between anabolic and catabolic activity. Anabolic activity is traditionally thought to be stimulated by IGF-I, TGF-β, and BMPs, while catabolism is mainly induced by the proinflammatory cytokines IL-1β and TNFα [155]. While IL-1β seems to responsible for cartilage matrix destruction TNFα drives the inflammatory cascade [156]. Catabolic markers for cartilage degeneration are Matrix Metalloproteases (MMPs) and A Desintegrin And Metalloprotease with Trompospintin Mofifs (ADAMTS). MMP-13 (collagenase-3) is an important collagenase in cartilage degradation while ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) highly active aggrecanases [10, 157]. Inhibition of these is a compelling strategy to improve cartilage repair, but a recent study have shown beneficiary roles of transient activation of MMP-13 and ADAMTS-4 in cartilage-to-cartilage integration *in vitro* [158]. Naturally occurring inhibitors

of both MMPs and ADAMTS such as Tissue Inhibitor of MMPs (TIMPs) represents an endogenous regulatory mechanisms in tissue homeostasis. The complete picture of cartilage homeostasis and especially the regulation of the catabolic processes and their stimulation and inhibition is highly complex and outside the scope of the present thesis. However, it is important to note while the anabolic processes are the main focus in the repair and regenerative processes, the challenge of maintaining cartilage integrity after cartilage after injury or treatment such as seen in microfracture is challenging and is dependent of the homeostatic balance [159, 160]. The evaluation of these proteins and their gene expression patterns were however not included in the present experiments but may have been able to show the catabolic involvement during *in vitro* culture [41, 42].

TISSUE ENGINEERING AND ACI

For cartilage tissue engineering, biomaterials are often combined with stem cell instead of chondrocytes as these intuitively represent an attractive cell source due to the combination of multipotency and relatively easy accessibility from sources such as bone marrow or fat [161]. Investigations of the use of MSCs from different sources encounter an approach for optimization of step 1 in the ACI procedure (Fig. 3), but this has not been studied in the thesis. In terms of tissue engineering and ACI, the use of stem cells represent a different area and may well require different properties of the biomaterial in respect to induce differentiation etc. While obvious commercial motivations exist for development of improved biomaterials with improved outcome in preclinical testing [78], no one have been able to demonstrate differences in clinical trials. Additional targets for optimization of the ACI and the related treatments within tissue engineering are the cell culture, determination of cell quality before implantation, patient selection, and rehabilitation regimes. Treatment with stem cells of different origin is not addressed here, as it by definition is not an ACI treatment.

Targets for optimization of the *in vitro* culture of chondrocytes for ACI treatments include use of autologous serum [162], addition of growth factors [163], hypoxic culture [41], and different culture surfaces [164]. Combinations of these have been combined in bioreactors such as in the NeoCart® treatment (Histogenics, Waltham, MA), which has been reported on in two clinical studies by Crawford *et al*. [165, 166]. Cell quality assessment prior to implantation are seen in both characterized chondrocyte implantation (CCI®) (ChondroCelect®, TiGenix®, Leuven, Belgium) [167-169] and less obviously marketed in CartiCel® and MACI® treatments (Genzyme®, Naarden, The Nederlands & Cambridge, MA). Collectively none of these approaches have resulted in improved outcomes in ACI, which may be explained, at least in part, by the lack of comparative studies of the different ACI methods.

EVIDENCE FOR SURGICAL CARTILAGE REPAIR

There are a wide range of available methods for surgical repair of articular cartilage defects such as smoothening of the cartilage defect area (débridement); abrasion arthroplasty [170]; bone marrow stimulation such as microfracture [171]; microfracture with scaffold support (AMIC® [172] and BST-CarGel®); transplantation of autologous (OAT) [173], allogenic [174], or synthetic osteoshondral plugs such as TruFit® [175]; transplantation of particulated autologous cartilage (CAIS®) [176] or allogenic juvenile cartilage (DeNovo NT®) [177]. Of the numerous treatment methods ACI represents the most costly procedure due to expenses associated with the *in vitro* culture of the autologous chondrocytes. Comparative studies in cartilage repair are sparse. Few studies have compared ACI treatments with microfracture. Knutsen *et al*. [178, 179], found no difference after 2 and 5 years follow-up. In two studies comparing CCI® and microfracture, Saris *et al*. [167, 168], reported that CCI® performed significantly better than microfracture after 18 and 36 months. In a small randomized controlled trial Crawford *et al*. found a trend in favor of Neocart® treatment compared to microfracture at 6-24 month follow-up [165]. Basad *et al.* [180], compared MACI® (40 patients) to microfracture (20 patients) in a randomized clinical trial. Both treatments improved significantly from baseline to 24 month follow-up but the improvement in the MACI® group was significantly larger than in the microfracture group. Zeifang *et al.* [181], 1st and 3rd generation ACI techniques in a randomized clinical trial. In the 21 patients enrolled in the study they were not able to show differences in scores International Knee Documentation Committee score, Tegner Activity Score, and Short Form-36. They did however show significantly better results in Lysholm and Gillquest score in the 1st generation ACI group. Six month postoperative MRI results were significantly better for the 3rd generation ACI, but at 1- and 2-year follow-up the difference were no longer significant. There are currently no additional comparative studies of the different ACI-treatments and there are apparently no registered ongoing studies on this topic [182]. Transplantation of particulated allogenous juvenile cartilage (DeNovo NT®) is a promising new recent approach for treatment of cartilage defects. It is cheaper than ACI because it does not require *in vitro* culture. The treatment has successfully been approved and marketed without any thorough clinical investigation [177]. Hence, it remains unknown how this treatment performs over time and compared to other treatments options. The treatment naturally requires donors and there may be moral concerns for the patients receiving tissue from children autopsies.

There are several issues related to the conduction of clinical trials in cartilage repair. The issues related to quality and level of evidence of the studies have been evaluated by different authors. Of them, many argue that there are little or no evidence for ACI treatment of cartilage injuries compared to other treatment methods or evidence for surgical treatment for cartilage defects at all, which may be a results of insufficient and poor quality data [56, 183-186]. Having no studies directly comparing ACI treatments, there is need for standardized evaluation methods allowing for comparison of the outcomes between studies. In a review by Benthien *et al*., the authors found that the 133 included studies used 27 different clinical scoring systems [184]. Dr. Lars Engebretsen's group in Oslo, Norway, cleverly addresses another very important issue in a recent publication [187]. They found that patients included in clinical studies on cartilage repair do not represent the typical patients seen in clinical practice. In 137 patients with cartilage defects in their clinic, only 6 patients (4%) matched the inclusion criteria in all randomized controlled trials. The strict inclusion criteria that have been determined necessary to avoid possible confounders have not only made patient inclusion in trials difficult, but it has also limited the translation of the results from cartilage repair trials to clinical practice.

TREATMENT OPTIMIZATION: CLINICAL PERSPECTIVE OF THE FINDINGS FROM THE THESIS STUDIES

Improvements of the cell culture method have been demonstrated by hypoxic culture and by altering the spatial environment. In this thesis we showed there was a positive effect of culturing chondrocytes for only 6 days in hypoxia and on a 3D

scaffold. The data requires experimental *in vivo* verification to determine the potential clinical benefits. This would however be easily applicable for companies producing 3rd generation ACI products. Yet to be determined is also if just a short period (hours) of hypoxic culture before implantation would provide a regenerative benefit as physiologic priming.

While the our approach with addition of DS to the MPEG-PLGA scaffold did not provide *in vivo* benefits in regenerating cartilage, other types of recently developed biomaterials still provides promising outlook for future clinical applications. Self-assembling scaffolds [188] and other injectable materials such as hydrogels [79] are recent advances that may well provide significant improvements to existing techniques as *4th generation ACI* products.

Coupling preclinical findings to clinical practice is a challenging endeavor. Several biomaterials and tissue engineering approaches have shown promising results but very few have been introduced into the clinic. This is of course, at least in part, due to regulatory restrictions and challenges, but may also be due to the multifactorial nature of cartilage repair and challenges related to running clinical trials.

Determining the optimal chondrocyte seeding density would have been an easy applicable finding in ACI treatments. In our literature review in study 4 we were not able to determine such a density. In addition, an optimal density may be dependent on the scaffold in 3rd generation ACI, the patients' age, and cell proliferation capacity. *In vivo* mimicking of *in vitro* culture conditions through bioreactors are being investigated for the in vitro expansion of the harvested chondrocytes. It may be speculated what makes this approach advantageous over *in vivo* expansion in the defect where many of the parameters being introduced by the bioreactor culture are already present. Avoiding *in vitro* expansion would lower the cost of the treatment significantly, but would of course also make issues related to seeding densities redundant.

CONCLUSION

William Hunter was right in most of his "predictions". Cartilage damages are hard to manage, but although difficult to restore I do not believe that is an impossible task to restore the articular cartilage. Certainly, to date, no one has been able to fully regenerate articular cartilage in a consistent and predictable manner. In this thesis I have focused on advances of the ACI treatment and addressed and investigated specific targets eligible for optimization. There are limitations to all five studies. These have been addressed in the papers with additional discussions in this summery.

We successfully identified hypoxia-stable reference genes and showed that commonly used reference genes were among the most unstable. Our second study investigating the effects on chondrocytes by combining hypoxic- and 3D-culture have added to the existing knowledge in terms of possible benefits of culturing cells under physiologic conditions. The study has limitations regarding lack of mechanistic studies and protein assays, and the relatively short observation time. Regardless, the findings are interesting and may be easily applicable in clinical treatments. In study 3 we evaluated a non-invasive method for tracking of chondrocytes with intracellular labeling with VSOP. Although the study did not lead to results that encouraged further implementation and *in vivo* investigation it highlighted important issues related to pursuing methods for chondrocyte tracking by intracellular labeling agents. In study 4 we reviewed chondrocyte seeding densities in clinically available ACI treatments for cartilage repair. These were discussed in relation to preclinical studies. We showed high variances in the densities used in the included studies, and we were not able to show any correlation between the density of implanted cells and the outcome. Study 5 basically depicts that small differences observed *in vitro* are not always enough to show differences *in vivo.* This was demonstrated with the addition of DS to an MPEG-PLGA scaffold that showed an early and small increase in fibronectin gene expression in *in vitro* cultured human chondrocytes but had no effect on osteochondral repair in a rabbit model.

The work performed during this PhD may hopefully contribute to the overall picture and understanding of management of articular cartilage injuries using ACI, and have hopefully provided results that are eligible for implementation in clinical and manufacturing practices.

PERSPECTIVES

There are several surgical approaches for articular cartilage regeneration and repair, but good explanations of why some treatments are more successful than others and why some completely fail are rarely presented. While some approaches target inhibition of angiogenesis or stimulation of chondrogenesis others primarily focus on optimization of the biomechanical properties. I do however believe, that key answer lie in the understanding of how and how cartilage repair fails.

The uses of MSCs for cartilage repair are still considered of promising value, but despite very promising pre-clinical findings with stem cell therapies there is still a lack of significant positive findings in orthopaedic applications *in vivo*, and this definitely justifies questioning the role of these in cartilage repair as well as other areas of regenerative medicine. Do MSCs represent a cell source for differentiation into chondrocytes for regeneration of articular cartilage or do they simply exist as nursing cells? While current attempts have to demonstrate the former have largely failed clinically, there is evidence that at least co-culture of MSCs and chondrocytes provides several benefits that could improve future cartilage repair techniques with MSCs as an adjuvant to mature chondrocytes[189], and the interplay between MSCs and differentiated host cells are definitely essential in the understanding of this.

Recent studies have shown that molecules normally associated with the basement membrane, such as laminin and collagen type IV, are found throughout the regenerative tissue in the early cartilage repair in an shallow osteochondral defect in goats [190]. We have also recently described the localization of these molecules the pericellular matrix in healthy and degenerative cartilage [191] as well as their presence in chondrogenesis [192]. However, what remains to be determined is the role of these molecules in cartilage repair, degeneration and homeostasis.

Due to rising costs of health care in general and increase in life expectancy, there is a definite need for cost-effective treatments for cartilage repair, symptom relief, and prevention of the development of post-traumatic osteoarthritis. While years of exploring advanced and expensive treatment modalities have provided little clinical benefit, development of simple cost-effective surgical treatments for larger cartilage defects is essential. For smaller defects it is possible that non-surgical treatments with injection of stimulating or modulating factors are potential solutions. These include factors providing suppression or stimulation specific pathways in the regenerative response.

ACI does not represent a treatment of the past. Several advances and developments arising from this treatment have been made, and I believe that the ACI and related treatments will represent a backbone cartilage repair in many years to come. The existence of the ACI treatment will depend not only on current and new competitors but also its ability to adjust to recent findings such as improved culture methods, new biomaterials and improved rehabilitation regimens.

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Research is a result of inspiration, curiosity, persistence and dedication. At times these criteria for success are hard to fulfill, and I want to thank my wife and son for providing me with an environment that allows this quest.

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ABSTRACT

Articular cartilage is a specialized tissue exhibiting low intrinsic capabilities of regeneration or healing after injury. Autologous Chondrocyte Implantation (ACI) and scaffold-supported ACI are often used for treatment of larger chondral defects (>2cm^2). These utilize open surgery re-implantation of *ex-vivo* cultured autologous chondrocytes harvested as a biopsy arthroscopically in a prior surgery. This two-step procedure is an advanced and expensive treatment that despite high expectations have failed to regenerate articular cartilage in a consistent and predictable fashion, and as many as 25% the operated of patients have dissatisfactory outcomes.

The objective of the present thesis was to address and investigate methods for optimizing the steps involved in the ACI and scaffold-supported ACI treatment including chondrocyte culture environment, chondrocyte labeling and tracking, improved biomaterials, and cell seeding densities. We hypothesized that these areas were eligible for targeted optimization, which has been addressed in the five papers constituting the work performed in the present thesis.

The first two studies address the *in vitro* cell expansion of chondrocytes before re-implantation. After validation of hypoxia-suitable housekeeping genes for quantitative gene expression analysis using previously validated algorithms (study 1) the effect of combined hypoxic- and 3D culture on human chondrocytes gene expression was investigated (study 2). An *in vitro* experiment was performed to determine the effect on gene expression of an intracellular superparamagnetic labeling agent for 1.5T MRI-tracking of alginate-embedded human chondrocytes (study 3). We further performed a literature study, reviewing the cell seeding densities of the implanted chondrocytes used in clinically available cell transplantation-based treatments for cartilage repair (study 4). Finally, we tested the addition of dermatan sulfate to a clinically approved methoxy-polyethen-glycol (MPEG) substituted polylactide-co-glycolic acid (PLGA) scaffold by implantation of cell-free scaffolds in an osteochondral rabbit model (study 5).

We determined a set of hypoxia-stable reference genes in study 1 that were then used in study 2. We observed that there was a positive effect on chondrogenic gene expression in human chondrocytes when culturing in 3D compared to monolayer and in hypoxia compared to normoxia and that there was an additional positive combined effect of 3D and hypoxia. Using a clinical MRI-system we were able to track labeled chondrocytes for up to 4 weeks, but we found that the labeling agent had significant effects on chondrocyte gene expression, which could potentially confound results when used *in vivo.* In our review of chondrocyte seeding densities we found large variability between commercial products and a very limited preclinical basis for the applied densities. Lastly, we found that there was no positive effect *in vivo* of adding dermatan sulfate to MPEG-PLGA scaffold in osteochondral repair.

We conclude that while the outcome of ACI-related treatments certainly is multifactorial it may be improved by optimizing the *in vitro* culture by hypoxic and 3D culture and by adjusting the chondrocyte seeding density. Our studies on biomaterials and potential system for cell tracking *in vivo* did not show results that justified further studies and clinical trials.

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