CARTILAGE TISSUE ENGINEERING
A STUDY ON HOW TO IMPROVE CARTILAGE REPAIR

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To my family

"Success is not final, failure is not fatal, and it is the courage to continue that counts"

Winston Churchill (1874-1965)

ABSTRACT

One of the first examples of musculoskeletal tissue engineering is autologous chondrocyte implantation (ACI).The first patient with a cartilage lesion was operated with ACI in 1987 and at that time suspension implantation was used. Today, we use the third generation of ACI where scaffolds are employed to support redifferentiation and neocartilage formation *in vitro* and further maturation *in vivo* after implantation to treat the cartilage defects.

A great deal of information is still needed to clinically improve cartilage production. Variables such us the cell seeding density, the cell culture media formulation, the degree of redifferentiation and the material and biological properties of the scaffold used remain to be investigated further.

In the work reported in **paper I** we aimed to elucidate whether mesenchymal stem cells (MSC:s) are better than committed chondrocytes in producing cartilage in vitro, whether the co-culture of MSC:s and chondrocytes play a role in enhancing cartilage production in vitro and if different biomaterials affect the differentiation capacity *in vitro*.

The effect of the cell seeding concentration was evaluated in **paper 2** by culturing human adult chondrocytes in chitosan scaffolds. After 14 and 28 days in a 3D culture, the constructs were assessed for collagen, glycosaminoglycans and DNA content. The mechanical properties of the constructs were determined using a dynamic oscillatory shear test.

In **paper III** we studied whether the degree of redifferentiation of chondrocytes in *in vitro* cultured scaffolds had an effect on the neocartilage formation after implantation. It was studied whether redifferentiation of the chondrocytes was accomplished by recapitulating the signaling pattern used by chondrocytes during fetal development.

In **paper IV** we tried to determine the effect of different culture conditions on the *in vivo* chondrogenic capacity and integration properties of human tissue engineered chondrocyte constructs.

In **paper V** we evaluated the biomimetic properties of different materials. Materials with good biomimetic properties may influence the initial phases of tissue regeneration by inducing a strong migration of cells into the pores of the scaffold.

Materials and Methods

MSC:s and human adult and pig chondrocytes were cultured in different materials in order to prove the different hypotheses. The chondrocyte differentiation *in vitro* and *in vivo* was evaluated using real time PCR to asses the expression of different genes. The total amount of collagen and proteglycans was determined biochemically. Inmunohistochemistry and different histological scores were used to evaluate the presence of cartilage specific proteins and to semiquantify the histological aspect of tissue engineered constructs after in vitro or in vivo evaluation. A novel transmigration assay was designed to evaluate the biomimetic properties of different biomaterials. To evaluate the in vivo chondrogenic potential, tissue engineered constructs produced *in vitro* were subcutaneously implanted in nude mice or into cartilage defects in human osteochondral plugs.

Results

Related to the number of chondrocytes used, coculture with MSC:s led to a strong increase in collagen type IX mRNA expression, an indicator for long-term stability of cartilage. Chondrocytes had better redifferentiation potential as compared to MSC:s. Tissue glue Tisseel® provided slightly better chondrogenic conditions than Tissue Fleece®.

We determined that concentrations of 12–25 million cells/cm^3 are needed in a chitosan scaffold to increase the matrix production and mechanical properties of human adult chondrocytes under static conditions.

We were able to determine that the *in vitro* chondrogenesis in scaffolds induce a signalling pattern similar to the one seen in fetal development. Furthermore the results indicates that redifferentiation of *in vitro* expanded articular chondrocytes is needed at the time of implantation for neocartilage formation. However, 14 days of preculture *in vitro* used clinically today might be reduced.

Conclusion

It is possible to significantly improve cartilage repair by using the right amount of cell concentration in seeded scaffolds, chondrogenic cells co-cultured and by choosing the right type of biomimetic scaffolding material. The future of cartilage repair lies in further development of suitable materials and good quality cells expanded under the most ideal conditions.

LIST OF PUBLICATIONS

I- Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes.

*Hildner F**, Concaro S**, Peterbauer A, Wolbank S, Danzer M, Lindahl A, Gatenholm P, Redl H, van Griensven M.*

Tissue Eng Part A. 2009 Dec; 15(12):3961-9.

II- Effect of cell seeding concentration on the quality of tissue engineered constructs loaded with adult human articular chondrocytes.

***Concaro S**, Nicklasson E, Ellowsson L, Lindahl A, Brittberg M, Gatenholm P.*

J Tissue Eng Regen Med. 2008 Jan;2(1):14-21.

III- The *in vivo* chondrogenic potential of chondrocytes seeded in hyaluronic acid based scaffold is triggered by the degree of redifferentiation *in vitro*.

Stenhamre H, **Concaro S***, Brantsing C, Enochson L, Gatenholm P, Lindahl A, Brittberg M.*
∗ These authors contributed equally and should both be considered first authors.

Submitted after revision to Cells, Tissues and Organs.

IV- How to improve the in vivo chondrogenic properties of chondrocyte seeded scaffolds; A study on the effect of different nutrition media compositions and culture time.

***Concaro S**, Concaro C, Brantsing C, Lindahl A and Brittberg M.*

Submitted after revison to Tissue Engineering.

V- A study on how different biomimetic material properties influence the proliferation and migration capacity of porcine articular chondrocytes.

***Concaro S**, Lönnqvist C, Gatenholm P , Lindahl A and Brittberg M.*

Submitted.

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ABBREVIATIONS

3D	Three-dimensional
ACI	Autologous chondrocyte implantation
AGC1	Gene coding for aggrecan
ASC	Adipose-derived stem cells
BMP	Bone morphogenic protein
cDNA	Complementary deoxy ribonucleic acid
COL 2A1	Gene coding for collagen type II
COL10A1	Gene coding for collagen type X
COL1A1	Gene coding for collagen type I
COL9A2	Gene coding for collagen type IX
COMP	Cartilage oligomeric matrix protein
CRTL1	Gene coding cartilage link protein
CSPG2	Gene coding for chondroitin sulphate proteoglycan II
DDA	Degree of deacetylation
DMB	1.9-dimethylmethylene blue
DMEM-hg	Dulbeccos modified Eagles medium- high glucose
DMEM-lg	Dulbeccos modified Eagles medium- low glucose
DMEM/F12	Dulbeccos modified Eagles medium/F12 media 1:1
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorter
FAM	6-carboxyfluorescein
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycans
GDF 5	Growth and differentiation factor 5
HA	Hyaluronic acid
HAC	Human articular chondrocytes
HES	Hairy and enhancer of split
HP	Hydroxyproline
IGF-1	Insulin-like growth factor 1
ITS	Insulin transferrin selenium
KI 67	Kiel protein 67
MACT	Matrix associated chondrocyte transplantation
MCS	Mesenchymal stem cells
MIA	Melanoma inhibitory activity
ML	Monolayer
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging

mRNA	Messenger RNA
N-CAM	Neutral cell adhesion molecule
OC	Osteochondral
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-BB	Platelet derived growth factor BB
PG	Proteoglycan
PGA	Poly glycolic acid
PLA	Poly lactic acid
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RGD	Amino acid code abbreviation for "Arginine-Glycine-Aspartic acid"
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
SOX 9	SRY-box containing gene 9
SRY	Sex determining region Y
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
WNT 14	Wingless-related protein type 14

BACKGROUND

Articular cartilage structure

Composition

Chondrocytes are the cells that make up cartilage.[7] The number of chondrocytes in cartilage is less than 10% of the full tissue volume. Chondrocytes produce an extra cellular matrix (ECM) which is composed of a dense network of collagen fibers (collagen II) and proteoglycans (PGs). The collagen content in cartilage is about 10-30% while the PG content is 3-10% (wet weight). The remaining component is water. Other compounds are inorganic salts and small amounts of other matrix proteins, glycoproteins and lipids. It is the collagen and PGs that provide the structure for the tissue and together with water determine the biomechanical properties and functional behavior of cartilage.[12,23]

The articular cartilage can be divided into different zones consisting of a superficial tangential zone (10-20%), a middle zone (40-60%) and a deep zone (30%). There is also a calcified zone close to the bone.[16,194]

Collagen

Collagen is a protein that is very common in the body. There are three procollagen polypeptide chains (Alfa-chains) that are coiled into left-handed helices, which are then coiled in a right-handed helix around each other, forming the basic biological unit of collagen called tropocollagen. The tropocollagen then assembles into larger collagen fibrils. This crosslinking of tropocollagen is responsible for the tensile strength of collagen. The diameter of the collagen varies but the average diameter in articular cartilage is 25-40nm.[42,80,88,123,148] The distribution of collagen in articular cartilage is not homogenous and varies through the different zones of the cartilage. In the superficial tangential zone the collagen fibers are densely packed and randomly orientated in planes parallel to the articular surface.[2,12,32,167]

Proteoglycans

Proteoglycans (PG) are polysaccharide-protein compounds that can exist as either monomers or as aggregates. The polysaccharide part is about 95% with the other 5% being proteins. The PG monomer is a protein core, about 200nm long, covalently bond to several glycosaminoglycans (GAGs) and oligosaccharides (both O-linked and N-linked). About 150 GAG chains are attached to the protein core. Some of the polysaccharides found in the articular cartilage are keratan sulphate, chondroitin sulphate, hyaluronic acid, dermatan sulphate and heparan sulphate.[27,40,132,247] The length of the keratan sulphate chain is about 13 disaccharide units while the length of the chondroitin sulphate chain is 25-30 disaccharide units. The GAGs are negatively charged, at least one per disaccharide making the GAGs

repel each other. However, this also leads them to attract cations and interact with water.[130] The amounts of these GAGs change when the cartilage age and in degenerative diseases.[14] The chondroitin sulphate decreases while the keratan sulphate increases. Most of the proteoglycan monomers form aggregates with hyaluronate.[194]

Tissue fluid

About 80% of the wet weight of the cartilage is fluid. This fluid is primarily made up of water but also contains gases, metabolites and a large amount of cations which stabilizes the negative charges of the GAGs. The nutrient and oxygen transport and waste removal in the cartilage take place through diffusion exchange between the tissue fluid and the synovial fluid. Only a small percentage of the fluid is intracellular. About 30% of the fluid is believed to have a strong association with the collagen fibers and is thus very important for the structural organization of the ECM. This interaction with the ECM gives the ability to resist and recover from compression. The rest of the fluid (about 70%) can move freely during loading.[161-166]

Articular cartilage mechanobiology

Interaction between components in cartilage

The negatively charged GAGs attract mobile cations in the tissue fluid such as sodium and calcium which creates an osmotic pressure (Donnan Osmotic Effect) of approximately 0.35MPa. The collagen network inhibits the swelling, leading to a pre-stress in the collagen network. When the cartilage is compressed the internal pressure in the matrix exceeds the osmotic pressure and the fluid begins to flow out of the cartilage.[125,152,162] When this happen the charge density from the GAGs increases which increases the osmotic pressure and the charge-charge repulsion. This finally leads to equilibrium with the external stress. This property complements the tensile strength of the collagen fibres. The compression strength of the proteoglycans is derived from the osmotic swelling pressure and from the PG aggregates that are entangled in the collagen network. The elastic modulus for the collagen-PG matrix is approximately 0.78MPa.[152,161,163,165,166]

Biomechanics of articular cartilage

To understand the mechanical response of cartilage it can be seen as being a biphasic tissue.[152,163,165] One fluid phase is the interstitial fluid and the inorganic salts and the other is the solid phase which is represented by the extra cellular matrix. The cartilage can then be seen as a fluid-filled, porous and permeable material. Since cartilage has a fluid part and a solid part its mechanical response is viscoelastic, a combination of the viscous response from the fluid and the elastic response of a solid. Owing to this viscoelasticity the response of a constant load or constant deformation is time dependent. These responses are called creep and stress

relaxation. It is called creep if the response to constant mechanical load is a quick, initial deformation followed by a slow but increasing deformation until equilibrium is reached. The other type of response is defined as a high initial stress followed by a slow decrease of the stress; this happens when a viscoelastic material is exposed to constant deformation and it is called stress relaxation.[161,164,165]
The viscoelastic behaviour during compression is essentially related to the flow of interstitial fluid. With shear, however, it is primarily due to the motion of the collagen and PG chains. The part of the viscoelasticity that is a result of interstitial fluid is known as biphasic viscoelastic behaviour and the part that is due to the macromolecules of the matrix is known as flow independent or intrinsic behaviour.[145,212-214]

Permeability of articular cartilage

A porous material becomes permeable if the pores are interconnected, and this maks possible for the fluid to flow through the material. The porosity (β) is defined as the fluid volume divided by the total volume. The permeability (k) is a description of how easy it is for the fluid to pass through the material and is inversely proportional to the frictional drag (K). Thus it is a measure of the force that is needed to move the fluid at a given velocity through the porous material. The frictional force is caused by the interaction of the fluid and the walls of the pores. The permeability k is related to K in the relationship:

$$k = \frac{\beta^2}{K}$$

There is also Darcy's law reference:

$$k = \frac{Qh}{A(P_1 - P_2)}$$

Here, Q is volume per unit time through the sample with the area A. P_1-P_2 is the pressure difference between the different sides of the sample and h is the height of the sample.[152,161-163,214]

Joint development and joint formation phases

It is very important to understand the biology of articular cartilage and the chondrocytes, their genesis is especially critical in developing biological approaches to the treatment of cartilage injury and degeneration. Most of articular cartilage and joint development occurs post-natally.[11,160]
The site of joint formation is determined by the balanced expression of different genes. Early events that occur in joint formation establish the joint as a centre of chondrogenic modulation that provides inhibitory signals at the ends of future bones to balance the vascular in growth and ossification that will later occur in the

middle of those bones. There are three important stages during the joint embryological process: *condensation, interzone formation and cavitation*[8]

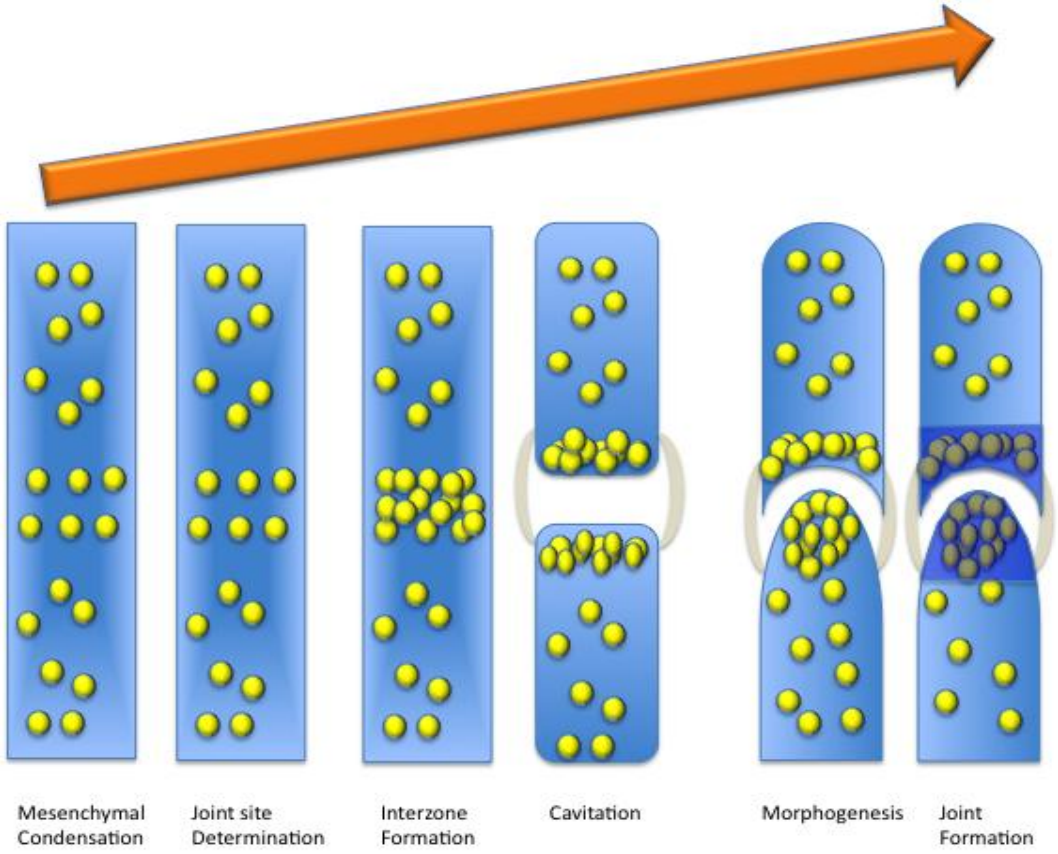


Figure 1
Graphic showing the developmental process in a synovial joint
Adapted and modified from Pacifici et al. [185]

Condensation

The first event during the development of a joint is the condensation phase.

Cellular condensation is associated with increased cell to cell contact. Several factors are involved in this phase such us the neural cell adhesion molecule (N-CAM), N-cadherin, tenascin, versican, fibronectin and several conexins.

At this stage the matrix is composed mainly of collagen type I and IIA and hyaluronan. After this stage an enzyme called hyaluronidase diminishes the amount of hyaluronan allowing cell cell interactions.

During the condensation phase the cells start to express collagen II, IX and XI, aggrecan, link protein and Gla protein.[44,91,105]

Interzone

The second event in joint formation is the appearance of the interzone which consists of two chondrogenic, perichondrium-like layers that cover the ends of the anlagen. Between them remains a dense condensation of cells that forms the proper

interzone. Mechanospatial changes and cell migration occur, which are facilitated by increased levels of hyaluronan and this surface receptor CD 44.
Articular chondrocytes derive from a special subpopulation of chondrocytes from the interzone that do not switch on the Matrilin 1 gene.[116]
These early events establish the interzone as a signalling center. This command point provides inhibitory signals at the ends of the future bones to balance vascular ingrowth and ossification.[8,158]
Wnt 14 is necessary in the earliest steps of joint specification. It negative regulates chondrogenic differentiation at the sites of future joints.[105]
GDF-5 (growth and differentiation factor 5) plays a very important role and is part of the transforming growth factor β superfamily. It is believed that GDF-5 positive cells give rise to articular chondrocytes. GDF-5 is an early marker of joint formation and has pro-chondrogenic properties such as chondrogenic cell mesenchymal cell recruitment and chondrogenic differentiation.[84,138]
Antagonists such as Noggin and Chordin are required for normal joint development.[8,158,209]

Cavitation

Cavitation is the last of this chain of steps. Apoptosis along the joint line has been described as an important event during cavitation, although local changes in the composition of the ECM and mechanical influence are also involved.[76,185]

Tissue organization

The matrix and cells do not behave as independent entities. The dynamic process that maintains the matrix is preserved by the interactions between collagen, proteoglycans and chondrocytes. The cells synthesize and degrade the matrix in response to different factors such as changes in the mechanical environment, concentration of different growth factors and cytokines.

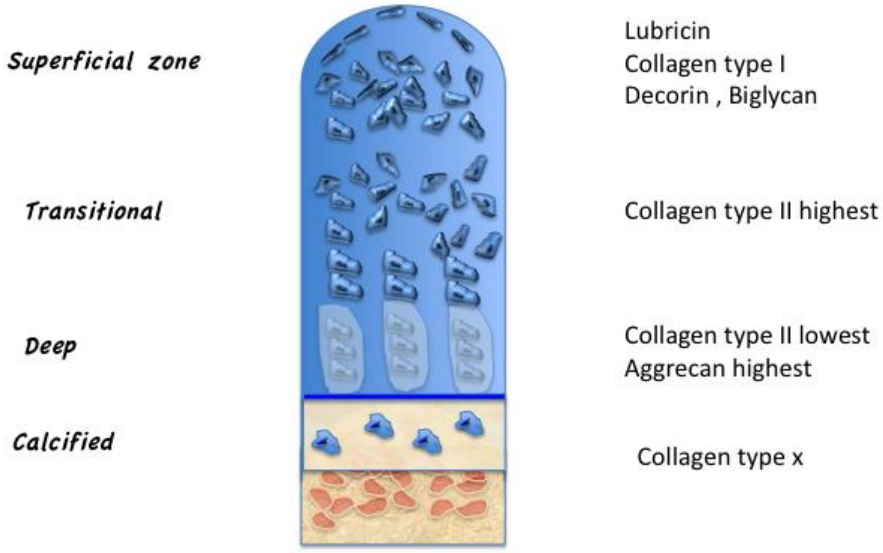


Figure 2
The graphic is showing the organization of the articular cartilage and the matrix distribution.

The superficial cells are surrounded by a very polarized close-knit organization of thin collagen fibrils that generally run parallel to each other and to the articular surface. The matrix organization is dominated by the fibrillar network. The content of aggrecan is at its lowest here. The superficial zone provides the highest tensile properties found in articular cartilage endowing it with the ability to accommodate the shear, tensile, and compressive forces encountered during articulation.[16] The fibril-associated small leucine-rich decorin and proteoglycans biglycan are most concentrated in the superficial zone.[194]
Below the superficial zone is the midzone where cell density is lower. This area has the more typical morphologic features of a hyaline cartilage with more rounded cells and an extensive extracellular matrix rich in the proteoglycan aggrecan. Here, the collagen fibrils are of a larger diameter and arranged more randomly. Situated between this zone and a layer of calcified cartilage is the deep zone. Cell density is at its lowest here while the aggrecan content and fibril diameter are maximal, although the collagen content is minimal.[58,89,194,205,213]

The partly calcified layer provides a buffer with intermediate mechanical properties between those of the uncalcified cartilage and the subchondral bone. The chondrocytes in this calcified zone usually express the hypertrophic phenotype. They reach a stage of differentiation that is also achieved in the physis and in fracture repair in endochondral bone formation. These hypertrophic cells are unique in that they synthesize type X collagen and can calcify the extracellular matrix. Unlike in bone formation, this calcified matrix is not resorbed fully during development and ordinarily resists vascular invasion.This interface provides excellent structural integration with the subchondral bone. Cells from different layers may have different function and redifferentiation potential.[8-10]

Molecular aspects of chondrogenesis

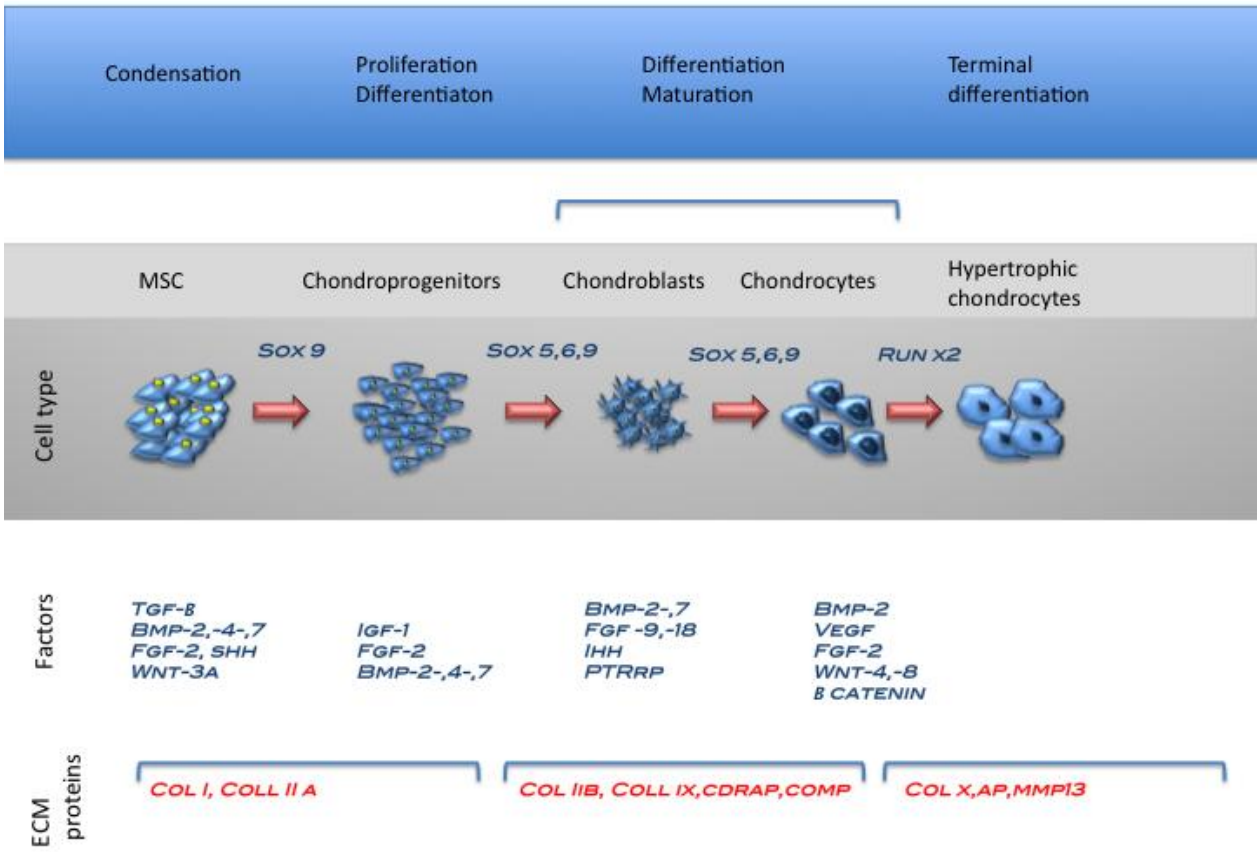


Figure 3
Molecular aspects of the differentiation process of human articular chondrocytes
Adapted and modified from Goldring et al. The control of chondrogenesis. [91]

The starting point of chondrogenesis

Cartilage is the first step in the development of almost every skeletal element.
The first step in chondrogenesis is mesenchymal condensation, first mediated by paracrine factors and later by Sox 9, a transcription factor that belongs to the SRY box and contains the high mobility group box DNA binding domain.
N-cadherin is upregulated at the beginning of the cell condensation phase.[70,232]
Sox 9 is regulated by members of the transforming growth factor β (TGF β), fibroblast growth factor (FGF) and Wnt families.[91] Sox 9 is responsible for the expression of some of the key genes in chondrogenesis. Sox 5 and 6 and collagen IIa are regulated by Sox 9 (Figure 3).[28,144,174]

Chondrocyte maturation

After the initiation of chondrogenesis by the activation of N Cadherin and Sox 9, the mesenchymal cells become chondroblasts and continue their differentiation path towards the mature phenotype. ECM molecules like COMP and proteoglycans are secreted only after Sox 9 expression.[149] Collagens type II, VI, IX and XI are expressed after the onset of these events.[26,28,254]
After the ECM of the cartilage is synthesized the chondrocytes enter hypertrophy to render calcified cartilage or bone. Hypertrophy is regulated by the Indian Hedgehog/parathyroid hormone related protein signaling. Collagen type X represents the ECM component of this phase (Figure 3).[131]

Cartilage pathophysiology

Cartilage lesion biology

Cartilage lesions represent a common problem in orthopedics. The frequency of these lesions has been documented by several authors. One study reported the prevalence of these lesions to be of 63% of 31.516 arthroscopies. Patients under 40 years of age with grade IV lesions accounted for 5% of all arthroscopies; 74% of those patients had a single chondral lesion (4% of the arthroscopies).[62]
Articular cartilage lesions do not heal or they repair transiently but imperfectly. These lesions are usually related to disability and symptoms such as pain, swelling, locking and malfunction of the joint. The progression of these lesions to osteoarthritis is unknown and there is no scientific basis to predict how an isolated articular cartilage lesion may progress and lead to secondary osteoarthritis.[208] This progression depends on several factors such as age, sex, and weight. Approximately one of every six people in America is affected by osteoarthritis making it one of the most prevalent diseases in the United States of America.

Articular cartilage lesions are found during the course of osteoarthritis, or as a result of articular trauma. Posttraumatic cartilage lesions occur as a result of an alteration of the matrix components and cell death. Matrix components are

disrupted by mechanical and enzymatic means. Chondrocyte death is by apoptosis.[3,150] In the course of osteoarthritis a loss of proteoglycans is the first detectable event followed by the disruption of the collagen network. The initially small focal lesions may gradually increase in depth and length. Chondrocytes react to these changes by increasing their metabolic activity; however catabolic processes predominate over the anabolic ones.[4]

Partial thickness defects

Partial articular cartilage lesions do not heal spontaneously. This failure is thought to be due to the fact that there is no communication between the cartilage defect and the bone marrow that contains stem cells which have potential to generate cartilage. It has been shown that partial defects heal without scarring in a fetal lamb model. Whether this is a true full regeneration or a filling owing to growth is a matter of debate.[168]

Different types of repair response have been described. Cells at the margins of cartilage defects undergo cell death. This is followed by an increase in cell proliferation or cluster formation as well as matrix synthesis and catabolism. This response is short and fails to repair the lesion.[153] Synovial cells can migrate to these partial defects but in the absence of fibrin and, growth factors and due to the anti-adhesive properties of the proteoglycans, they fail to populate and provide adequate repair.[112-115]

Full Thickness defects

Full thickness defects are considered to be those that pass through the zone of calcified cartilage and penetrate into the subchondral bone communicating the cartilage lesion to the bone marrow that contains mesenchymal stem cells. The repair response in these defects leads to the formation of fibrocartilage. The tissue adjacent to the wound becomes necrotic. The integration between the repair tissue present in these types of defects and the native cartilage showed no true integration in a study reported by Shapiro et al.[210]

Cartilage repair and regenerative techniques

The aim of the surgical cartilage repair procedures is to provide pain relief and to improve joint function. Malalignment, meniscal deficiency and ligament instabilities should be addressed and treated prior to cartilage treatment.

Bone marrow stimulation techniques

The microfracture technique is one technique for bone marrow stimulation technique. In this technique the subchondral bone is penetrated with an awl in order to promote the migration of stem cells and factors from the bone marrow and

generate a superclot. The best outcome is seen in young patients and small lesions.[101,135,136]

The location of the lesion appears to be important when using this technique. Femoral condyle lesions seem to show better outcomes after microfracture.[139]

Autologous osteochondral transplantation

This technique consists of transplanting one or more cylindrical autografts into a cartilage defect to provide a congruent surface. The autografts are harvested from the periphery of the trochlea or the intercondylar notch as these are low weightbearing areas. The technique is limited mainly by the amount of tissue available to harvest. Donor site tissue morbidity might be a problem if multiple grafts are harvested. This technique is best for lesions smaller than 2 cm^2. Good results have been reported with lesions between 2 cm^2 and 4 cm^2.[104]

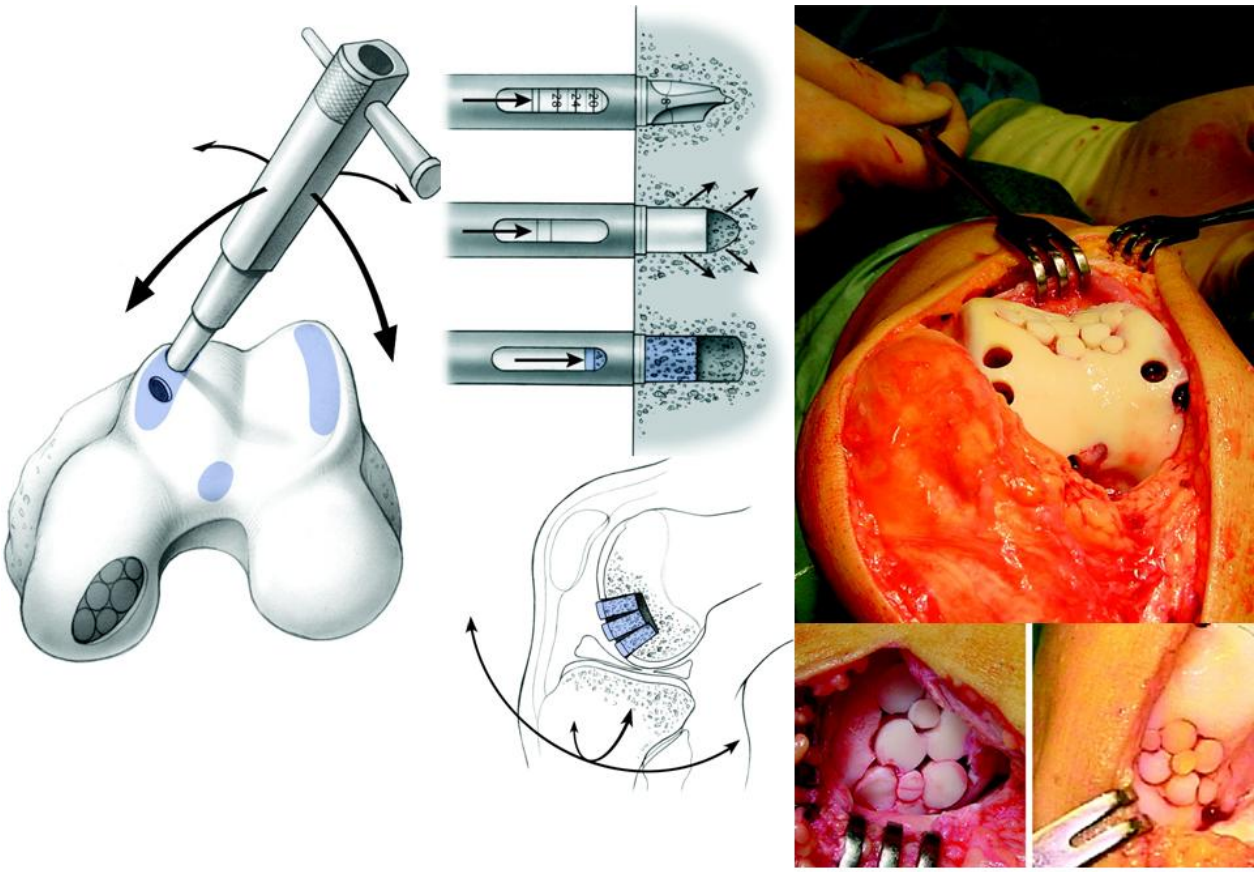


Figure 4
Osteochondral cartilage transplantation.
With permission of Dr. László Hangody *J Bone Joint Surg Am.* 2004;86:65-72.

Allogeneic cartilage transplantation

Allogeneic cartilage transplantation provides an option for treatment of lesions larger than 2-3 cm^2 with significant bone loss.[41,64,100] Tissue matching or inmunosupression are not needed because chondrocytes are isolated by the ECM and are therefore not exposed to the host´s immunological system.[63] Fresh allografts are maintained in medium for no longer than 48 days. This allows chondrocytes to survive after transplantation.[18,63,120,238-241] Frozen allografts are maintained at - 40 º C.[1]

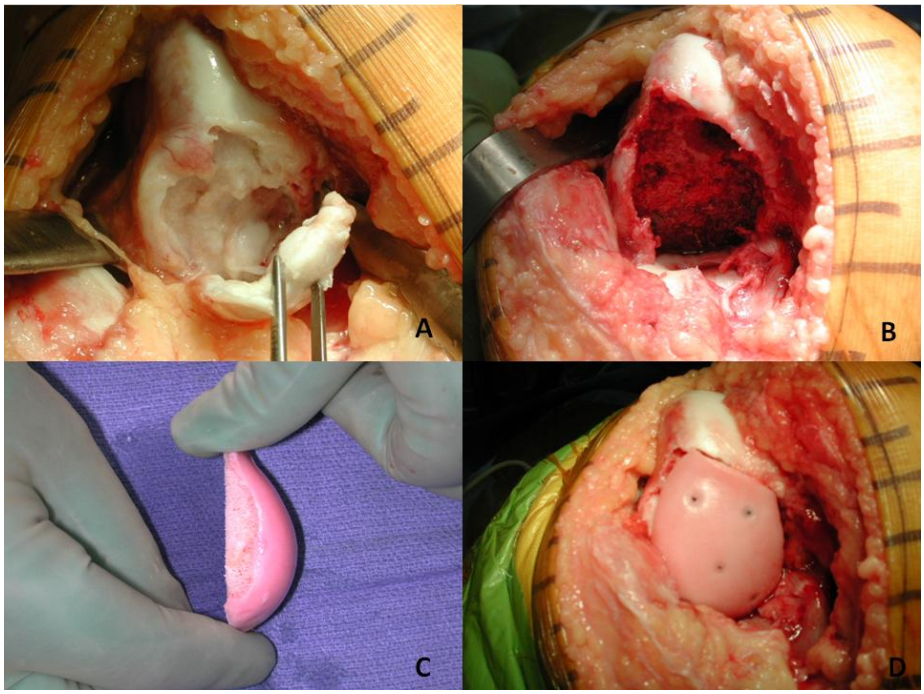


Figure 5
Large osteochondral lesion in the medial femoral condyle treated with a fresh osteochondral allograft.
A: Osteochondral lesion B: After debridement C: Shell allograft preparation and D: Osteochondral allograft in place.
Reproduced with permission of Bugbee B and Görtz S. Department of Orthopaedics, UCSD, Ca, USA.

Cell based therapies

Cell based techniques such as autologous chondrocyte implantation (ACI) have emerged in recent years as a potential therapeutic option. Brittberg et al.[26] were the first to apply cell transplantation techniques in orthopaedics.[35] There are different generations of those techniques. In the classic ACI technique, chondrocytes are isolated from small slices of cartilage harvested arthroscopically from a minor weight-bearing area of the injured knee. The extracellular matrix is removed by enzymatic digestion, and the cells are then expanded in monolayer culture. Once a sufficient number of cells have been obtained, the chondrocytes are implanted into the cartilage defect, using a periosteal patch over the defect as a method of cell containment. The major complications are periosteal hypertrophy, delamination of

the transplant, arthrofibrosis and transplant failure.[36,189-191] Further improvements in tissue engineering have contributed to the next generation of ACI techniques, where cells are combined with resorbable biomaterials, as in matrix-associated autologous chondrocyte transplantation (MACT).[21,22,24,154,171,172,188,229] These biomaterials secure the cells in the defect area and enhance their proliferation and differentiation.

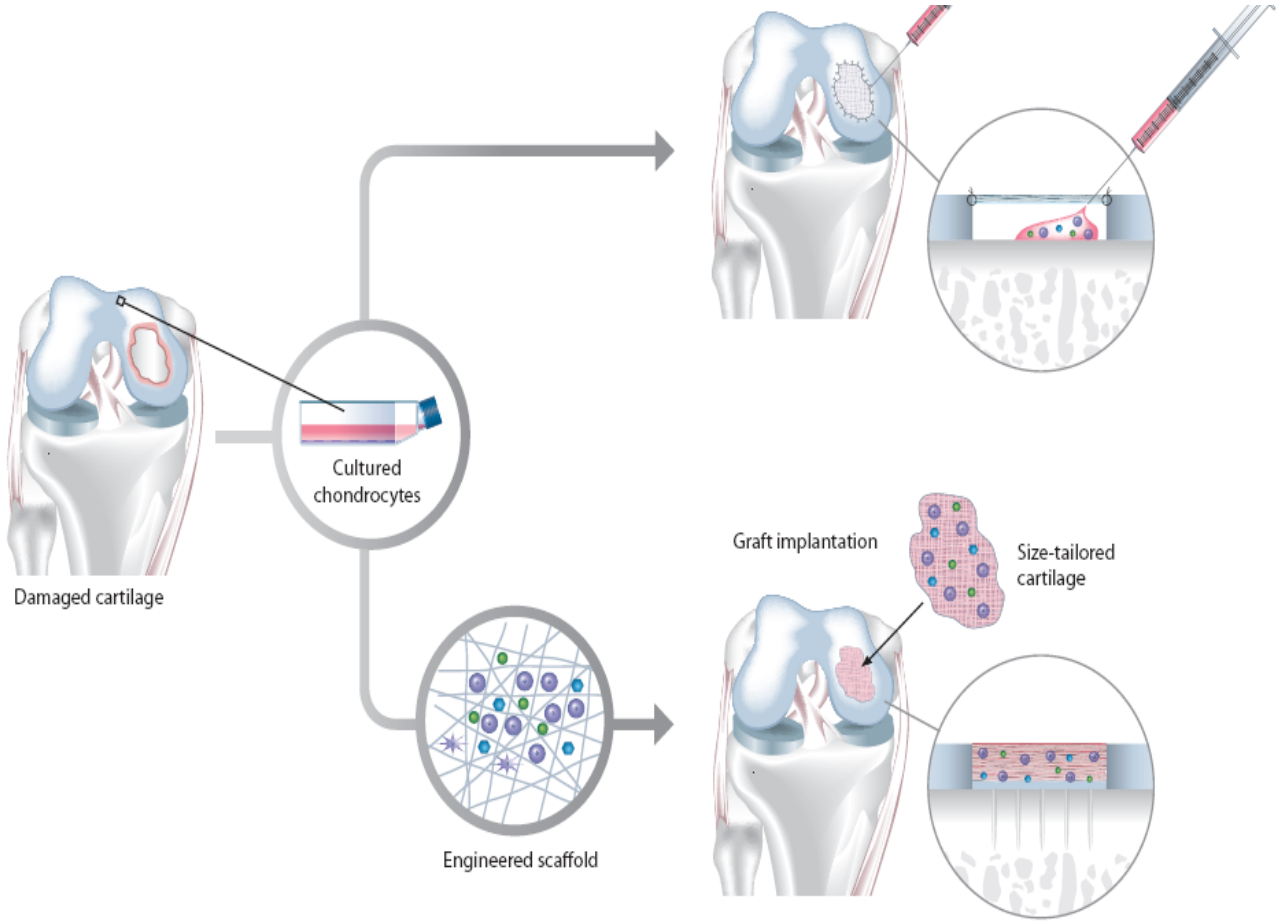


Figure 6
Illustration of the different techniques of chondrocyte cell implantation. The upper part of the figure shows the technique in which the cells are injected underneath a periosteum patch (1st generation) or a collagen membrane (2nd generation**)**. The 3rd generation which uses cells, scaffolds and growth factors is seen in the lower part. Art by Pontus Andersson.

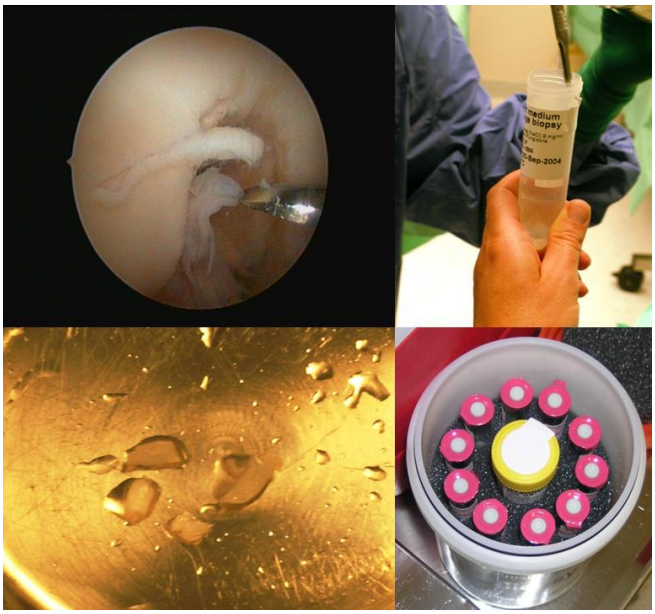


Figure 7
Biopsy technique and transportation recipient.

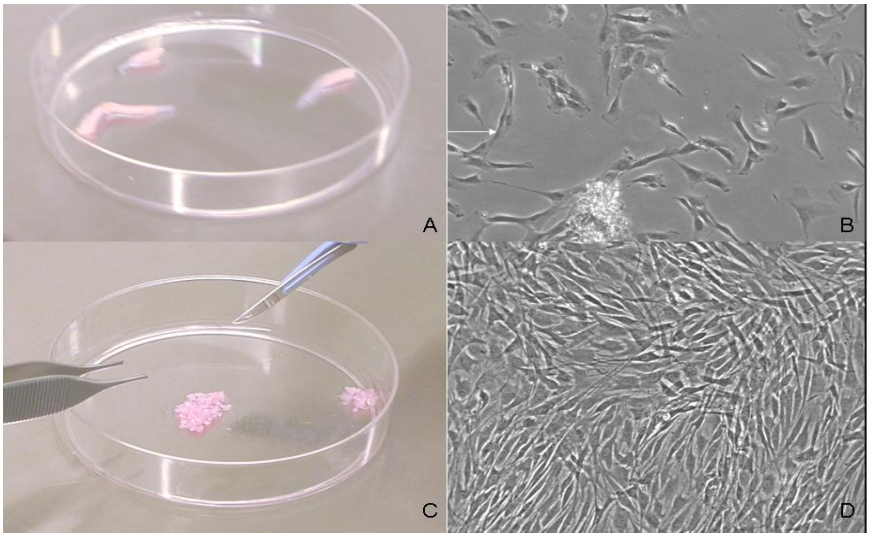
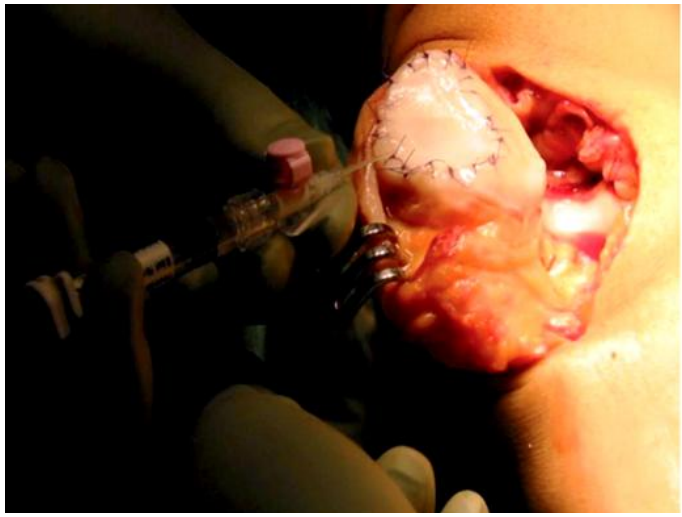


Figure 8
The initial biopsy before and after mincing (A-C) and the chondrocytes during monolayer expansion (B-D).

A



B

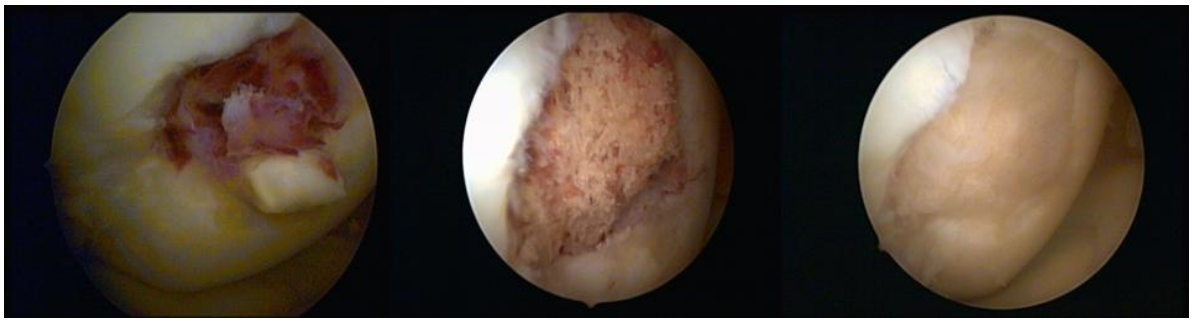


Figure 9
A: Injection of human expanded chondrocytes underneath a collagen membrane.
B: Osteochondritis dissecans treated with arthroscopic third generation chondrocytes implantation.

Long term results and a MRI follow up were recently published showing the durability and long term performance of the implants.[192,236] Saris et al.[159] showed in a randomized multicenter trial that characterized chondrocyte implantation for the treatment of articular cartilage defects of the femoral condyles of the knee results in significantly better clinical outcome at 36 months than microfracture. In this study the authors could determine that time to treatment and chondrocyte quality affected the postoperative outcome.[204] In a systematic review Bekkers et al.[25]concluded that patients who are active show better results after autologous chondrocyte implantation or osteochondral autologous transplantation when compared with microfracture. Younger patients (<30 years) seem to benefit more from any form of cartilage repair surgery than patients over 30 years of age.
This review also concludes as well that lesions larger than 2.5 cm^2 should be treated with sophisticated techniques, such as autologous chondrocyte implantation or osteochondral autologous transplantation, while microfracture is a good first-line treatment option for lesions smaller than 2.5 cm^2.[25]

Tissue engineering based techniques

The use of classic ACI has been associated with several limitations related to the morbidity of the surgical procedure. This technique requires an arthrotomy that may be associated with a higher morbidity. Periosteum hypertrophy has been reported in 10% to 25% of the cases and may require a revision surgery.[37,189,190]

Other potential disadvantages of the first generation are that the implanted cells are undifferentiated and it is unclear whether the cells can reexpress the hyaline phenotype after the implantation. Other concerns are the distribution of the cells in the cartilage defect after injection and the effect of gravity.[181,215] The 3rd generation of tissue engineered based techniques were developed in order to overcome these problems.

Tissue engineering relies on many factors such as obtaining the right cell type, directing the development of those cells towards a chondrogenic pathway using growth factors, supporting the growing cells on a three-dimensional matrix and having that matrix remain in the defect at least until healing is complete.

(Figure 10)

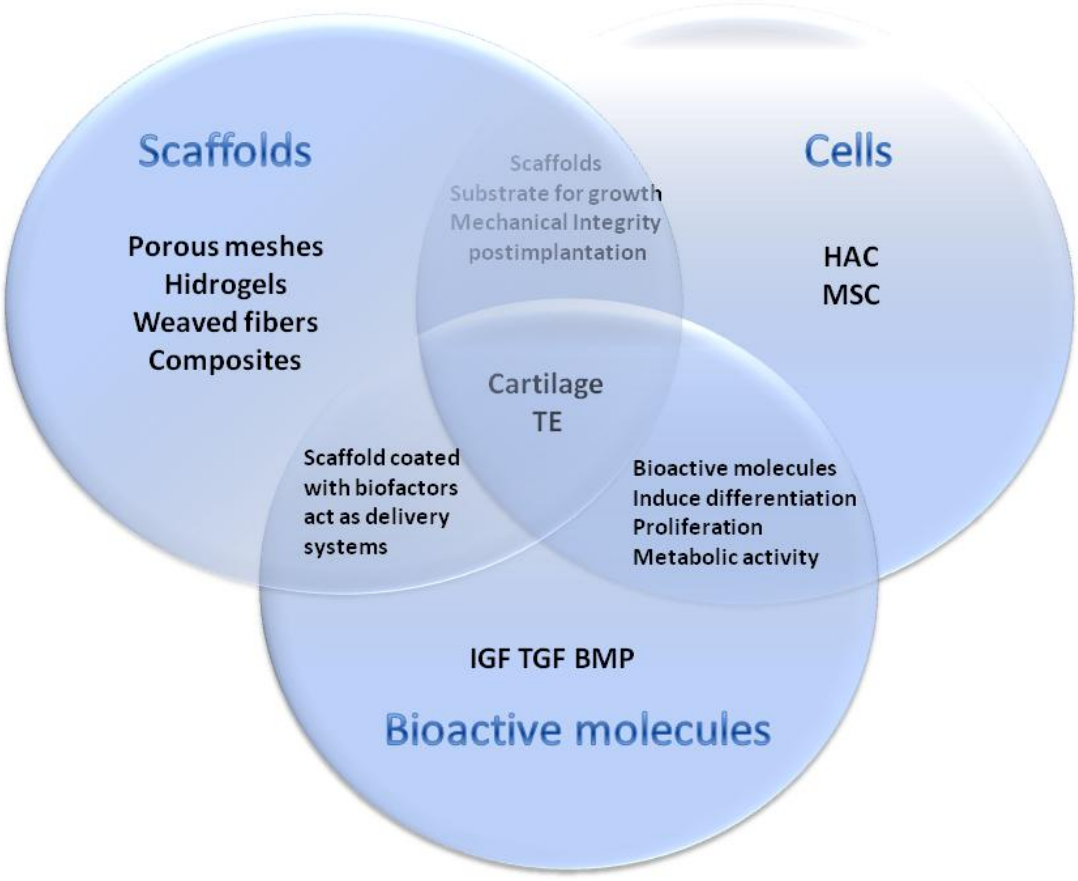


Figure 10
Tissue engineered based approaches involve the use of cells, bioactive molecules and scaffolds.

Cell sources

Among all the cell sources considered for tissue engineering, chondrocytes represent the logical cells of choice.

While chondrocytes are obtained mainly from the articular cartilage, there are other sources such as nasal chondrocytes.

The potential limitations are the instability of these cells in monolayer and the limited amount of cells present in the donor tissue.[35] Topographic variations regarding the redifferentiation potential have been postulated.[219]

Chondrocytes in monolayer express mesenchymal like markers.[66,129] It has been postulated that the surface of the articular cartilage contains a progenitor cell population that may be ideal for cartilage repair.[10,75]

Recently mesenchymal stem cells have been considered as an attractive source of cells for cartilage tissue engineering because of their availability and their high capacity in *in vitro* expansion. They are characterized for their capacity to adhere to the plastic and, their potential to differentiate into adipocytes, chondrocytes and osteoblasts.[38,46,47,49,50,74,87,216,252] These cells express a specific phenotype.[74,184]

However, it is still debatable whether the implantation of fully differentiated MSC-derived chondrocytes or of precommited cells is indeed required for successful cartilage repair.

Scaffolds

It is well established that cells reside, proliferate and differentiate inside the body within a complex three dimensional (3D) environment. In articular cartilage, chondrocytes are surrounded by an abundant ECM, which is composed of a highly hydrated complex network of molecules. In contrast, chondrocytes in monolayer shift towards a fibroblast-like phenotype that is characterized by an increased expression of type I collagen and the adoption of a spindle shape. The dedifferentiated chondrocytes can recover their differentiated phenotype when they are relocated into a 3D environment. This observation confirms that the 3D environment is a pivotal factor that have a significant role in supporting or in restoring the chondrocytic phenotype.[224]

Biomaterials serve as scaffolds and can be classified as natural biomaterials, which are further distinguished as protein-based and polysaccharide-based biomaterials, and as synthetic biomaterials. Among the protein-based biomaterials, membranes formed of type I and III collagens are clinically available for autologous chondrocyte implantation; such membranes include MACI (Genzyme Biosurgery, Boston, MA, USA).[22,91,155,231] Atelocollagen (Koken Co. Ltd, Tokyo, Japan) is a gel made of type I collagen from which telopeptides containing antigenic determinants have been removed.[180] This collagen gel enables the 3D culture and in vivo implantation of human autologous chondrocytes and of bone marrow MSCs.

Hyalograft C®, a tissue-engineered graft, consists of autologous chondrocytes that are associated with a hyaluronic-acid-based matrix termed HYAFF-11® (Fidia Advanced Biopolymers, Abano Terme, Italy).[73,154,172,188]

Among the synthetic biomaterials, Bio-Seed® (BioTissue Technologies, Freiburg, Germany) is a porous 3D scaffold made of polyglycolic acid (PGA), polylactic acid (PLA) and polydioxanone seeded with autologous chondrocytes embedded within a fibrin gel. Bio-Seed® has been reported to induce the formation of hyaline cartilage, which is associated with a significant clinical improvement of joint function.[79,173,175,176]

Despite encouraging clinical results, the above-mentioned matrices suffer a major limitation in that that they all require a surgical incision into the joint to be implanted. In this context, the development of injectable biomaterials suitable for mini-invasive transplantation of chondrogenic cells remains challenging to researchers.[78,146,211] Hydrogels are a new class of biomaterials that could potentially be injected transcutaneously into the joints. These biomaterials are composed of a viscous polymer made of synthetic or natural hydrophilic macromolecules, that are able to form a hydrogel after physical, ionic or covalent cross-linking. Hydrogels exhibit a high water content close to that found in cartilage and therefore mimic the 3D environment of cells in cartilage. The chondrogenic differentiation of MSCs has also been demonstrated with most of the above-mentioned scaffolds. Future advances in the development of 3D scaffolds, such as hydrogels that might be able to support in vivo chondrogenesis, could help to address this issue and should be studied further.

Table I
Cells and materials used in cartilage tissue engineering

Type	Material	Chondrocyte	MSC
Protein Based	*Collagen*	17,21,24,55,106	140
	Fibrin	106	77
Polysaccharide Based	*Alginate*	187	15
	Chitosan	234,248,249	195
	Hyaluronic Acid	154,171,172,188,229	59,86,217
	Cellulose	221	NA
Synthetic	*PLGA*	54,228	217
	Self assembling peptides	133	134

Growth factors

Growth factors that induce specific differentiation pathways and the maintenance of the chondrocyte phenotype are needed in tissue engineering. Several growth and differentiation factors that are involved in regulating cartilage development and homeostasis of mature articular cartilage have been identified.

Transforming growth factor family

The transforming growth factor family of polypeptides includes TGF-β, bone morphogenetic proteins, activins and inhibins.

These molecules initiate signaling from the cell surface by interacting with type I and type II receptors, depending on the ligands they bind. Upon ligand binding, the type II receptor activates the type I receptor, which phosphorylates the downstream mediators: Smads 1, 5 and 8 after BMP activation and Smads 2 and 3 after TGF-ß and activin-binding, respectively.

The phosphorylated Smads associate with Smad 4 and translocate into the nucleus, where they participate in gene transcription.[45,97] The TGF-ß family includes five members (TGF-ß 1–5), which are predominantly produced in bone and cartilage. Active TGF-ß 1, 2 and 3 are generally considered to be potent stimulators of proteoglycans and of type II collagen synthesis in chondrocytes and are able to induce the chondrogenic differentiation of MSCs *in vitro*.[97]

Fibroblast growth factor family

This family represents 22 related proteins. The importance of FGF signalling in skeletal development is highlighted by the number of dysplasia´s that are present if their encoding genes demonstrate a mutation.[183,235,237] One of the best factors within this family in terms of the effect on chondrocytes and mesenchymal stem cells is FGF-2, which has been shown to influence cell proliferation and redifferentiation in three dimensional systems.[217]

Insulin and Insulin-like growth factor 1

These proteins are not from the same family but act jointly in cartilage. Insulin at higher levels than are present in human serum promotes differentiation and stabilizes the chondrocytes phenotype.[43] Insulin-like growth factor 1 (IGF-1) supports chondrocyte differentiation at a low concentration.[82,83,220,246]

Insulin is a typical component of chemically defined chondrocyte culture media and it is used as an alternative to IGF-1.

AIMS OF THE STUDY

The objective of the present investigation was to study different methods for improving different parts of the techniques for cartilage repair with autologous chondrocytes and/or mesenchymal stem cells.
The following questions were raised:

- 1. Could a partial exchange of chondrocytes with autologous adipose-derived stem cells (ASC) reduce the number of chondrocytes needed for a cell implantation in cartilage repair?

- 2. How many chondrocytes or chondrogenic cells are needed for a secure chondrogenic induction?

- 3. How much time of *in vitro* redifferentiation is needed to obtain a secure cartilaginous production?

- 4. Do the dedifferentiated chondrocytes imitate the behaviour of interface mesenchymal stem cells responsible for joint formation during foetal development?

- 5. What is the best composition of nutrition media when to produce a cell-scaffold construct with the best properties for integration after implantation?

- 6. To which degree can the culture conditions influence the results of the repair?

- 7. The choice of scaffolds for the chondrogenic cells is difficult. How much can the biomimetic properties of a matrice influence the cells ability to migrate? Further, is it possible to improve the cells migration capacity?

- 8. How important is it for the results of the repair that the cells increase their migration capacity?

MATERIALS AND METHODS

Cell culture techniques

Cell type

In the work reported in paper I adipose stem cells (ASC) were isolated from three different donors as previously described.[255] Briefly, liposuction material was washed with phosphate-buffered saline (PBS) to remove most of the blood and tumescence solution. Afterwards, tissue was digested with collagenase at 37ºC for 1h. To eliminate red blood cells, the isolated fraction was incubated with erythrocyte lysis buffer for 10 min. Remaining cells were filtered through a 100 µm filter and cultured in an expansion medium containing DMEM-low glucose and HAM's F12 (60:40), 1% fetal calf serum, 1% insulin, transferrin, and selenium, 2 mM L-glutamine,100 U/mL penicillin,10 ng/mL epidermal growth factor, and 10 ng/mL platelet derived growth factor-BB (PDGF-BB).

In papers I to IV, adult human articular chondrocytes obtained from patients that were to undergo an ACI procedure were used. The harvested cartilage biopsies were transported to the cell culture laboratory in sterile saline solution supplemented with antibiotics (gentamicin sulphate) and antimicotic (amphotericin B).

The transportation medium holds a pH of 5.4 (measured at 4°C) and preserves the viability of cartilage biopsies for up to 48 hours.

On arrival at the cell culture laboratory the chondrocytes were isolated in a two step procedure as previously described.[35] After removal of contaminating subchondral bone, the chondrocytes were isolated from their surrounding matrix by mechanical mincing using a scalpel followed by overnight collagenase digestion at 37°C and 7% CO_2 / 93% air.[35] The clostridial collagenase degrades the collagen fibers. After the enzymatic digestion the cells are released from the matrix and exist as single cells or small cluster of cells in the suspension.

The study reported in paper V used chondrocytes from skeletally mature pigs. To ensure as sterile conditions as possible, whole pig knees were obtained from the experimental department at the University of Gothenburg. Cartilage from the femoral condyle was harvested and only animals with no macroscopic signs of degeneration were used in the study.

Cell expansion

Directly isolated human chondrocytes were seeded at a concentration of 10-16 x10^3 cells/cm^2 on Primaria® (Falcon, BD, New Jersey, USA) flasks. Isolated chondrocytes were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with L-ascorbic acid, gentamicin sulphate, amphotericin B and L-Glutamine. The medium was supplemented with 10% human serum when expanding human chondrocytes. Subculture was made with trypsin-EDTA solution when the cells reached 80% confluence. Afterwards, the cells were seeded at a confluence of 3-4 x 10^3 into new polystyrene flasks (Costar,Corning,USA). Trypsin is a protease that is highly specific to positively charged side chains with lysine and arginine and degrades the matrix that has built a bridge between the cells and the negatively charged culture flask resulting in a single cell suspension.

The culture conditions during the expansion in culture flasks are called monolayer (ML) culture in this thesis.

The human serum used consists of serum pooled from at least six donors in order to minimize batch-to-batch variation. The ethics committee of the medical faculty of Gothenburg University approved the use of human serum.

The fetal calf serum (FCS) used in this thesis was tested with respect to proliferation and subsequent chondrogenic, osteogenic and adipogenic differentiation.

Chondrogenic differentiation and three dimensional differentiation systems

The matrix production capacity of the expanded chondrocytes studied in paper 1 was analyzed in a pellet mass culture system (also called pellets).[124,222] The pellet mass culture system provides important conditions for recovering a differentiated phenotype from the *in vitro* expanded cells, namely a defined medium with certain growth factors and a three dimensional environment at high cell density. It has been reported that hyaline cartilage engineered by chondrocytes in pellet mass culture shares similarities with native cartilage in cellular distribution, matrix composition and density, and tissue ultrastructure and is thus a good system for chondrogenic differentiation.[253]

To achieve a three-dimensional environment, the cells were centrifuged at 500 g for 5 minutes. The chondrocytes were cultured in defined medium, consisting of DMEM-high glucose supplemented with ITS+ (insulin, transferrin and selenium), linoleic acid, human serum albumin, TGF-β1, dexamethasone, ascorbic acid and penicillin-streptomycin. The addition of TGF-β1 increases the expression of cartilage extracellular matrix proteins.[90,118] Dexamethasone, similar to TGF-β1, enhances chondrogenic differentiation as demonstrated by the expression of collagen type II, aggrecan, SOX9 and linkage protein.[122,206]

In paper I, adipose stem cells (ASC) from three different donors were mixed with human articular chondrocytes (HAC) at various ratios and $5x10^5$ cells were seeded in 100 mm^3 fibrin matrix Tisseel® (Baxter, Immuno, Vienna, Austria) or a COL1A1 Tissue Fleece® scaffold (Baxter, Immuno, Vienna, Austria). (Figure 11)
The following ratios were used for the study: 0% HAC (100%ASC), 5% HAC (95% ASC), 10% HAC (90% ASC), 20% HAC (80% ASC) and 100% HAC (0% ASC).
(Figure 11)

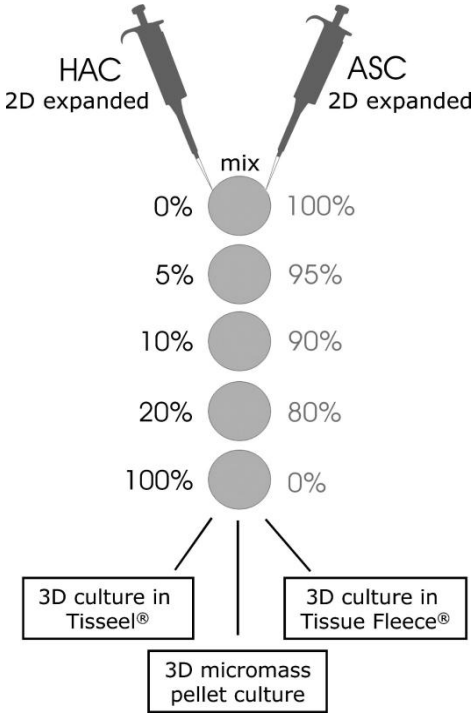


Figure 11
Schematic figure of how the HAC and the ASC were mixed in paper I.

Scaffold based differentiation

In papers I-IV the redifferentiation capacity of *in vitro* expanded human chondrocytes was studied in a scaffold mediated chondrogenic assay. The scaffold provides a three-dimensional environment that is one of the essential factors for redifferentiation to take place. The scaffolds used in this thesis are listed in Table II.
The work reported in paper I used a fibrin gel and a collagen matrix as three dimensional differentiation systems. The aim in this case was to compare the effect of the biomaterials to a scaffold-free system; cells were also cultured as three-dimensional micro mass pellets.
The chondrocytes were prepared by centrifugation of 3 x 10^5 cells at 350 g for 5 min in 1.5mL screw cap tubes. Cell seeding to Tisseel® (Baxter Immuno, Vienna,

Austria) was performed by diluting 25 mL of the cell suspension (2 x10^7 cells/mL) 1:2 with the fibrinogen compound.
Clot formation was induced at 37ºC using 50mL thrombin (4U/mL). Tissue Fleece® (Baxter, Immuno, Vienna, Austria) was conditioned with human serum for 2 h at 37ºC. Discs with a diameter of 8mm were punched and seeded statically from the top.
After 2 h, Tisseel® and Tissue Fleece® scaffolds were covered with 700 mL of differentiation medium consisting of DMEM-hg (PAA laboratories, Linz, Austria) supplemented with 5.0 mg/mL linoleic acid (Sigma-Aldrich, Stockholm, Sweden), 1 % insulin, transferring selenium (ITS-G concentrate, Life Technologies, Paisly,UK), 1mg/mL human serum albumin (Equitech-Bio, Kerrville, TX, USA), 10 ng/mL transforming growth factor-β1 (TGF-β1) (R&D Systems, Barton Lane, United Kingdom), 10 $^{-7}$ M dexamethasone (Sigma-Aldrich, Steinheim, Germany), 14 µg/mL ascorbic acid (Sigma-Aldrich, Steinheim, Germany), 1% penicillin–streptomycin (PAA laboratories, Linz, Austria) and 100 IU/mL aprotinin (Trasylol®; Bayer, Leverkusen, Germany) and incubated at 37ºC with 5% CO_2.
RNA for quantitative RT-polymerase chain reaction (PCR) was isolated on day 14, and histological evaluations were performed after 28 days of culture. The medium was replaced three times per week.

In paper II chitosan powder (Primex, Haugesund, Norway) with 90% DDA (degree of deacetylation) was dissolved in 1.25% acetic acid in a glass beaker at a concentration of 3% (w/v). The solution was mixed with a magnetic stirrer for about an hour to assure complete dissolution of the powder. Molds (ϕ= 10 mm, depth= 2 mm) were filled with the solution and placed in a -34 °C freezer. After 24 hours in the freezer, the scaffolds were placed in a freeze dryer (Heto Power Dry PL3000), for at least 48 hours. The scaffolds were rehydrated in a graded ethanol series, starting with 99.9% ethanol, and then put in distilled water.

Before cell seeding, the scaffolds were autoclaved for at least 20 min at 121°C.
Four groups (A, B, C and D) were created and seeded with different cell concentrations. After the first passage the cells were seeded into chitosan scaffolds with a diameter of 10mm at a density of 0.5x10^6, 1 x10^6, 2 x10^6 and 4x10^6 cells per cm^2 in a volume of 50 µl of media. The final cell seeding concentration per cm^3 of chitosan scaffold was group A: 3 x $10^{6,}$ group B: 6 x 10^6, group C: 12 x 10^6 and group D: 25 x 10^6 cells.
We used a defined culture differentiation media for chondrocyte differentiation in 3D consisting of DMEM-High Glucose (PAA Laboratories, Linz, Austria) supplemented with ITS-G (Life Technologies, Invitrogen, Paisly, UK), 5.0 µg/ml linoleic acid (Sigma-Aldrich, Stockholm, Sweden), 1.0 mg/ml human serum albumin (HAS-Equitech Bio,TX,USA), 10 ng/ml TGF-β1(R&D systems, UK), 10^{-7} M dexamethasone (Sigma-Aldrich, Stockholm), 14 µg/ml ascorbic acid (Sigma-Aldrich, Stockholm) and 1% penicillin-streptomycin (PAA Laboratories, Linz, Austria).

In papers III and IV prior to the seeding of the cells the scaffolds were pre-coated with human serum for 1.5 hours. The scaffolds were subsequently seeded with a cell density of 2 x 10^6 cells/cm^2 and incubated over night at 37° C in 7% CO_2/93% air in a humidity chamber. The scaffolds were then cultured in a defined media promoting differentiation of chondrocytes.

In paper III, the cells were cultured in the scaffolds for 1, 7, 14 and 21 days and the medium was changed three times a week. Half of the cell-scaffold constructs were implanted subcutaneously in a Balb C nu/nu mice model and the rest of the scaffolds were further analyzed using histological and immunohistochemical analyses, and at the mRNA level. After 8 weeks *in vivo* the scaffolds were harvested and analyzed in the same way.

In paper IV, different experiments were conducted as described in Figure 12.
The effect of *in vitro* pre-culture was evaluated by implanting constructs subcutaneously in BALB C mice either directly without pre-culture or after allowing the cells to re-differentiate in culture using the media composition described below for group C.
In another experiment, to determine the effect of different media conditions on the *in vitro* and *in vivo* redifferentiation capacity, the scaffolds were cultured for 14 days in three different media conditions. After 14 days, the scaffolds were evaluated using histological analyses, biochemical composition and at mRNA level or implanted subcutaneously into BALB C mice for 8 weeks. After this period, the scaffolds were evaluated as described above.
The cell seeded scaffolds were divided into three groups (A-B-C). A control group, D, was used with a non cell seeded scaffold.
The media used were:
Group A: DMEM/F12 media (Gibco, Invitrogen, Paisly, UK) supplemented with 10% pooled human serum, 0.1mg/ml Ascorbic acid (Apotekets production unit, Umeå, Sweden), 2 mmol/ml L-Glutamine (Gibco, Invitrogen, Paisly, UK), 0.05µg/ml gentamicin sulphate (Gibco, Invitrogen, Paisly, UK) and 0.5µg/ml Amphotericin B (Gibco Invitrogen, Paisly, UK).
Group B: DMEM/F12 media (Gibco, Invitrogen, Paisly, UK) supplemented with 10% pooled human serum, 0.1mg/ml Ascorbic acid (Apotekets production unit, Umeå, Sweden), 2mmol/ml L-Glutamine (Gibco, Invitrogen, Paisly, UK), 1% ITS (Invitrogen, Paisly, UK,), 10 ng/mL transforming growth factor B_1 ($TGF\beta_{1)}$ (R&D systems), 10^{-7} M dexamethasone (Sigma-Aldrich, Stockholm, Sweden), 0.05µg/ml gentamicin sulphate (Gibco, Invitrogen, Paisly, UK), 0.5µg/ml Amphotericin B (Gibco Invitrogen, Paisly, UK)

Group C: DMEM high glucose (PAA Laboratories, Linz, Austria) supplemented with 1% ITS (Life Technologies, Invitrogen, Paisly, UK), 5µg/ml Linoleic acid (Sigma-Aldrich, Stockholm, Sweden), 1.0 mg/ml human serum albumin (Equitech-Bio TX USA), 10ng/ml TGFß$_1$ (R&D systems, UK), 10^{-7} M dexamethasone (Sigma-Aldrich, Stockholm, Sweden), 14,1µg/ml ascorbic acid 2-phosphate (Sigma-Aldrich, Stockholm, Sweden), and 1% Penicillin-Streptomycin (PAA Laboratories, Linz, Austria).
Group D: An empty scaffold.

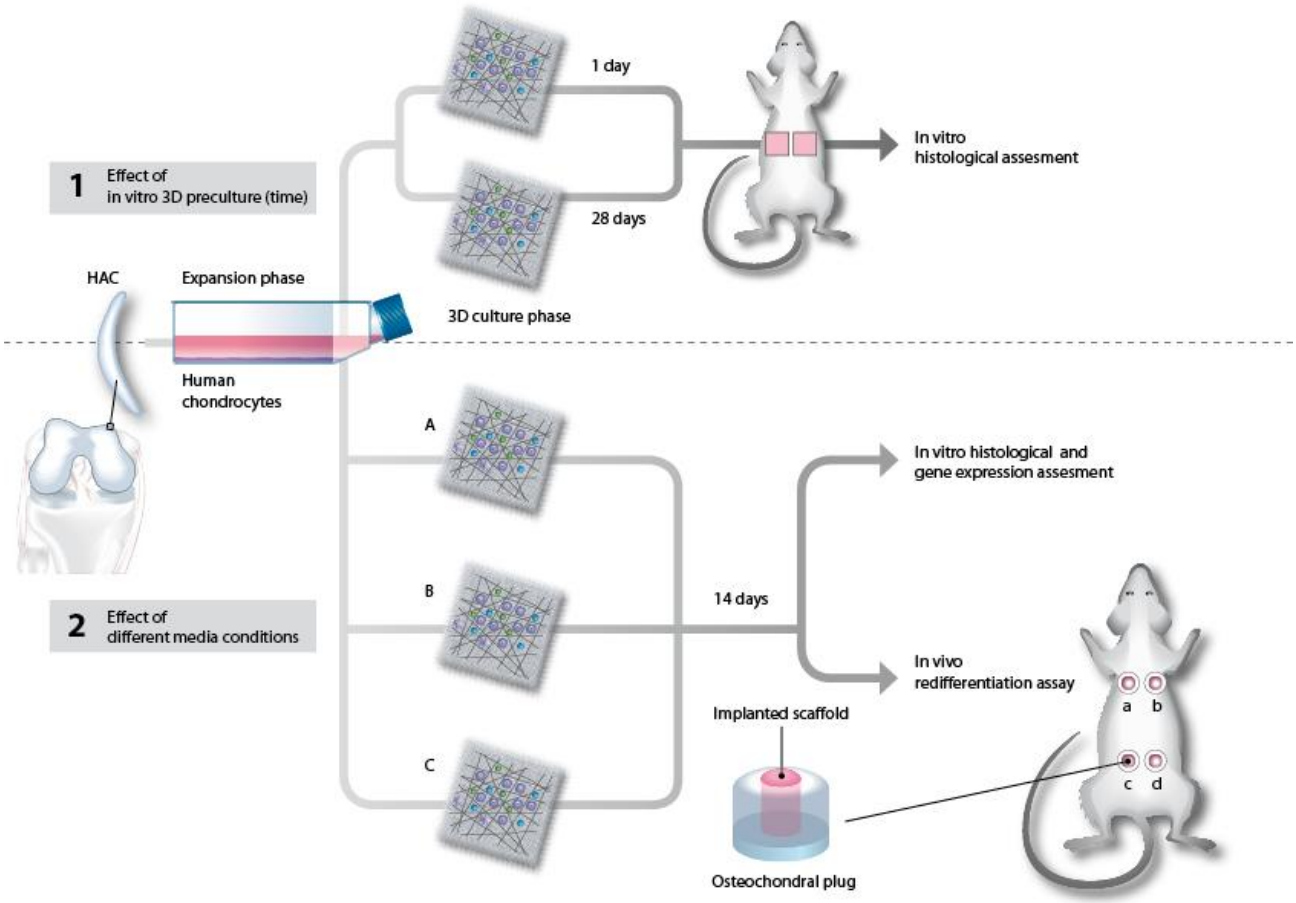


Figure 12
Experimental design paper IV. Art by Pontus Andersson.

Table II: Materials used in this thesis

Material	*Collagen*	*Fibrin*	*Chitosan*	*Esterfied-hyaluronan*	*Alginate-Agarose*	*RADA 16*
Commercial name	Tissuefleece	Tissel	NA	Hyaff 11™	Cartipach®	Puramatrix®
Scaffold fabrication	Mesh like	Gel	Foam	Non-woven	Gel	Gel
Porosity	>95%	>93%	>95%	>95%	NA	NA
Pore size	>150µm	NA	>150µm;150-300µm; 300-500µm	100-300µm	NA	NA
Fiber size	30µm	~300-500 nm	N/A	~10-20µm	50-100 nm	50nm
Sterilization	Electron beam irradiation		Peroxide	γ-radiation	γ-radiation	γ-radiation
Degradation	Hydrolysis	Hydrolysis	Hydrolysis	Hydrolysis	Hydrolysis	Hydrolysis
Provided by	Baxter	Baxter	Chalmers Biopolymer department	Fidia Advanced Biopolymers	TBF	3DM

In vivo differentiation model

In papers III and IV, where the re-differentiation capacity of human articular chondrocytes was studied in a more biologically environment, HA scaffolds were implanted subcutaneously into female Balb C nu/nu 12-week-old mice.[197] At the time of implantation the mice were anesthetized with 3% Isofluorane. The mice were sacrificed 6-8 weeks after implantation by cervical dislocation.

The work reported in paper IV evaluated the integration capacity of the scaffolds by implanting the constructs into osteochondral defects and then subcutaneously into Balb C mice as described below. Two sections of human femoral condyles were obtained from donors undergoing total knee replacement. The condyles were stored at 4º C in DMEM/F12 media for 4 weeks and the medium was changed three times a week according to Williams et al.[240] At the time of implantation, Ø8 mm full thickness cartilage discs were punched out from the condyles. A chondral defect (4 mm in diameter) was made in the center of the cartilage plugs. Discs with Ø4 mm were punched from the different constructs and placed in the defect. The constructs were fixed with a drop of fibrin sealant (Tissucol duo S 0.5 ml immuno®, Baxter, Immuno, Germany). There was a complete filling of the defect with the construct. The constructs were implanted subcutaneously in the backs of Balb C mice (Figure 13).

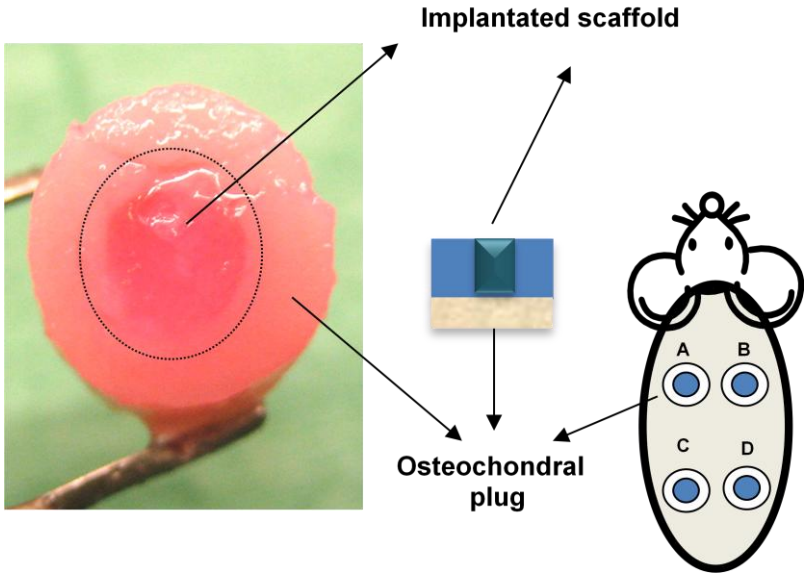


Figure 13
Osteochondral defect model.

Semi-quantitative histological analysis

The chondrogenic capacity of the pellets and scaffolds was analyzed with the Bern score.[98,159,193,203] The O Driscoll system was used to evaluate the integration of the scaffolds into the host tissue.[159,177] The Bern score is a visual histological evaluation system for neocartilage formation based on Safranin-O staining (minimum value 0 and maximum 9). Safranin-O stained sections from pellets or scaffolds were scored according to the uniformity and intensity of Safranin-O staining, the distance between cells/amount of matrix produced and the cell morphology.

Three dimensional transmigration assay

In paper V a three dimensional transmigration assay was used to determine the migration potential of different biomaterials.

A Hyaff-11® scaffold (Fidia Advanced Biopolymers, Albano Terme, Italy) measuring 0.5×0.5 cm was used as a scaffold in all groups.

Two different gel coatings were used in two groups. One group was left uncoated. All groups were assembled using pig cartilage fragments obtained as previously described.

Three different groups were assembled with 24 samples each and placed in 24-well tissue culture plates (Falcon).

Group A: In this group, Hyaff 11® was placed alone in a cell culture insert with a 0.4 µm pore size (Falcon, 0.4 µm pore size, γ-irradiated).

A total of 12.37 ± 2.41 mg of cartilage fragments per cm^2 was placed on the scaffold and covered with media.

Group B: In this group the Hyaff 11® scaffold was precoated using a self assembling peptide PuraMatrix® (RADA 16, 3DM, Cambridge, MA, USA).

The total amount was 15.20 ± 4.71 mg of cartilage fragments per cm^2 of scaffold. The coated scaffold loaded with the cartilage fragments was placed in a cell culture

insert with the same pore size as previously described. After this, medium was added and changed after 10 minutes. The change in medium was made three times in order to achieve a homogenous assembly and to raise the pH of the sample according to the manufacturer's instructions.

Group C: This group was prepared as described above using 13.50 mg ± 4.33 per cm^2 of scaffold but in this case, the Hyaff 11® scaffold was coated with a gel solution consisting of 1.5% alginate and 1% agarose (Cartipatch® - TBF Tissue Engineering, Bron, France).

Before adding the minced cartilage, 500 μl of DMEM-F12 (Life Technologies, Invitrogen, Paisly, UK), 10% pooled human serum, 50 µg/ml ascorbic acid (Sigma-Aldrich, Stockholm, Sweden) and 1% penicillin/streptomycin (PAA Laboratories, Linz, Austria) were added to the wells.

All samples were then incubated at 37°C with 7% CO_2. The explants were cultured for a minimum of 19 days using DMEM-F12 (Life Technologies, Invitrogen, Paisly,UK), 10% pooled human serum, 50 µg/ml ascorbic acid and 1% penicillin/streptomycin (Invitrogen, Paisly, UK). This media composition was called proliferation media.

The cartilage pieces were separated from the scaffolds and the DNA content was measured in the different groups at 19, 33 and 41 days to evaluate the amount of cells that migrated to the biomaterials.

To evaluate whether these cells had a chondrogenic potential the medium was changed after 19 days to a defined chondrogenic medium composed of DMEM high glucose (PAA Laboratories, Linz, Austria) supplemented with 1% ITS+ (Life Technologies, Invitrogen, Paisly, UK), 5 µg/ml linoleic acid (Sigma-Aldrich, Stockholm, Sweden), 1.0 mg/ml human serum albumin (Equitech-Bio TX USA), 10 ng/ml TGFß$_1$ (R&D systems, UK), 10^{-7} M dexamethasone (Sigma-Aldrich, Stockholm, Sweden), 14.1 µg/ml ascorbic acid 2-phosphate (Sigma-Aldrich, Stockholm, Sweden) and 1% penicillin-streptomycin (PAA Laboratories, Linz, Austria). The chondrocyte differentiation phase lasted 4 weeks.

Migration properties of different cartilage layers

The system described above was used in the work reported in **paper V** to determine the origin of the cells that showed the best migration properties. Cartilage fragments from the superficial and deeper layers of the cartilage were sectioned and placed in culture as shown in Figure 5. Briefly, six 24-well tissue plates with culture inserts were used (Falcon). The PuraMatrix® (RADA 16) gel was sonicated for 30 minutes, and 600 µl of the gel was placed in the insert. DMEM-F12 medium was placed in the well outside the cell culture insert to start the assembly of the gel and to raise the pH. After three media changes, when the gel became assembled, the fragments were placed on top of the gel inside the insert. The migration of cells from the fragments was recorded using an inverted microscope. The number of cells per field was recorded by two independent observers.

The status of the fragments after culture was evaluated to assess the neocartilage formation around the fragments.

Biochemical analysis

Biochemical measurements were made to examine and quantify the amount of ECM in scaffolds. Scaffolds were digested with a papain solution. Papain is a cysteine protease that degrades the extracellular matrix and exhibits a broad specificity, cleaving peptide bonds of basic amino acids, leucine, glycine hydrolyzing esters and amides. The digested samples were then further analyzed for DNA, GAG and hydroxyproline (HP) content.

DNA

The amount of DNA was measured spectrophotometrically using Hoechst 33258.
Hoechst is a fluorescent staining for labeling DNA in fluorescence microscopy and fluorescence-activated cell sorting (FACS) and is a bisbenzyimidiazole derivate that is fluorescent when it binds to AT-rich regions of double stranded DNA. The concentration of DNA was calculated against a standard curve of serially diluted calf thymus DNA.

Proteoglycans

A dimethylmethylene blue (DMB) assay was performed to biochemically measure the proteoglycan content. DMB is a cationic dye that binds to sulphated GAGs and, by binding, changes the absorption spectra. The samples were measured spectrophotometrically at 515 nm, and the GAG content was calculated against a standard curve of chondroitin sulphate diluted in PBS.[81]

Collagen

Collagen can be studied by measuring the content of hydroxyproline (HP) since HP together with proline accounts for 25% of the amino acids in collagen. The hydroxyproline in the digested biopsy, scaffolds and pellets, was analyzed with a modified colorimetric method.[182]
The HP content was measured spectrophotometrically at 550 nm with a reference at 650 nm, and HP was used as a calibrator.

Electron microscopy

Transmission electron microscopy (TEM) (LEO 912 Omega, Carl Zeiss AB, Stockholm, Sweden), and scanning electron microscopy (SEM) (LEO 982 Gemini field emission, Carl Zeiss AB, Stockholm, Sweden) were done to study the structure of the scaffolds and the contact between the biomaterial and the chondrocytes.

Histological analysis

The biopsies, scaffolds and pellets were fixed with formaldehyde (Histofix™ Histolab, Gothenburg, Sweden), consisting of 6% formaldehyde buffered with PBS). With formaldehyde, the tissue is fixed by cross-linkages formed in the proteins, particularly between lysine residues. The stains used in the thesis are listed in Table III.

Table III: Stains used for histological evaluation.

Stain	ECM component analysed	Comment
Alcian blue van Gieson	**Proteoglycans** **This staining results in the articular cartilage staining blue, nuclei black and connective tissue red.**	**A cationic dye that forms reversible electrostatic bonds with the negative sites on polysaccharides.**
Safranin-O	**Proteoglycans** **Safranin O stains the sulphated proteoglycans orange to red, cytoplasm blue-gren and nuclei black.**	**A cationic dye that binds to a negatively charged group on chondrotin-6-sulphate or keratin sulphate, components of mature articular cartilage.**

Gene expression analysis

Gene expression analysis were made to study the process of redifferentiation *in vitro* and *in vivo* of adult articular chondrocytes on a regulatory level, reported in papers I, III and IV. Articular cartilage has a low cell to ECM ratio, which makes the RNA isolation a crucial step in achieving enough RNA for analysis of the cells. Furthermore, the ECM is rich in proteoglycans, which are large and negatively charged macromolecules that tend to co-purify with RNA.

Isolation of RNA

In paper I the total RNA was isolated according to the Tri Reagent protocol, and RNA content and integrity were assessed with an Agilent 2100 Bioanalyzer (RNA 6000 NanoChips Kit) (No. 5065-4476; Agilent Technologies, Böblingen, Germany). Isolated RNA was transcribed to cDNA according to the High Capacity cDNA Archive Kit protocol (Applied Biosystems, Brunn am Gebirge, Austria).

In papers III and IV, the total RNA was extracted by grounding the scaffold cell cultures to powder using MixerMill® (Qiagen, Hilden, Germany) and QIAzol® (Qiagen, Hilden, Germany). QIAzol lyses the cells and removes some of the proteoglycans of the ECM. Total RNA was isolated using the RNeasy® mini kit (Qiagen, Hilden, Germany). DNase mix was added to remove contaminating genomic DNA from the isolated RNA, and the RNA was finally eluted.

The amount and quality of total RNA were measured using a Nano Drop® (Thermo Scientific, Willmington, USA).

The ratio of λ260/λ280 was considered a measurement of the purity of the sample and a value between 1.9 and 2.1 was considered adequate. The λ260/λ230 was also measured to examine the nucleic acid purity. Values between 1.8 and 2.2 were considered acceptable. If the ratios described above are lower, they may indicate the presence of co-purified contaminants.

Quantitative real-time PCR analysis

Real-time PCR is a quantitative and very sensitive gene expression analysis.

In paper I, specific cDNA was quantified conducted using a LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) and TaqMan gene expression assays (Applied Biosystems, Brunn am Gebirge, Austria) for the following genes: sex-determining region Y (SRY)-box 9 (SOX9; Hs00165814_m1), COL2A1 (Hs01064869_m1), collagen type IX (COL9A2;Hs00899019_m1), aggrecan (AGC1; Hs01048724_m1), melanoma inhibitory activity (MIA; Hs01064456_g1), cartilage oligomeric matrix protein (COMP; Hs01561085_g1), cartilage link protein 1 (CRTL1; Hs00157103_m1), chondroitin sulfate, proteoglycan II (CSPG2; Hs01007932_m1), COL1A1 (Hs00164004_m1) and collagen type X (COL10A1;Hs00950955_g1).

The PCR was programmed as follows: initial denaturation at 95º C for 10 min, followed by 95º C for 10 s and 60º C for 45 s cycled 50 times.

Cooling to 40º C was done and this temperature was held for 30 s. The slope speed was 20º C per second. Standard curves were prepared for quantification, and expression values were normalized to the hypoxanthine–guanine phosphoribosyltransferase housekeeping gene. The efficiency-corrected quantification was made automatically using LightCycler 480 Relative Quantification Software (Roche Diagnostics, Mannheim, Germany).

In papers III and IV, the RNA was transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems, Brunn am Gebirge, Austria) and random hexamer primers.

The real-time PCR analyses were made as first described by Holland et al.[109]. Commercially available assay-on-demand mixes of primers and TaqMan MGB (FAM dye labelled) (Applied Biosystems, Brunn am Gebirge, Austria) probes were used in the work reported in this thesis.

The genes used to study the redifferentiation of the chondrocytes were e.g. collagen types I, IIA, IIB and X, COMP, Sox 9, tenascin C, fibronectin, aggrecan and versican, as well as enzymes controlling the matrix degradation, such as matrix metalloproteinases (MMP) 13 and tissue inhibitors of metalloproteinases (TIMP) 3. Additionally, the cell fate receptor Notch 1, its ligands Jagged 1 and Delta 1, and transcription factors hairy and enhancer of split (HES) 1 and 5 were examined. Additionally growth and differentiation factor 5 (GDF5) was studied. The 18S rRNA housekeeping gene labelled with VIC/TAMRA was used as an endogenous control.[13] The relative quantification of the target gene expression was made according to the standard curve method.[7,127,222]

Subjective evaluation of handling properties of the seeded scaffolds

Four orthopaedic surgeons with documented skills in cartilage repair evaluated implants that had been cultured in different ways. The surgeons classified the scaffolds from 1-4, where 1 was the best and 4 the worst.

Animal experiments

In paper II and IV we used a well established animal model to evaluate the re-differentiation potential of tissue engineered constructs. This system creates a milieu that provides adequate nutrients. Neocartilage formation depends on the intrinsic chondrocyte potency and commitment rather that than the micro-environmental conditions in the mouse model. This model, however, does not replicate the assorted biomechanical milieu present in the joint. Female Balb C *nu/nu* mice with an age of 12 weeks were used in our studies. (Charles River laboratories, Germany) At the time of implantation the mice were anesthetized with 3% Isofluorane. All animals were cared for and processed according to guidelines from the Experimental Biomedicine department at the University of Gothenburg. The procedures were approved by the ethical committee of the University of Gothenburg. (Ethical approval: 245-2008 and S40-01)

Statistics

Statistical analyzes were made with different tests. The work reported in paper I used one way analysis of variance and Tukey´s *post hoc* test. Statistical significance between biomaterials was evaluated using paired Student's *t*-test.

The significance of the difference between the different cell concentrations was evaluated in the work presented in paper II with the paired Student's *t*-test.

The work reported in paper III to V used the Wilcoxon paired signed rank. Nonparametric tests are often used when two or more independent samples are compared without assuming that the difference between the samples is normally distributed. The test considers whether each observation is above or below the chosen value of interest and is often used to examine the difference before and after a treatment. It is designed to test a hypothesis about the location (median) of a population distribution.

Papers III and IV used the Mann-Whitney U test. This test compares two independent groups of observations and assesses whether two samples of observations come from the same distribution. P-values lower than 0.05 were considered significant.

RESULTS

Paper I

The relative mRNA expression of cartilage matrix proteins COL2A1, AGC1, MIA, CRTL1, and COMP was significantly higher in pure HAC than in co-cultured cells and pure ASC in both scaffolds. The same results were obtained for MIA, which is also present physiologically in cartilage tissue. No significant differences between the coculture groups and the pure cultures were observed for COL9A2 and SOX9. However, in Tisseel® (Baxter, Immuno, Vienna, Austria) two of three donors demonstrated strongly enhanced COL9A2 expression in the co-culture groups and in the pure ASC group compared to the pure HAC group. Markers for fibrous cartilage (CSPG2 and COL1A1) and hypertrophic cartilage (COL10A1) were expressed at approximately the same level in both cell types and co-cultured cells. Overall a high donor variability was noticeable.

To illustrate whether ASC could principally contribute to chondrogenesis in co-culture with HAC, gene expression data are also presented as ‘‘relative mRNA expression per initial percent of chondrocytes’’.
COL2A1 was expressed equally in all groups in Tisseel®. In contrast, COL9A2 demonstrated an increased expression (97.3 ± 78.6) in the 5% HAC group compared to purely cultured HAC. A lower but still significant increase was demonstrated for COMP (6.1 ± 3.1), AGC1 (2.2 ± 1.5), MIA (2.3 ± 0.76), and CRTL1 (2.2 ± 0.7). In Tissue Fleece® COL2A1 was down-regulated in co-culture groups, and no up-regulation was observed for AGC1 and MIA.
To evaluate the type of cartilage engineered we also included markers for fibrous (CSPG2 and COL1A1) and hypertrophic (COL10A1) cartilage. Expression in the 5% HAC group demonstrated 22.8 ± 6.0-fold of induction for COL1A1 in Tisseel® and 26.3 ± 6.2 for CSPG2 compared to 100% HAC.
Although the ratio of up-regulation of CSPG2 exceeded the increase in AGC1, absolute expression values of AGC1 were approximately 10 to 40-fold higher compared to CSPG2. For COL10A1, a 67.3 ± 57.8-fold increase was measured for 5% HAC compared to 100% HAC. However, because of the high donor variability, the up-regulation was not significant.
A comparison of the biomaterials used revealed a significantly higher expression of COL2A1 when ASC were cultured in Tisseel®. However, the gene was expressed very weakly and the results should therefore be viewed with care. Other markers (COL9A2, MIA, CRTL1, AGC1, and COL10A1) demonstrated the same tendency toward a higher level of expression in Tisseel® but there were not significant differences.

SOX9, COMP, and COL1A1 showed equal expression in both materials. Purely cultured HAC did not show a significant difference in chondrogenic marker gene expression between the biomaterials tested.

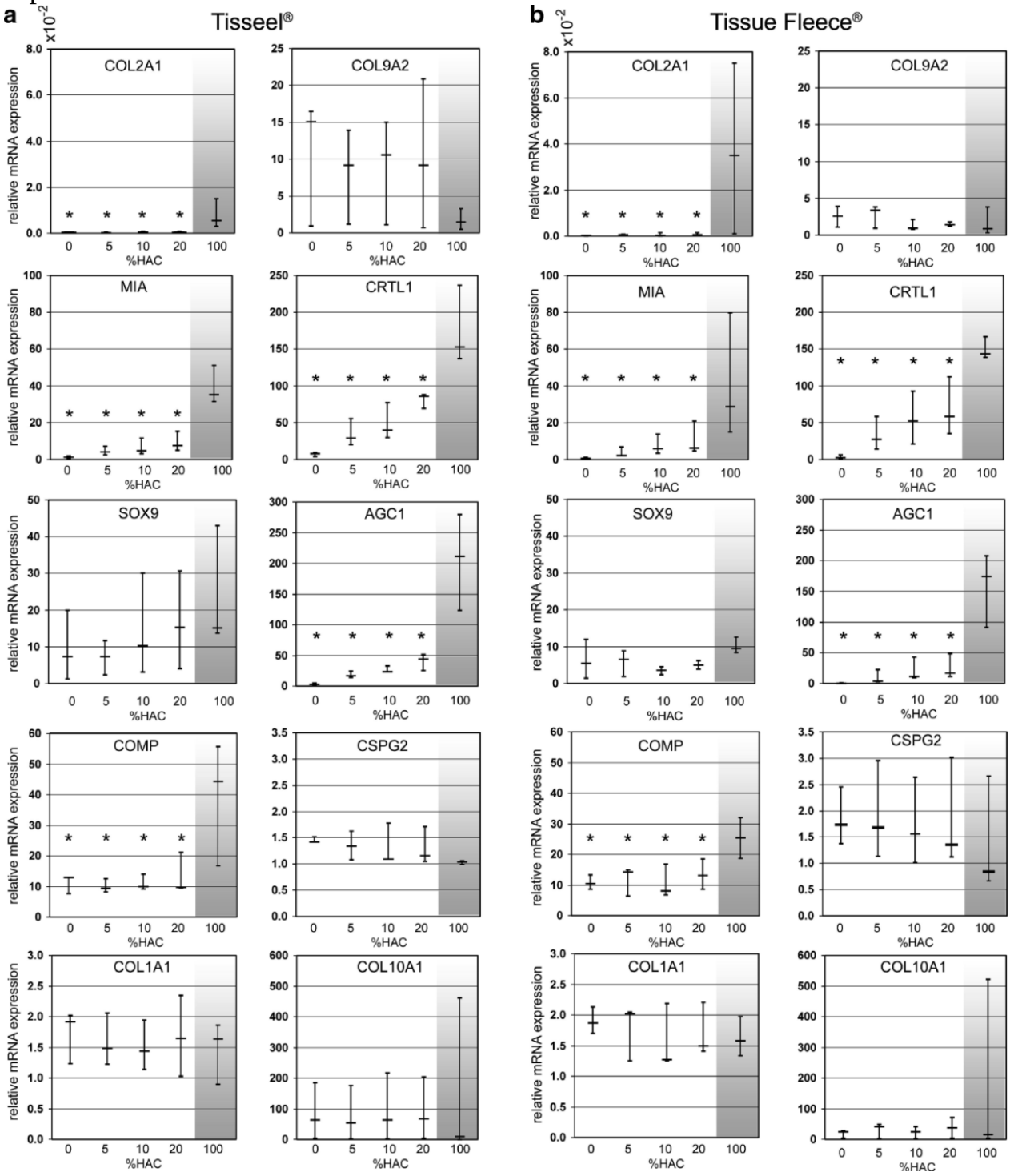


Figure 14

Quantitative reverse-transcriptase (RT)-polymerase chain reaction of co-cultured ASC and HAC in Tisseel® (a) or Tissue Fleece® (b), values represent the relative mRNA expression of marker genes coding for collagen type II (COL2A1), collagen type IX (COL9A2), SOX9, aggrecan (AGC1), melanoma inhibitory activity (MIA), cartilage link protein (CRTL1), cartilage oligomeric matrix protein (COMP), chondroitin sulphate proteoglycan II (CSPG2), collagen type I (COL1A1) and collagen type X (COL10A1). Data are presented as median and range (median, min, max), n=3 donors for each cell type. *Significantly different compared to 100% HAC.

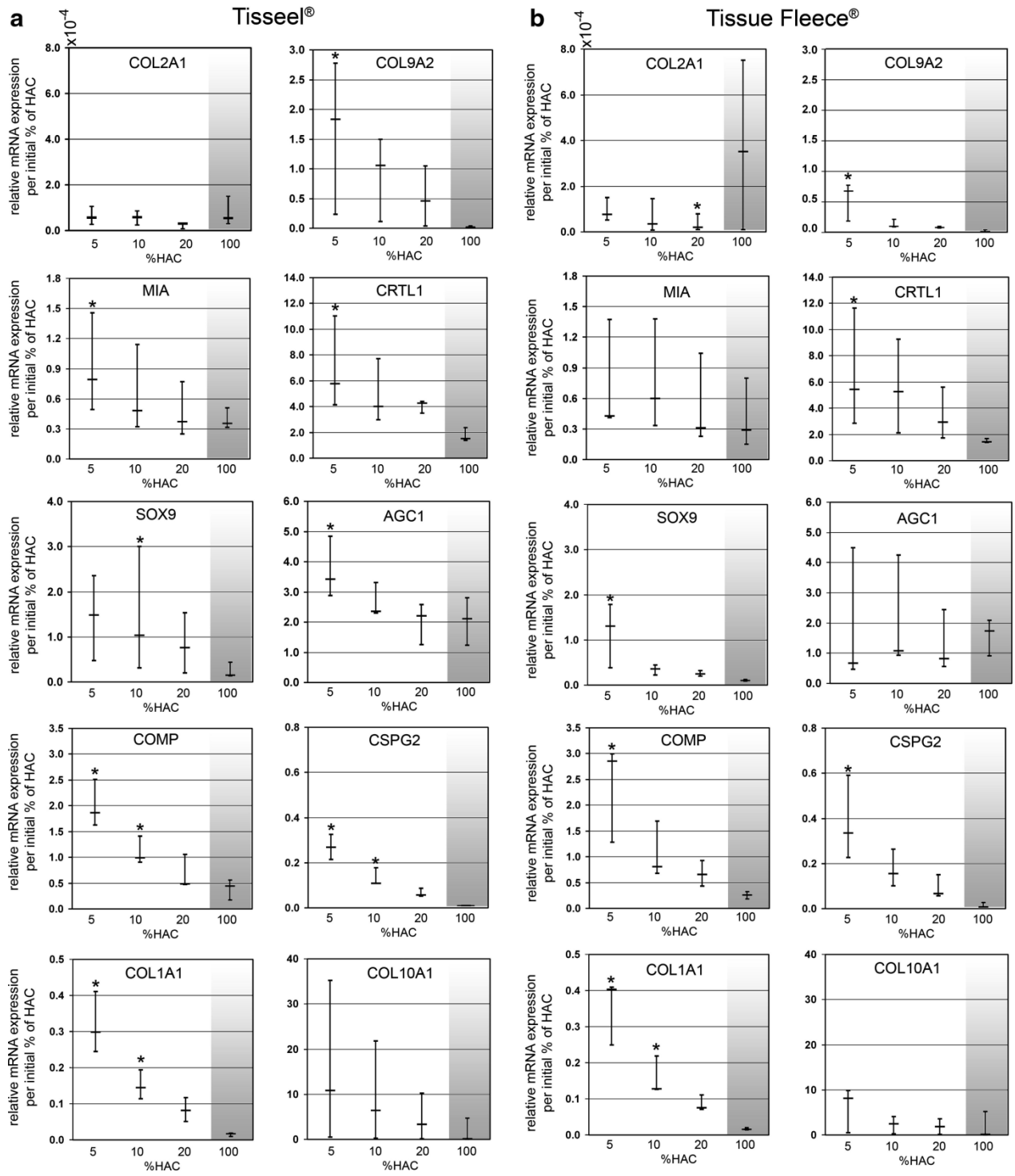


Figure 15

Quantitative RT-polymerase chain reaction of co-cultured ASC and HAC in Tisseel® (a) or Tissue Fleece® (b), values represent the relative mRNA expression per initial percent of HAC of marker genes coding for COL2A1, COL9A2, SOX9, aggrecan, MIA, CRTL1, COMP, CSPG2, COL1A1 and COL10A1. Data are presented as median and range (median, min, max), n=3 donors for each cell type. *Significantly different compared to 100% HAC.

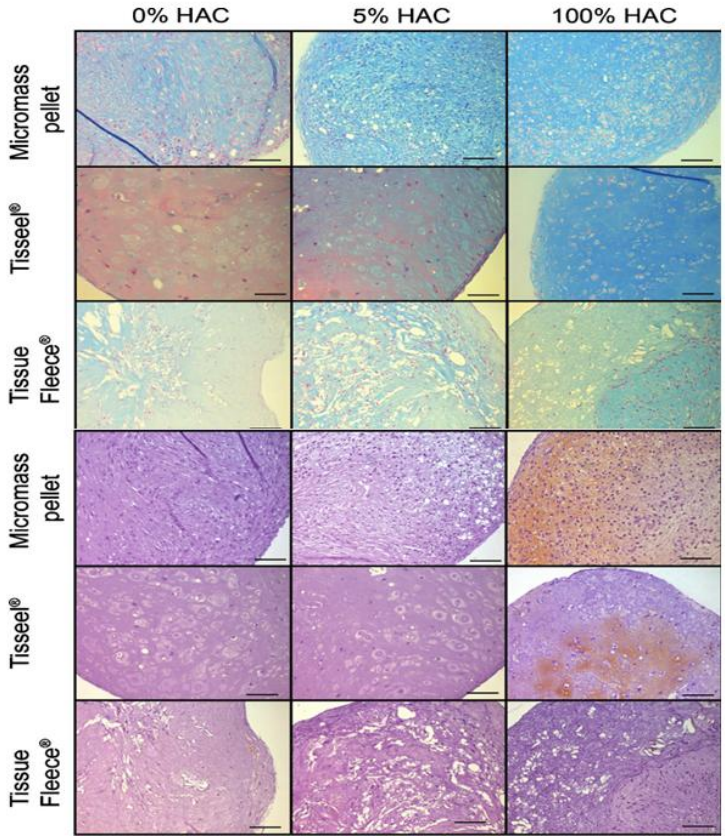


Figure 16
Histological evaluation of pure ASC (0% HAC), pure HAC (100% HAC) or mixtures (5% HAC) cultured as micromass pellets in Tisseel® or Tissue Fleece®. Sections were stained for glycosaminoglycans with Alcian blue and collagen-IIa1 (c). Scale bar 100 µm.

Alcian blue staining demonstrated a strong glycosaminoglycan (GAG) expression by HAC when cultured as micromass pellet cultures or in Tisseel®. A less pronounced staining was observed for Tissue Fleece® cultures.
The 5% HAC group showed GAG expression in micromass pellets and also when seeded in biomaterials. In Tisseel®, GAG staining is found homogenously around uniformly distributed, spherical cells. Tissue Fleece® cultures appear as loose networks with cells producing moderate amounts of GAG.
The lowest GAG expression was observed for the 0% HAC group, whereas more intense staining was shown for micromass pellets compared to Tisseel® and Tissue Fleece® (Tisseel® < Tissue Fleece® << micromass pellets).
Collagen-Ia1 was expressed throughout all samples and most intensively in the 100% HAC group. Collagen-IIa1 staining was visible only in purely cultured HAC and was restricted to micromass pellet cultures and Tisseel® (Figure 16).
Collagen-IIa1 expression of purely cultured ASC did not reach the detection limit of immunohistochemistry.

Paper II

The chondrocytes induced an active matrix synthesis and several points of interaction with the chitosan scaffold and the surrounding extracellular matrix. The pores varied from 50-100 μM in diameter (75 μM on average). SEM confirmed these findings. The chondrocytes grew along the porous structures and built aggregates within the material. A homogeneous distribution of the seeded chondrocytes was seen in the chitosan scaffold (Figure 17).
Histomorphometric analysis showed that, with a cell seeding concentration of 12-25 x 10^6 cells/cm^3 chondrogenic differentiation is triggered in areas of chondrocyte condensation (Figure 18).
The analysis of these condensation sections using the Bern score supported this observation (Figure 19). The cells in the constructs with high cell seeding densities showed increased Alcian blue staining, a rounded cell shape and a higher matrix production.
There was a significant difference in the number of cells between the groups A and D, containing 3 x 10^6 and 25 x 10^6 cells per cm^3 (p= 0.0019). There was a correlation between the number of cells evaluated by these two different methods.
The collagen and GAG concentrations increased with a variation of the cell seeding concentration.
The difference in the increase in total GAG concentration was significant between groups C and D (12 and 25 x10^6 cells /cm3) (p=0.03) (Figures 20 B).
We found a significant difference in the increase in total collagen production between groups A and C (3 and 12 x 10^6 cells /cm^3 respectively) (p=0.019)
(Figure 20-A).
The sulphated GAG content increased notably in the 25 x10^6 cells per cm^3group as compared to the other groups (Figure 20-B).
The mechanical properties of the different constructs increased over time. This increase was from 9.6 kPa at 14 days of 3D culture to 14.6 kPa at 28 days under the same culture conditions. These values correspond to a frequency of 5Hz. (Figure 20-C) The group D showed significantly higher mechanical properties compared to the other groups; group D showed 15 kPa and group C 6 kPa
(Figure 20-D)**.**

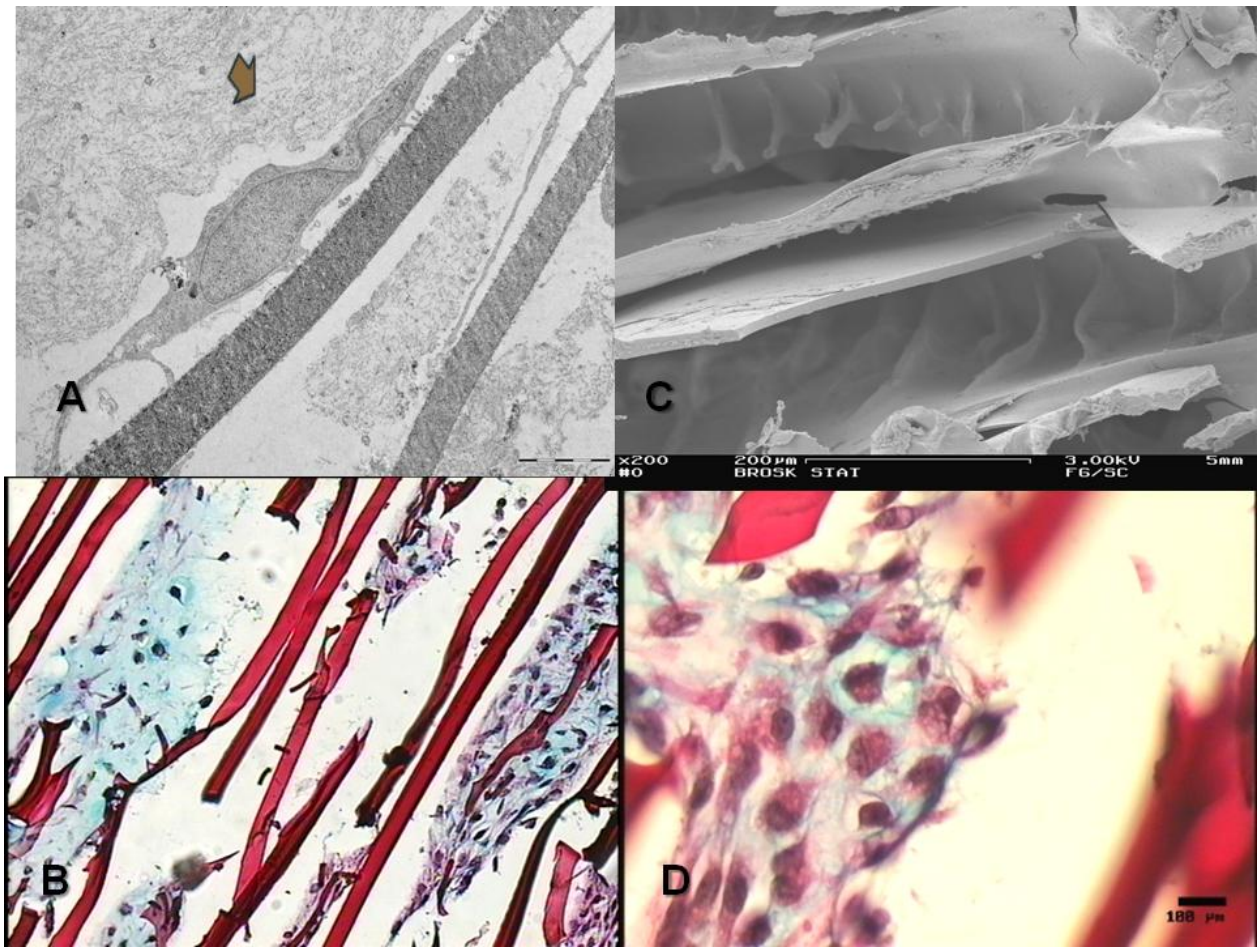


Figure 17
A: Chondrocyte attached to the chitosan scaffold. The arrow shows the extracellular matrix around the scaffold.
B: Scanning electron micrograph of a chitosan scaffold.
C: Chondrocytes growing in a chitosan scaffold; note the cells growing in micro-mass (Alcian blue/van Gieson, ×20).
D: Chondrocyte interaction; note the Alcian blue positive staining.

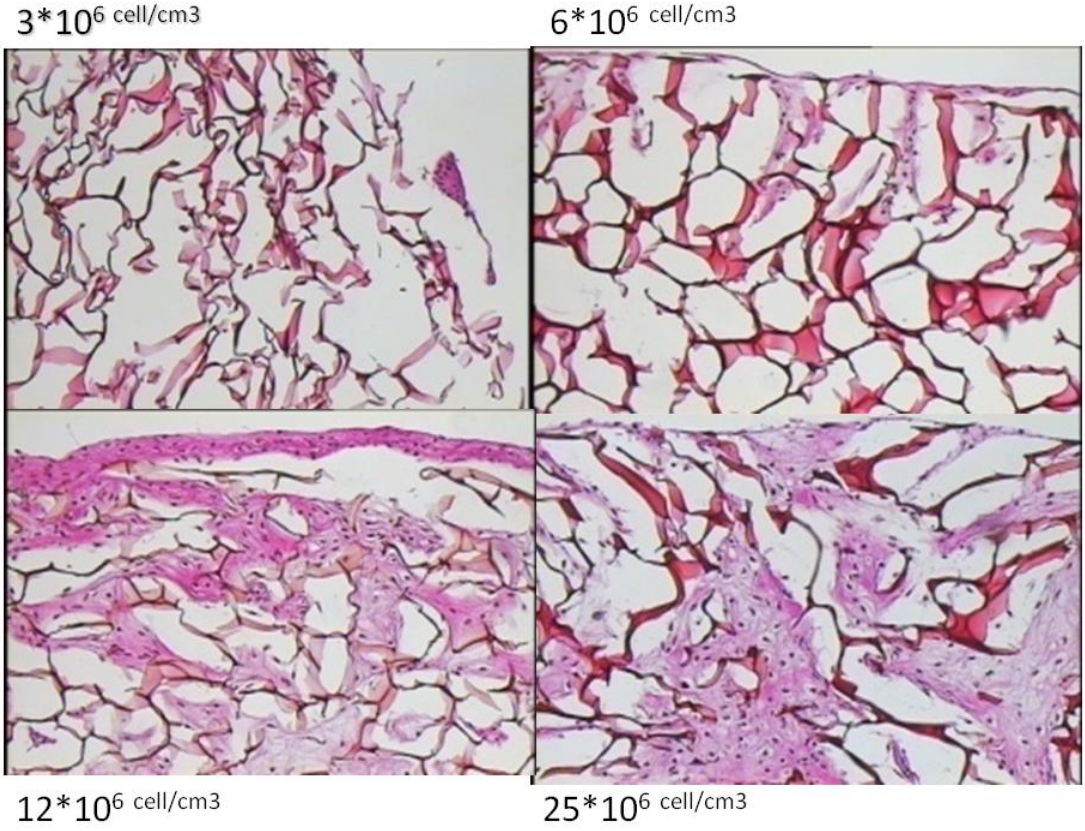


Figure 18
Histology pictures of chitosan scaffolds seeded with different cell densities corresponding to groups A, B, C and D. Note that the cell interaction and matrix production increase at higher seeding densities.

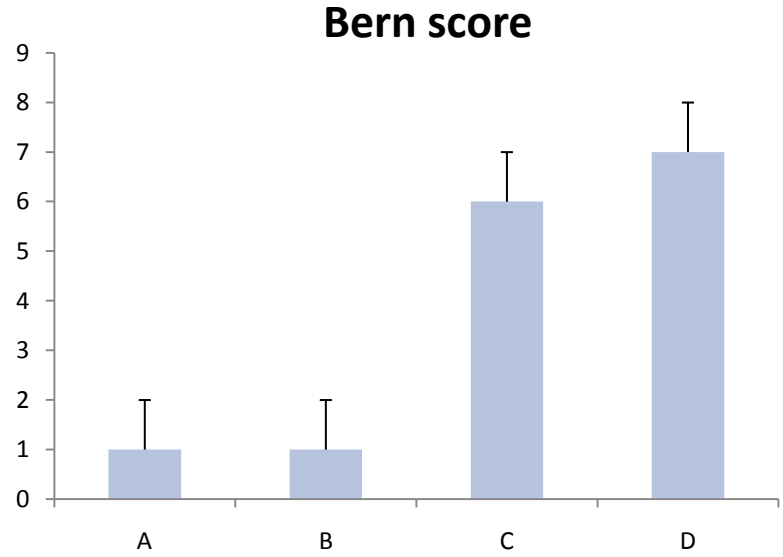


Figure 19
Bern score assessment of chitosan scaffolds seeded with different cell densities corresponding to groups A, B, C and D. The higher seeding concentrations show higher scores.

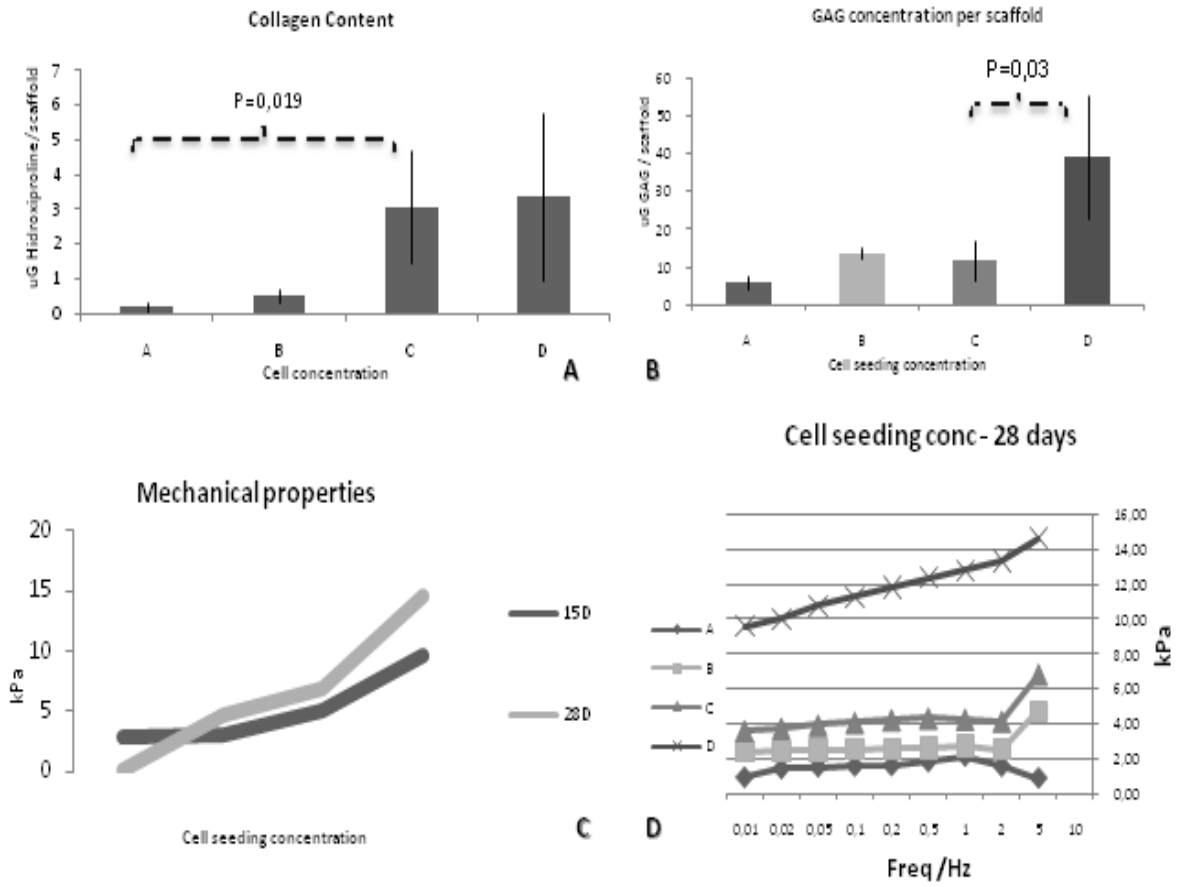


Figure 20

A: Hydroxyproline quantification.
B: Glucosaminoglycan quantification.
C: Mechanical property increments between 14 and 28 days.
D: Shear mechanical properties of constructs seeded with different cell concentrations.

Paper III

Chondrogenic differentiation *in vitro*

Histology and immunohistochemistry of the cell-scaffold construct

The matrix production increased over time as indicated by the increased Alcian blue van Gieson and Safranin-O staining. No matrix production had taken place after 1 day in culture but a nice even cell distribution was observed throughout the cell-scaffold construct. After 7 and 14 days in culture a rather poor matrix production was seen, as indicated by a weak chromatographic staining. The cells in the constructs had a fibrous morphology. Maturation was seen in the constructs after 21 days in culture and a moderate matrix was observed in some areas. Moreover, a mixture of rounded and spindle shaped chondrocytes was seen. The collagen production in the cell-scaffold construct was analyzed using immunohistochemistry. A weak collagen type I expression was detected after 7 days in culture and increased over the culture time. After 21 days in culture, an even collagen type I expression was seen throughout the scaffolds. Hardly any collagen type II expression was detected during the *in vitro* expansion. A week expression could be observed in some areas in the scaffolds cultured for 21 days. The increase in matrix production and maturation of the chondrocytes over time was confirmed by the Bern score. The cell-scaffold constructs cultured for 1, 7, 14 and 21 days had a Bern score of 0.67 ± 0.29, 3 ± 0, 3 ± 0 and 4.3 ± 0.58 respectively. Cell-scaffold construct cultured for one day in chondrogenic media displayed a significantly lower Bern score than constructs cultured for a longer time. A significant difference was also seen between the constructs cultured for 21 days and 7 and 14 days.

Gene expression analysis of the cell-scaffold constructs

The collagen type IIA/collagen type I (CIIA/CI), collagen type IIB/collagen type I (CIIB/CI) and aggrecan/versican (agg/vers) ratios were examined to investigate the grade of re-differentiation of the cell-scaffold constructs. An increase in all three ratios was observed. When all the patients were compared a large standard deviation was observed due to patient variation. However, all the patients had the same maturating trend with increasing CIIA/CI, CIIB/CI and agg/vers ratios although the actual number varied giving the large standard deviation. The COMP and SOX9 expression were lowest after one day in culture and then peaked at 7 days and thereafter decreased and stabilized over the culture time. Collagen typeX (CX) was analyzed to investigate whether the phenotype of the cells was affected by the culture period. An increase in collagen type X was seen during the culture period.

Table III

	CIIA/CI	std	CIIB/CI	std	Agg/Vers	std	SOX9	std	COMP	std	CX	std
Preculture in vitro												
1 day	UD	± -	UD	± -	0,6	± 0,3	4,2	± 3,7	45	± 27	11	± 4
7 days	62	± 32	192	± 79	6,6	± 3,7	8,7	± 4,8	293	± 96	115	± 58
14 days	521	± 446	1569	± 1182	9,0	± 3,2	4,1	± 1,9	212	± 31	210	± 63
21 days	807	± 659	3141	± 2983	13,0	± 7,5	3,6	± 0,1	182	± 55	344	± 497

Additional markers were investigated to further investigate the process of chondrogenesis *in vitro*. The highest expression of these genes was seen in the monolayer (ML) culture, as expected. The HES5 expression dramatically decreased from ML to day 1 in culture and was totally abolished at day 7. GDF5 and Notch1 both decreased dramatically from ML to day 1 and then had a small expression throughout the culture period. The KI67, fibronectin and tenascin C expression peaked at 7 days of culture. A high standard deviation is seen in Tables III and IV that was caused by the patient variation. However, the same trend in gene expression is generally seen in the patients studied.

Table IV

	GDF5	std	KI67	std	HES5	std	Notch 1	std	Fibronectin	std	Tenasin C	std
Preculture in vitro												
ML	1	± 0	1	± 0	1	± 0	1	± 0	1	± 0	1	± 0,00
1 day in scaffold	0,09	± 0,04	0,07	± 0,07	0,59	± 0,78	0,35	± 0,22	2,31	± 1,61	0,95	± 0,67
7 days in scaffold	0,18	± 0,17	0,20	± 0,13	UD	± UD	0,50	± 0,26	4,51	± 2,97	1,56	± 0,87
14 days in scaffold	0,14	± 0,14	0,07	± 0,05	UD	± UD	0,20	± 0,06	2,54	± 1,41	0,91	± 0,60
21 days in scaffold	0,16	± 0,10	0,03	± 0,02	UD	± UD	0,33	± 0,16	1,69	± 0,61	0,81	± 0,37

Chondrogenic differentiation *in vivo*

The pre-cultured cell-scaffold constructs, cultured for 1, 7, 14 and 21 days, were implanted subcutaneously in a mouse model for further maturation in a milieu that provided an adequate supply of nutrients. The neocartilage formation in the mouse model depends on the intrinsic chondrocyte commitment rather than the microenvironmental conditions.[197]

Histology and immunohistochemistry of the cell-scaffold constructs

The cell-scaffold constructs that had been precultured for one day in a chondrogenic inducing media did not show any signs of hyaline-like cartilage matrix production after the implantation time and had a fibrous morphology. A maturation of the constructs was however observed with longer culture time. The

constructs cultured *in vitro* for 7 days showed a mixture of an even and dark chromatic staining with an extensive matrix between the rounded chondrocytes and areas with less staining and a more poorly produced matrix.
Furthermore, there was a mixture of fibrous and more rounded chondrogenic morphology. A further maturation of the *in vitro* cell-scaffold constructs cultured for 14 days was seen, indicated by larger areas in the constructs that had an even and dark chromatic staining with an extensive matrix between cells with a chondrogenic morphology. In the constructs cultured *in vitro* for 21 days, slightly larger areas had a mixture of fibrous and rounded cells than areas where the majority of the cells exhibited rounded chondrogenic morphology compared to the 14-day-old constructs. No mineralization or bone formation was observed in the cell-scaffold constructs as assessed by von Kossa staining.
The collagen production in the constructs was analyzed using immunohistochemistry. A significant difference in collagen type II expression was seen between the constructs pre-cultured for only one day and the constructs pre-cultured for a longer period of time *in vitro*. A collagen type II expression was detected in the cell-scaffold constructs that had been pre-cultured for 7 days. An increase in the collagen type II expression was seen in the constructs cultured for 14 days *in vitro* and thereafter a slight decrease in the expression after 21 days.

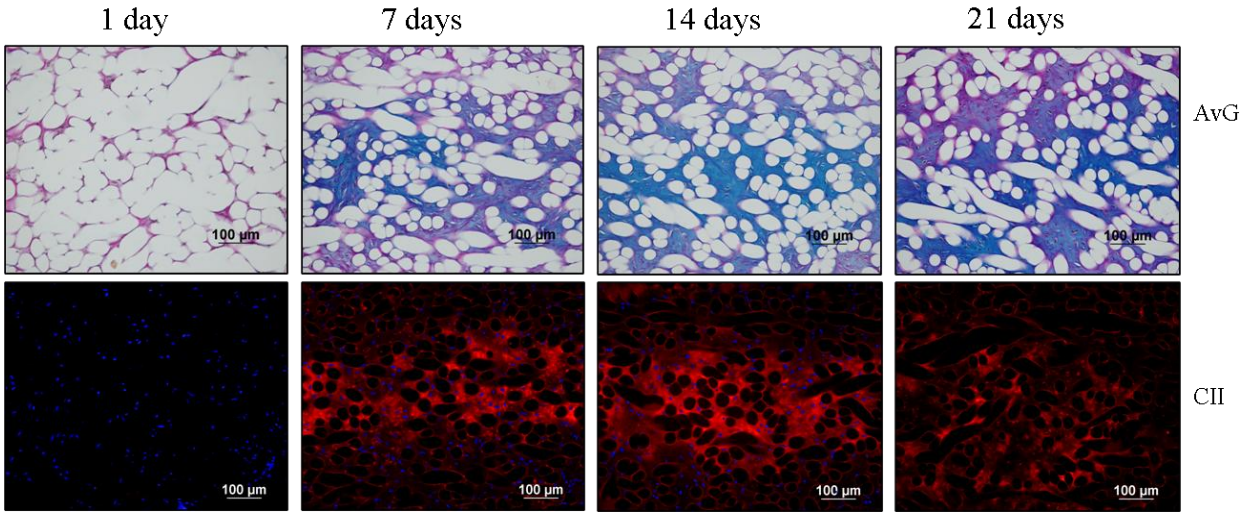


Figure 21
Constructs stained with Alcian Blue and anticollagen type II Immunohistochemistry.

The maturation of the neocartilage in the cell-scaffold constructs was also examined using the Bern score[98]. The constructs cultured for one day *in vitro* had the lowest Bern score and there was a significant difference between day 1 and longer culture periods. A nice neo-cartilage formation was observed in the constructs that had been cultured for 7 days *in vitro* before implantation. The Bern score was highest in the constructs pre-cultured for 14 days whereas a decrease was observed in the constructs pre-cultured for 21 days.

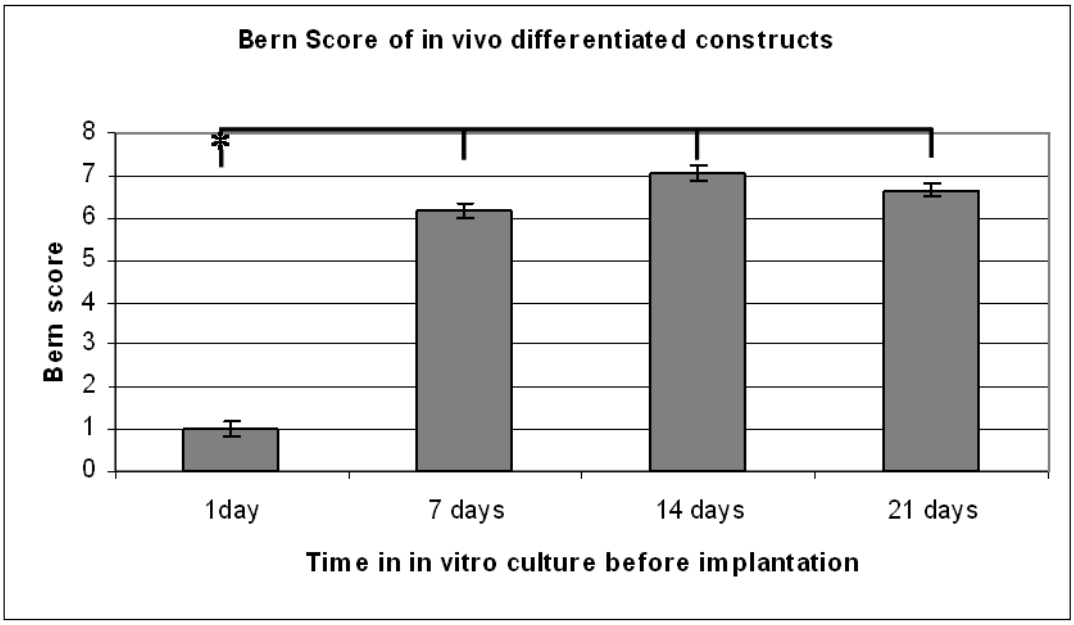


Figure 22
Bern score of *in vivo* differentiated constructs

Gene expression analysis of the cell-scaffold constructs

A minor expression of collagen type I was detected at all time points in all patients, although, the relative gene expression value was in general <1% of the collagen types IIA and IIB expression. Collagen type IIA was detected in samples from day 14 and 21 in culture. Collagen type IIB was expressed from day 7 and then in general increased until day 21 in culture. No expression of collagen type X was observed except for at day 21 in one patient. The relative gene expression of collagen types I, IIA and IIB was 1% of the values obtained in the *in vitro* cell-scaffold constructs. A high expression of the investigated genes was observed on the protein level. The low expression of the genes studied *in vivo* might be due to a negative feedback loop.

Table V

Gene expression of cartilage differentiation markers related to time.

	CIIB/CI	std	CIIA/CI	std	Agg/Vers	std	CX	std
Days in cultre								
1	0	± 0	0	± 0	0,00	± 0,00	0,00	± 0,00
7	216	± 298	0	± 0	0,14	± 0,12	0,00	± 0,00
14	847	± 1109	18	± 16	0,10	± 0,17	0,00	± 0,00
21	1555	± 2410	33	± 36	0,49	± 0,23	3,82	± 6,61

Paper IV

Effect of pre-culture on *in vivo* differentiation

The effect of pre-culture was evaluated by implanting constructs subcutaneously in a Balb C mouse without pre-culturing or after allowing the cells to re-differentiate *in vitro* for 4 weeks in media. The histology of these constructs gave no evidence of sulphated GAGs in the ECM in the group without pre-culture. In this group the chondrocytes had a fibroblastic shape. On the other hand, a significantly higher amount of sulphated GAGs was found in the explants that were pre-cultured for 28 days *in vitro* prior to *in vivo* implantation. The chondrocyte aspect in this group had a round shape as seen in adult chondrocyte differentiated phenotype. (Figure 23B-C). The Bern score was used to semi-quantify the histological aspect of the constructs. The group that was pre-cultured for 28 days had a significantly higher score than the group that was cultured for one day (Bern Score mean values: 1 day : 2.66 ± 0.57 and 28 days: 8.66 ± 0.57) (Figure 23A).

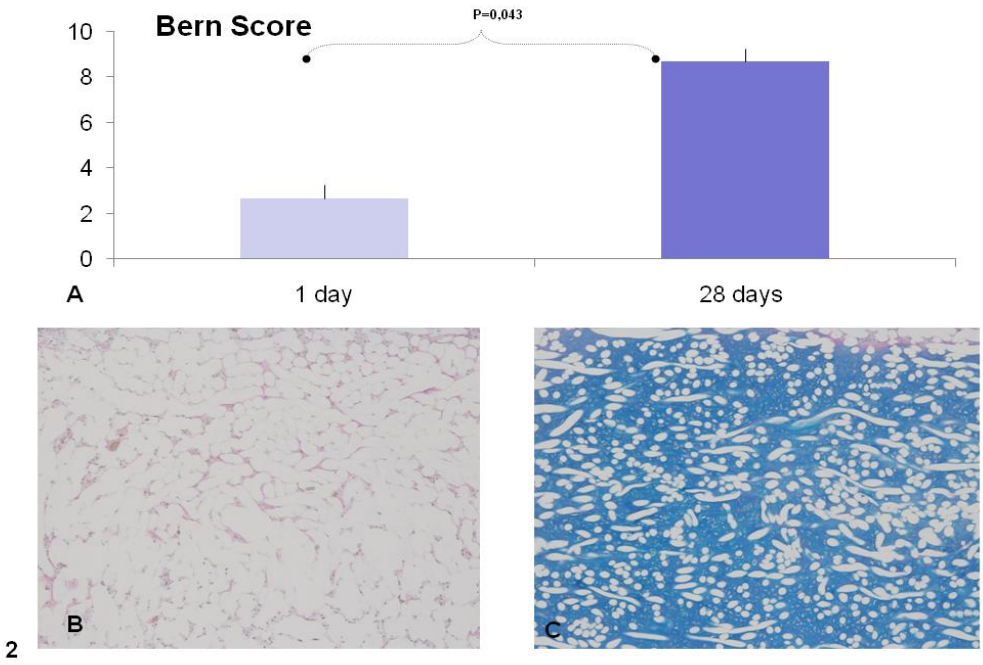


Figure 23

A: Histogram showing Bern score mean values and standard deviations corresponding to constructs implanted subcutaneously in Balb C mice after 1 or 28 days of *in vitro* culture in media C.

B: Picture showing the histological aspect of a representative construct cultured for 1 day prior to 8 weeks of subcutaneous implantation in a Balb C mouse. Note the absence of ECM and GAGs**.**

C: Picture showing the histological aspect of a representative construct cultured for 28 day prior to 8 weeks of subcutaneous implantation in a Balb C mouse. Note the extensive production of ECM and strong Alcian Blue van Giesson staining representing the presence of sulphated GAGs.

Variation in *in vitro* rediffentiation influenced by the media composition

The composition of the media affected the re-differentiation of the constructs. Histological analysis of the cell-seeded scaffolds after the *in vitro* culture revealed that all media tested had a similar cell distribution pattern with adequate cell binding and matrix production. A slightly higher degree of Alcian blue-Van Gieson positive areas was seen in group C.

The analysis of gene expression demonstrated that the constructs in group A were more undifferentiated as measured by the relative gene expression of collagen type I (Group A: 21.33 ± 22.26, group B: 8.84 ± 10.3 and group C: 7.61 ± 2.94).

Group C showed the highest values of Collagen type IIB (Group A: ud, group B: 28.46 ± 38.69 and group C: 113.83 ± 81.94) **(**Figure 24B).

Group B showed intermediate amounts of GAGs and collagen IB. (Figures 24 A-B)

The biochemical quantification showed that the constructs in Group C contained a significant higher GAG/DNA ratio (Group A: 0.09 ± 0.023, group B: 0.13 ± 0.03 and group C: 0.75 ± 0.112) (Figure 24A).

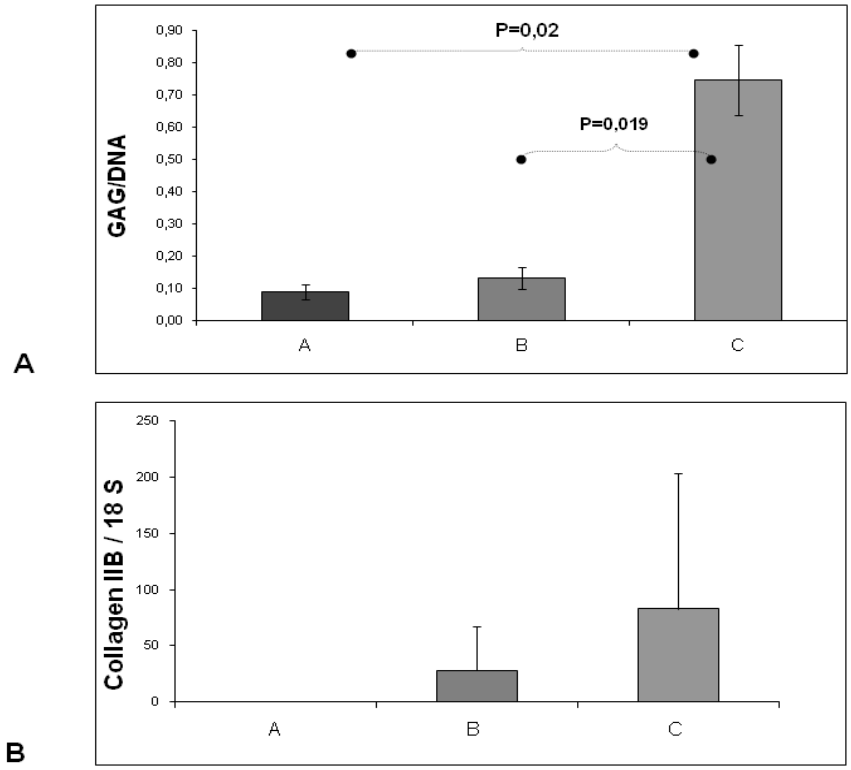


Figure 24

A: GAG per DNA ratio of constructs in groups A, B and C. Note the significant increase of GAG per DNA ratio in group C. Significant p values.

B: Relative gene expression of collagen type IIB. Note the increase in the expression of collagen type IIB in the presence of differentiation factors (Groups B and C).

Variation in *in vivo* re-diffentiation influenced by the media composition

After 8 weeks all constructs implanted in Balb C mice were found at their initial implant location with a distance of approximately 1cm between the different constructs. The histology sections from the constructs implanted in the Balb C mice resulted in positive histological staining with Saffranin O and Alcian blue van Gieson in all samples corresponding to media A, media B and media C; this was however negative in group D (empty scaffold). Pre-culture of constructs in media B and C generated an increased extra cellularmatrix production compared to the constructs pre-cultured in media A. Degrading Hyalograft C fibers (seen as white holes) were replaced by cells and matrix (Figure 25B).

The quality of the cartilaginous matrix formed was analyzed using the Bern score.[99] There were an inhomogenious cell morphology and staining intensity in some of the sections. To overcome this, the sections were divided in up to three different regions that were scored separately. The individual score was adjusted in relation to the percentage of the whole area and was then added together to get an overall single score. Statistically significant higher scores were found in media groups B and C as compared to groups A and D. The score for group A was 2.27 ± 1.72, group B: 4.68 ± 0.39, group C: 4.57 ± 1.19 and group D: 1.33 ± 0.58 (Figure 25A).

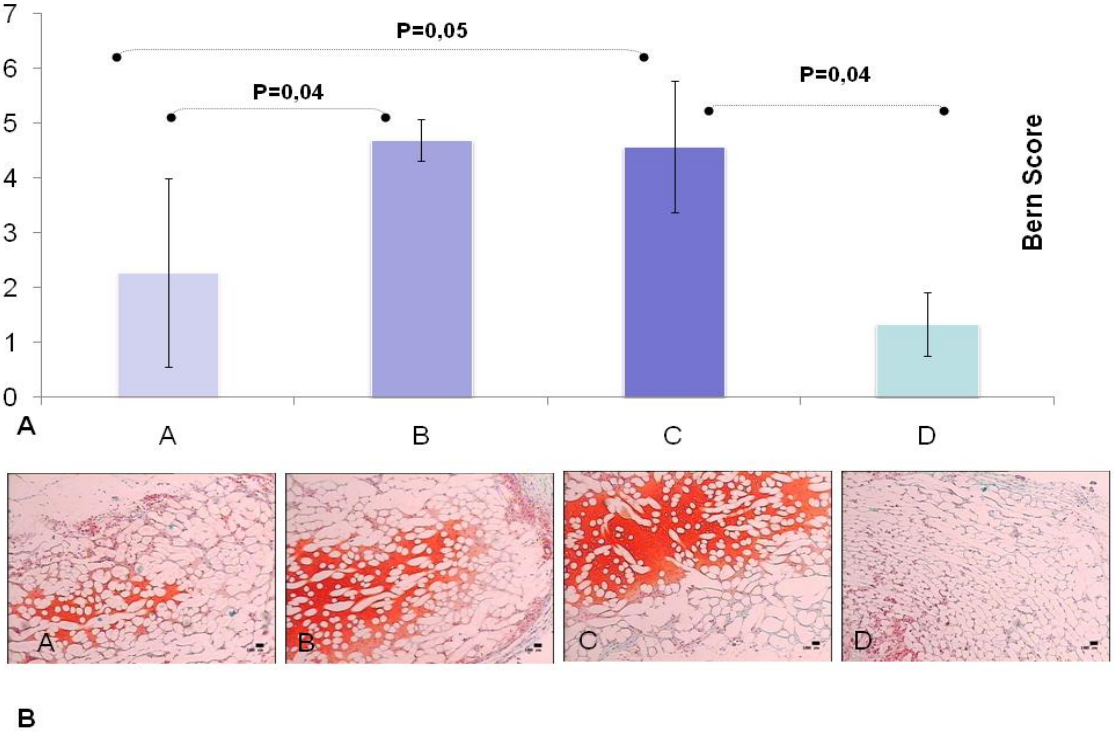


Figure 25

A: Bern score values corresponding to constructs after 8 weeks *in vivo*. Note the significant difference of values when the chondrocytes were cultured with media B and C.

B: The histological aspect of constructs corresponding to groups A, B, C and D. Note the increase in Safranin O staining area and intensity in groups B and C.

Variation in the *in vivo* integration influenced by the media composition

After 8 weeks all the osteochondral plugs were found at their initial implant location. The histology sections from the transplanted ostechondral plugs containing the tissue engineered constructs implanted in the Balb C mice resulted in positive histological staining with Saffranin O and Alcian blue van Gieson in the groups cultured with media B and media C while this was negative for the groups cultured in media A and in the empty scaffold (Figure 26B).

The integration of the tissue engineered constructs and human cartilage was evaluated using the integration subdivision of the O´Driscoll score. We were able to determine that the bonding of the graft to the adjacent native cartilage was higher in groups B and C compared to group A. The mean values of the O´Driscoll score were: group A: 0.33 ± 0.5, group B: 1.66 ± 0.5, group C: 1.66 ± 0.51 and group D showed no integration. Significant differences were found in comparisons of groups A and D to B and D (Figure 26A).

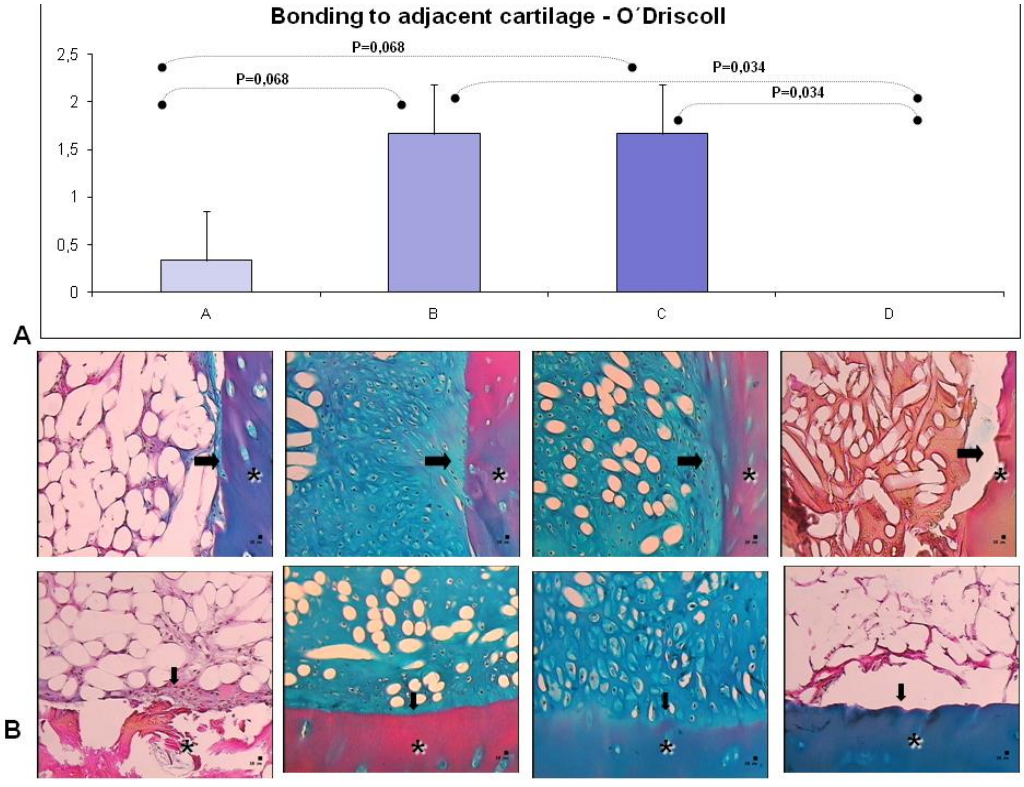


Figure 26

A: Figure showing the evaluation of integration of constructs to adjacent cartilage. O´Driscoll subdivision score values corresponding to constructs after 8 weeks *in vivo*. Note the significant increase in the integration values when the chondrocytes were cultured with media B and C. P values are shown when significant.

B: Figure showing the histological aspect of constructs corresponding to groups A, B, C and D. Pictures show the lateral and deep interfaces between constructs and native human cartilage. Note the increase in ECM and interface tissue bonding in groups B and C.

Surgeon evaluation

The surgeon evaluation showed that implants in group B presented the preferred implantation properties. All surgeons preferably chose B, scored as 1. These constructs showed good handling properties and could be sutured. They were also self-adherent to some extent and could be molded into the defect. The constructs cultured in media A had intermediate handling properties but were less stable than constructs cultured in media B or C. The empty scaffold was not preferred by any surgeon since it was too soft, lacked good handling properties and could not be sutured.

Paper V

Migration of chondrocytes towards different biomaterials

Histologically the groups that were coated with gels showed a greater number of cells that were distributed along the coated fibers. After 19 days there was a difference in the number of chondrocytes that migrated towards the different materials evaluated. After 33 and 42 days **group B** showed a significantly higher number of cells per scaffold than **groups A and C** (33 days: PuraMatrix® (RADA 16) > Cartipatch® p=0.0143; PuraMatrix (RADA 16)® > Hyaff 11® p=0.0143; 42 days: PuraMatrix® (RADA 16) > Cartipatch® p=0.0143; PuraMarix® (RADA16) > Hyaff 11® p=0.0286) (Figure 27).

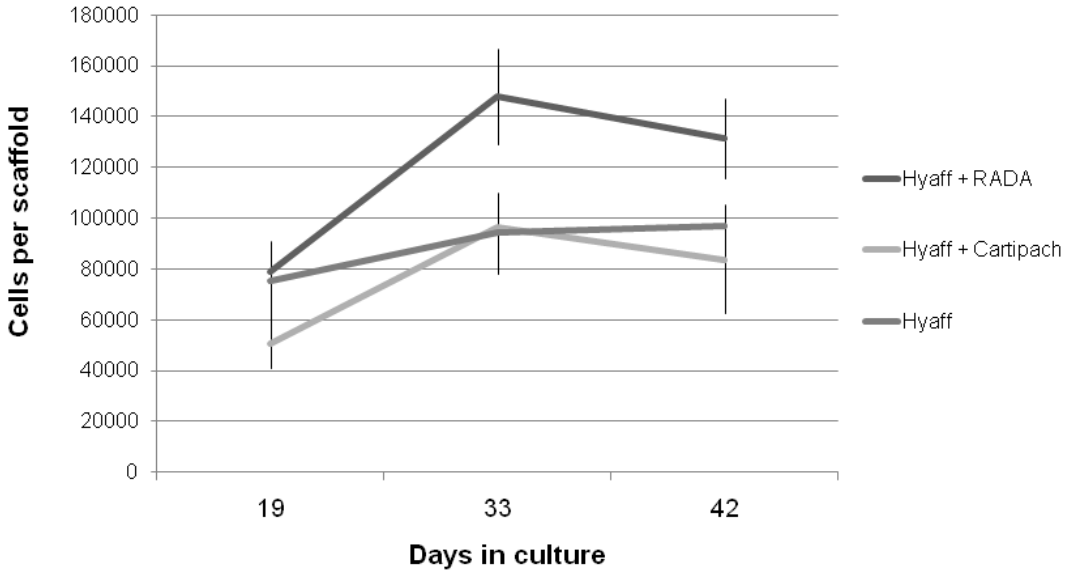


Figure 27

Figure showing the amount of migrating cells per scaffold in the different groups and within time.

Chondrocyte differentiation after culture in different materials

After 4 weeks of culture with chondrogenic media the degree of differentiation was evaluated by measuring the amount of GAGs and detecting the presence of collagen type II. In **Group B** the chondrocytes produced the highest amount of GAG and expressed collagen type II (Figure 28-C).

Chondrocytes in **Group C** had the highest amount of total collagen but did not express collagen type II (Figure 28-B).

Chondrocytes in **Group A** produced the lowest amount of collagen and GAGs and did not express collagen type II.

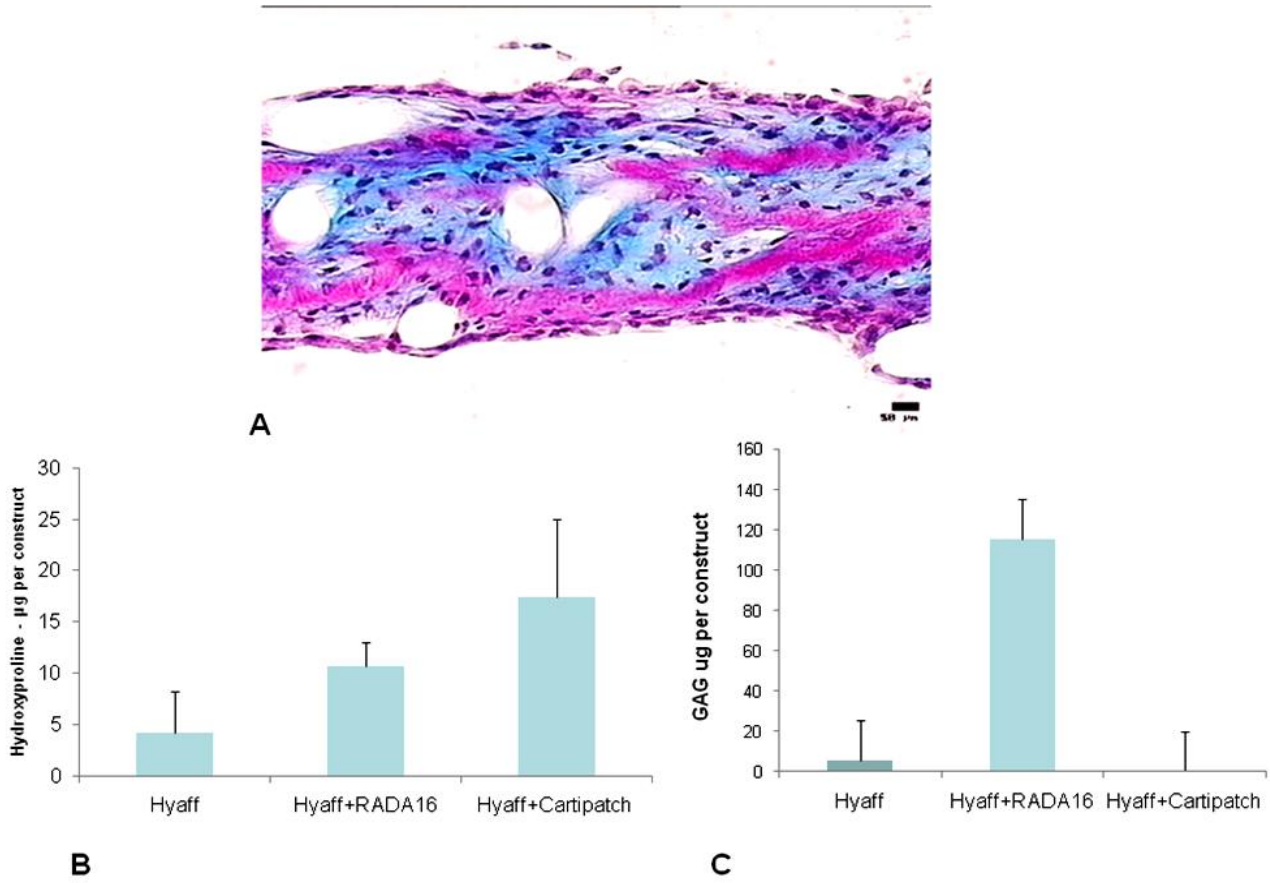


Figure 28

A: Picture showing a representative area of positive Alcian blue staining corresponding to group B (Hyaff coated with PuraMatrix® (RADA 16).

B: The total amount of hydroxyproline per scaffold after the differentiation phase. Note that group C showed the highest amount of total collagen.

C: Total amount of GAGs per implant. Note that group B showed the highest amount of GAG per implant.

Evaluation of migration in relation to the origin of the cartilage explants

The migration of chondrocytes from explants corresponding to the superficial and deep zones of the articular cartilage was evaluated. The number of cells that migrated towards the PuraMatrix® (RADA 16) coated Hyaff 11® scaffold was higher in the group corresponding to the superficial explants than in the group that contained the deep layer explants. Notch 1 positive cells were found only in the biopsies and migrating cells corresponding to the superficial layers (Figure 29).

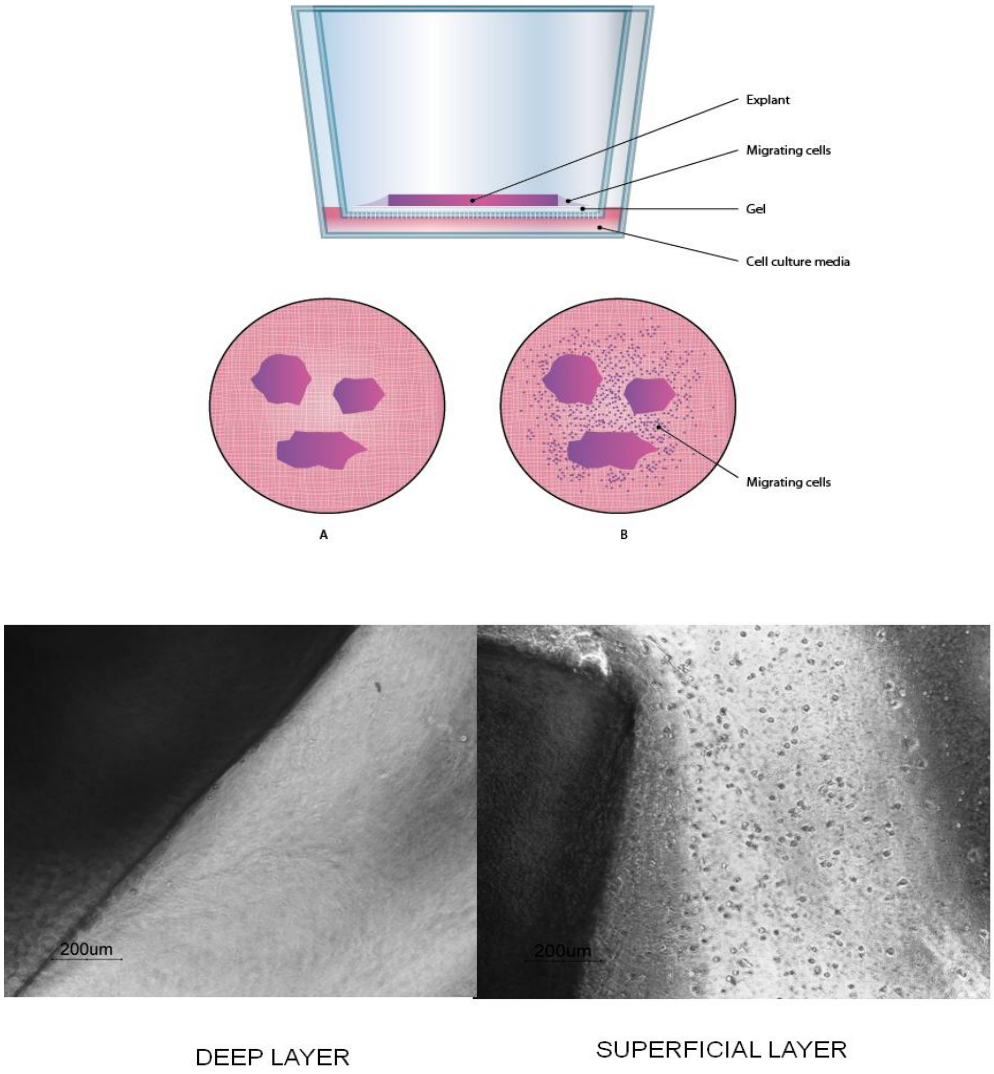


Figure 29
Explants from the cartilage deep (A) and superficial (B) layers were sectioned and placed in six well plates with culture inserts to determine the location of the cells that were showed the best migration properties. The lower part of this figure shows inverted microscopic pictures of the migrating cells corresponding to superficial layers. Note that there is no migration from the explants corresponding to the deep layer. Art by Pontus Andersson.

DISCUSSION

Cell source

It is known from the scientific literature that cartilage is derived from two different ontogenetic sources: the mesoderm and the neural crest[185] and due to this bilateral origin, it is possible that many cell types could become chondrocytes. Cells from either sources initiate skeletogenesis by the expression of SOX 9.[28,92,144,244]

The optimal cell for cartilage regeneration should be highly proliferative, easy available, and have multipotency capacity. In addition to chondrocytes, adult and embryonic mesenchymal stem cells have been postulated as potential cell sources.[29,39,48,51,87] Other sources with potential chondrogenic capacity that have been suggested are the bone marrow,[51,86,217,223,242] muscle,[251] synovium,[6,31,65,251] and adipose tissue.[251,255]

In this study we demonstrated that it is possible to partially exchange chondrocytes with adipose-derived stem cells to reduce the number of chondrocytes needed for implantation in cartilage repair.

Articular chondrocytes have a high chondrogenic capacity and it has been shown that they are capable to differentiate towards different lineages within the mesoderm sphere.[223] However, biopsies for ACI are harvested from healthy cartilage and their availability is limited. Furthermore chondrocytes dedifferentiate when they are expanded in monolayer and decrease their re-differentiation capacity with expansion.[71,72]

If it was possible to use a reduced initial amount of chondrocytes for cartilage regeneration, a direct isolation of chondrocytes as a one stage procedure would be interesting and would eliminate the cell expansion phase as well as the time, cost and risk associated with expansion. However, the reports about the chondrogenic capacity of directly isolated chondrocytes are controversial and articular cartilage biopsies provide low amount of cells.[85,121,156]

We evaluated in paper one the possibility of partly substituting chondrocytes with an easily available, alternative cell type with chondrogenic potential such as ASC. It has been reported that ASC posses a poorer chondrogenic capacity compared to MSC obtained from the bone marrow.[107]

However, in cocultures of ASC and chondrocytes a potent Alcian blue staining in the 5% group was found when cultured in Tisseel® (Figure 18).

Although gene expression of aggrecan was lower in cocultures compared to what was found in purely cultured chondrocytes, evaluation per initial percent of chondrocytes indicates that ASC contributed to aggrecan expression (Figure 17).

Cocultured ASC and chondrocytes clearly differ in their chondrogenic potential compared to purely cultured chondrocytes. ASC showed some contributory effect when our data was analyzed per initial percent of chondrocytes. This indicates that the system has a potential to enhance cartilage differentiation. Application of

directly isolated or differentiated chondrocytes and/or additions of growth factors could further improve this system.[107]

Chondrocytes have a differentiation capacity *in vitro* superior to that of ASC

In paper I human expanded chondrocytes showed a superior neocartilage formation in three dimensional cultures. The gene expression of chondrogenic markers was significantly superior in two different redifferentiation systems. Histological analysis of micromass cultures confirmed this data.

We found that collagen type IX was higher in ASC than in the pure chondrocyte group. Collagen type IX is present in late stages of degeneration and in high weight bearing areas. The chondrocytes from our study were obtained from low weight bearing areas which might explain the difference in results between the different cell types.

High initial cell seeding number affect the in vitro redifferentiation capacity of human adult chondrocytes

The redifferentiation capacity is related to different factors such as the age of the donor, the number of cells, cell doublings, the type of growth factors used during the expansion phase and the culture environment.[19,33,222,227] Cell to cell contact has a special significance during the condensation phase in *in vivo* joint development. Activation of N-cadherin occurs during condensation of mesenchymal cells *in vivo*.[69,70] N-cadherin is needed for the expression of Sox 9 and collagen type II.[243] It is established that the increase in cell to cell contact by aggregation induces chondrogenesis.[52,245] This process is mediated by a number of factors, among them cell adhesion molecules and matrix receptors such as CD44.[169] High seeding densities may facilitate cell interaction and promote chondrogenesis in the transplanted cells. It is then of great value to determine the number of cells needed to start chondrogenesis *in vitro*, especially when tissue engineered constructs are applied using adult human chondrocytes with less than two passages.

The results in **paper II** support the theory that chondrogenic differentiation starts in areas of cell condensation.

The cell seeding concentration where the cell-scaffold constructs showed a homogeneous cell distribution and chondrogenic differentiation was found to be between 12 and 25 x 10^6 cells per cm^3 (equal to 2 to 4 x 10^6 per cm^2).

The Bern score described by Grogan et al.[76] was a very useful tool for a rapid evaluation and quantification of the quality of the constructs.[76]

The collagen and GAG concentrations increased when the cell seeding concentration was varied. There was a significant increase in proteoglycan and hydroxyproline production when the constructs were seeded at high densities (12-25 x 10^6 cells/cm^3).

The group mechanically loaded with 40 x 10^6 cells per cm^3 showed a higher storage modulus after 15 and 28 days. The storage modulus gives an indication of

the stiffness of the sample. The collagen network primarily contributes to the mechanical properties of the ECM in shear; however, the interaction with the glycosaminoglycans may contribute to the storage modulus.[218]
Other studies showed that seeding densities of less than 10 million cells per ml, resulted in very little cartilaginous material, while seeding scaffolds at a density ranging from 20 to 100 million cells per ml resulted in the formation of clinically appropriate cartilage when subcutaneously implanted into nude mice.[196]
Mahmoudifar and Doran[119] recently found a relation between cell seeding density and matrix production using fetal human epiphyseal tissue and PGA scaffolds.[151]
In another study, chondrocytes seeded at high densities (10^7cells/ml) in Atelocollagen gels generated a cartilage-like tissue.[119]
Mauck et al.[124] showed that increasing cell seeding densities from 10 million cells/ml to 60 million cells/ml increased the mechanical properties of young bovine chondrocytes - agarose constructs only when they were cultured under dynamic conditions and supplemented with 20% FCS.[157]
All the publications that explore the appropriate cell seeding density have been done using different cell sources, scaffolds, cell seeding methods and growth media.
In the work reported in **paper II** we determined the importance of the initial cell seeding density. No studies have been published that evaluate the influence of the cell seeding concentration using human articular chondrocytes and clinically validated methods of culture in 3D.
The minimum effective cell seeding density may fluctuate as concerns, the porosity, effective volume and geometry of the biomaterial selected.
The ideal number of cells needed to start and retain a secure chondrogenesis is still not known, although many companies have as their goal to deliver 1 x 10^6 cells/ cm^2 on the commercial market.
Our study showed that concentrations between 12 and 25 million cells/cm^3 (2 to 4 x 10^6 per cm^2) are needed to increase the matrix production and mechanical properties of human adult chondrocytes under static conditions using chitosan scaffolds as the biomaterial. A seeding concentration of 25 million cells/cm^3 (4 x 10^6 per cm^2) showed an increase in the viscoelastic properties of the tissue engineered tissues. This seeding concentration may thus be desirable for human tissue engineering applications.

In vitro pre-differentiation affects the *in vivo* chondrogenic capacity

In **paper III** we were able to demonstrate that there was a biological relevance of *in vitro* precultivation of cell-scaffold constructs from expanded chondrocytes before implantation and neocartilage formation *in vivo* compared with freshly seeded scaffolds.
The results further revealed that a more differentiated cell-scaffold construct does not definitely provide a significantly enhanced cartilage formation *in vivo*.

It has been discussed wether the degree of maturation affects the integration of the cell-scaffold construct with the surrounding host tissue after implantation. In **paper IV** we found that the expression of collagen type II *in vitro* was related to higher histological bonding to the adjacent cartilage. Furthermore significantly higher scores were found using the O'Driscoll score.

We found that a more hypertrophic phenotype is developed during the culture period. This was recognized as collagen type X, a of marker for hypertrophic chondrocytes, increased during the culture expansion.[67,256]

The expression of collagen type X may occur owing to the use of the chondrogenic TGF-β_1 in the culture media.[198,207,225] We found that the increase in collagen type X was related to the culture time. The scaffolds cultured longer time *in vitro* were exposed for a longer period of time; thus an increase of the expression is seen in the current study. This could potentially be inhibited by the addition of factors such as parathyroid hormone related protein.[126] In addition, the results in the present study reveal that there was generally no expression of collagen type X and that no mineralization was present in the *in vivo* constructs.

The media composition used *in vitro* affects the re-differentiation capacity and the integration into the host tissue *in vivo*

Paper IV reported that the media composition used during the *in vitro* redifferentiation phase had an effect on *in vivo* cartilage formation. The histological semiquantitative analysis of the different groups showed that the groups re-differentiated with chondrogenic media (B and C) demonstrated a significant increase in the *in vivo* re-differentiation capacity.

These differences could be a result of the presence of chondrogenic factors in the media formulation of these groups such as TGF β_1, insulin, ascorbic acid and dexamethasone.

It has been shown that TGF-ß$_1$ treatment initiates and maintains chondrogenesis of mesenchymal progenitor cells.[122,232]

TGF-ß$_1$ plays an important role in inducing N cadherin which is necessary for cell condensation at the intial phases of *in vivo* differentiation[232] and in cartilage formation during embryonic stages and in both proliferation and matrix-forming ability of articular chondrocytes *in vitro*.[142,226,233]

Dexamethasone addition to the media increases cartilage such as ECM deposition.[30 122]

Ascorbic acid supplementation enhances collagen production and chondrogenesis at different levels.[57,117]

Human serum is often used as a culture media supplement during the *in vitro* expansion in ACI to support attachment and induce proliferation of the chondrocytes.[35,222] Human serum contains essential growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-ß$_1$), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF).[226]

Basic FGF and EGF stimulate the proliferation capacity but may have a negative effect on differentiation and chondrogenesis.[19,56,250]

In the work described in **paper IV**, the presence of human pooled serum did not disrupt the capacity of cartilage redifferentiation.
In contrast, Bilgen et al.[22,23] showed that the presence of 10% fetal bovine serum in synoviocyte cultures inhibited proteoglycan and collagen type II production even in the presence of TGF-ß$_{1.}$[22,31]
Differences found in the other studies could be related to the study design, cell type and the fact that bovine serum has a different growth factor composition than the human serum used in this study.
Little is known about how tissue engineered implants behave after implantation. A scaffold in which the cells have been programmed *in vitro* to differentiate towards the hyaline phenotype is desirable.
Chondrocytes seem to have a poor capacity to integrate or infiltrate the adjacent cartilage tissue. This integration deficit has been attributed to an insufficient degree of matrix secretion by the implanted cells and to soluble factors secreted by the host tissue.[111]
The degree of maturation affects the integration of the cell-scaffold construct with the surrounding host tissue after implantation. Furthermore, Obradovic et al.[138] demonstrated that the integration is dependent on the differentiation state of the chondrocytes, the less differentiated cell-scaffold constructs giving a better integration.[178]
In contrast Tognana et al.[180] showed that there was a correlation between GAG content and adhesive strength. They also demonstrated *in vivo* the fact that the time in culture increased the adhesive strength of the tissue engineered cartilage to the adjacent cartilage. They were able to determine that tissue engineered constructs integrate better to bone than to cartilage surfaces.[180]
Hunter et al.[88] showed that the native cartilage slows down the maturation of the engineered tissues. The authors suggest that the most successful tissue engineered cartilage repairs would rely on relatively mature tissues for implantation rather than maturation *in vivo*. Furthermore, the authors did not find any correlation between gross biochemical content and the interfacial strength.[111]
In **paper IV** we showed that a higher degree of integration was present in groups B and C. This confirms that the degree of differentiation affects the *in vivo* integration. Mechanical studies were not carried out in the present studies, but several authors postulate the difficulty of measuring adhesive strength and its importance in the clinical context.[111,179,230]
Understanding and predicting the behavior of engineered cartilages during and after implantation will greatly improve the potential for long-term functional restoration of the joint surface.

Biomaterials affect differently chondrocyte differentiation *in vitro*

Paper I and in **paper V** evaluated the effect of different materials on the *in vitro* re-differentiation. Materials can affect the behaviour of the cells cultured inside them. Factors such as the architecture of the scaffold and the pore size can affect the re-differentiation potential.[248] The material of the scaffold has been a matter of debate. Many materials have been used in cartilage tissue engineering.[93,94,96,143,147,181]

In **paper I** we compared the redifferentiation capacity of adipose stem cells and human chondrocytes in fibrin sealant (Tisseel® Baxter) and in a collagen sponge (Tissue Fleece® Baxter). We chose Tisseel® and Tissue Fleece® as representative samples for two classes of biomaterials: hydrogel and mesh-like material. We also processed histological preparations of micromass pellet cultured as a scaffold-free reference.

One of the advantages of Tisseel® is that cells can be easily be mixed in one of the liquid components before polymerization, which allows a homogenous distribution in the material. A complete surrounding of cells by the biomaterial promotes a spherical phenotype, which is characteristic for chondrocytes in the middle and deep zones of articular cartilage. Hence, two dimensional spreading of cells, which more easily occurs in large compartments of mesh-like scaffolds, might be avoided. A mesh-like scaffold on the other hand was assumed to provide better conditions for cell–cell interactions. Interestingly, qRT-PCR results do not indicate a clear difference between Tisseel® and Tissue Fleece®. Nevertheless, the histological results allow us to assume that Tisseel® provides better conditions for co-cultured ASC and HAC than Tissue Fleece®.

In **paper V** we compared the re-differentiation capacity of cells that migrate out from cartilage explants. Uncoated Hyaff-11® and Hyaff-11® coated with Cartipatch® or PuraMatrix® (RADA 16) were evaluated as materials. A significantly higher amount of GAGs was seen in the group that was coated with PuraMatrix® (RADA 16). These differences in rediffentiation capacity could be due to the chemistry or the architecture of the materials. However, it is also possible that the higher amount of cells in the PuraMatrix® (RADA 16) group is responsible for these differences.

Biomaterials affect chondrocyte migration

Cell adhesion to the extracellular matrix proteins is vital for development, organogenesis, and wound healing and tissue homeostasis.[199,201-202] The process of cell adhesion provides support for cell anchoring and cell migration.[61,141] Adhesion activates pathways that regulate survival proliferation and differentiation in many cell types.[102,103,137,200,202]

Fibronectin is a protein of the extracellular matrix that contributes to cell attachment. It has three globular domains where the arginine-glycine-aspartic acid recognition sequence is the major integrin binding site. This site mediates the adhesion of most cell types including chondrocytes.[102,108,110] Cell adhesion to the

extracellular matrix through integrin receptors plays an important function in many physiological and pathological processes.[20,53,75,80,108]
Cell migration is a critical cell process during wound healing and tissue development and different factors affect the migration activity of mammalian cells, e.g. the three dimensional environment and proteins. These factors can be added to the scaffolds to mimic the structure of the extracellular matrix and provide the proper environment to allow proliferation and differentiation.[53,91]
In **paper V** we found that after 33 and 42 days group B (Hyaff 11®-PuraMatrix®) showed a significantly higher number of cells per scaffold compared to groups A and C.
This might be due to the different three dimensional areas where the cells can attach and migrate. The chemistry and/or the 3D environment of the different materials had an impact on the migration efficiency.
Alginate and agarose have been extensively used to encapsulate chondrocytes and to study differentiation and clone forming ability. They have been applied clinically to deliver chondrocytes to patients with localized cartilage defects.[34,147] Coating Hyaff 11® with these materials did not show an increase in the migration of pig chondrocytes
PuraMatrix® (RADA 16) is an oligopeptide that has a structure similar to the native extracellular matrix (ECM) and it mimics the RGD sequence of fibronectin.[133]
In our study we were able to determine that the cells from the superficial layer had an increase in migration towards PuraMatrix® (RADA 16) and that these cells were Notch 1 positive. This might be due to the fact that the superficial layer of the articular cartilage contains a cell population that has a high affinity for fibronectin and shows characteristics that correspond to progenitor cells.[76] This cell layer is Notch 1 positive according to different authors. However, there is no agreement as to wether these cells represent a progenitor like population.[128,129] Selection of these cells might increase the differentiation potential *in vivo.*

In vitro chondrogenesis follows the same pattern as cartilage formation during development

The methods used to enhance *in vitro* chondrogenesis seek to reproduce chondrogenesis during the initial phases of joint formation. Articular chondrocytes expanded in monolayer *in vitro* display a phenotype characteristic for pre-chondrogenic mesenchymal stem cells at the early stage of limb development and, as such cells have a high proliferate phenotype, express collagen type I and the proteoglycan versican. The chondrocytes expanded *in vitro* have no or a low collagen types IIA, IIB and the proteoglycan aggrecan, as reported in **paper III**.

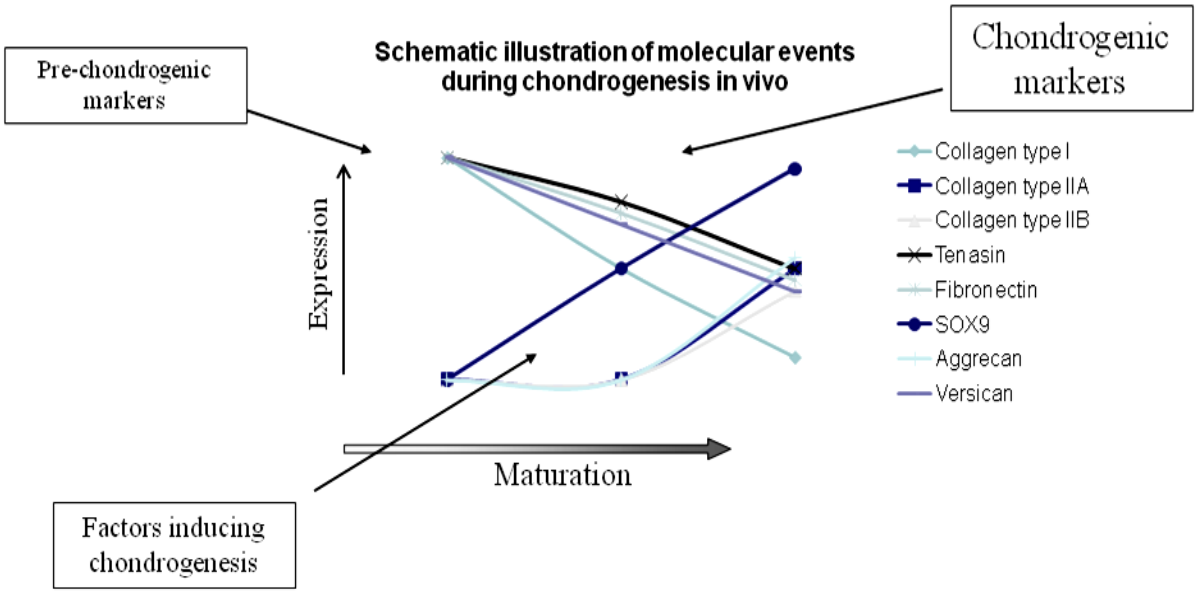
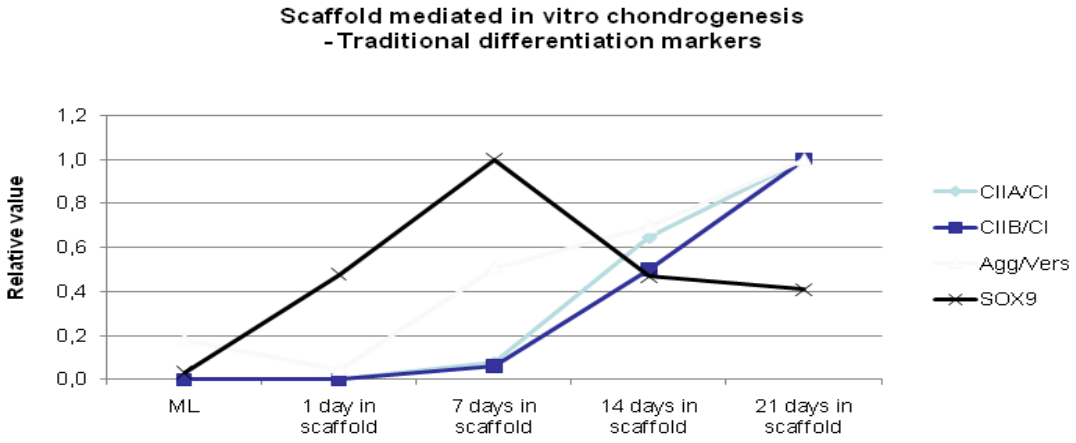


Figure 30
Graphic showing the most important molecular events during chondrogenesis.



CIIA/CI: collagen type IIA/ collagen type I

CIIB/CI: collagen type IIB/ collagen type I

Agg/vers. Aggrecan / versican

Sox 9: SRY-box containing gene 9

ML: Monolayer culture

Figure 31
Graphic showing the evolution of traditional chondrogenic markers in paper III.

The expression of HES5 and Notch1 in the monolayer culture in the current study also shows that the chondrocytes possess a dedifferentiated primitive phenotype at this stage. Chondrogenesis is a complex process involving several signalling

pathways and key factors, such as Notch 1 and HES5. It has been suggested that HES genes, which are controlled by the Notch receptor family, are important for cartilage differentiation.[127] During cartilage differentiation in humans, HES5 is suggested to function as a negative regulator and take part in the regulation of early phases of chondrogenesis.[127] In our study the expression of HES5 was abolished at day 7 in culture indicating that the early phase of chondrogenesis took place before 7 days of culture in a 3D environment. This is further confirmed by the reduction in proliferation of the chondrocytes and the collagen production.

The condensation phase is characterized by the switch from collagen type I to collagen types IIA and IIB as seen at day 7 in our study.[186] The additional expression of fibronectin and tenascin C at this time point further implies that *in vitro* condensation is taking place. During joint development *in vivo,* fibronectin and tenascin C are expressed at low concentrations pre-condensation and are up-regulated at condensation and play a role in increasing cell to cell contact.

The fibronectin expression then declined as differentiation proceeds, as seen during the culture in **paper III**.[68,91] SOX9 and COMP expression peak at 7 days of culture. SOX9 has been shown to play an important role in cartilage formation by influencing the MSC to commit to the chondrogenic lineage by for example ensuring the expression of collagen type II[5]. COMP has been demonstrated to play a pivotal roll in the activation of intracellular signal pathways that initiate the transition from chondroprogenitor cells to fully committed chondrocytes[91]. Notch signaling is involved in a wide array of developmental processes. It has been suggested that Notch signals are involved in cartilage formation.[60] The expression of the cell fate regulator Notch1 throughout the culture suggests that Notch is needed in the condensation phase *in vitro* and that redifferentiation of the chondrocytes is an ongoing progress throughout the *in vitro* culture. Taken together the results of the current study indicate that the chondrocytes cultured in the cell-scaffold constructs follow the chondrogenic lineage in a process analogous to chondrogenesis *in vivo* during the embryogenesis. Knowledge of molecular events and which signaling pathways that are involved during the redifferentiation *in vitro* may guide us when searching for potential markers that predict the chondrogenic potency of the cell-scaffold constructs to be implanted.

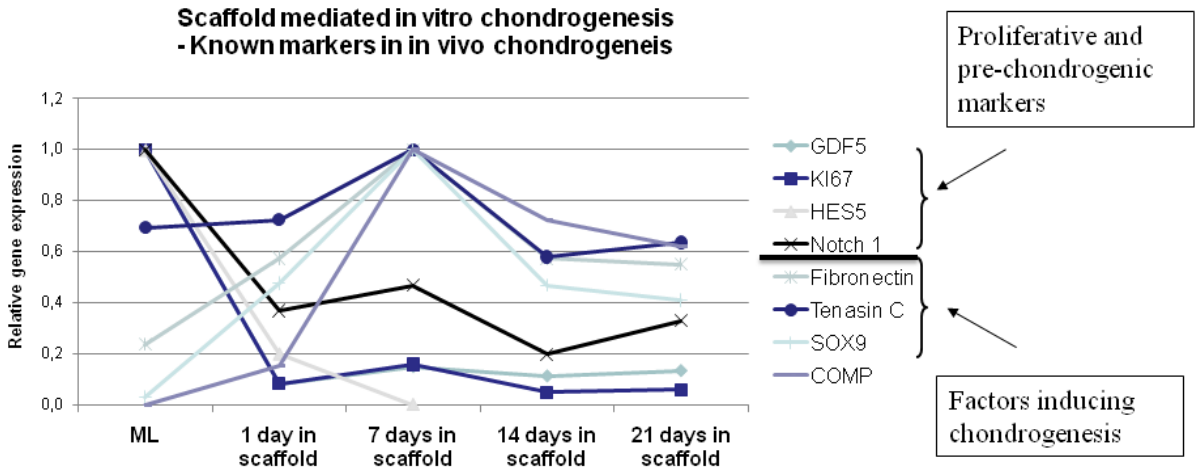


Figure 32
Graphic showing different molecular markers during in vitro chondrogenesis studied in paper III.

SUMMARY AND CONCLUSIONS

Different aspects of pre-implantation of *in vitro* expanded autologous chondrocytes for human cartilage repair were studied in the work reported in this thesis;
the cell type, cell seeding concentration, cell de- and re-differentiation, the culture media and cell migration capacity in scaffolds.
Our findings may be used as small pieces in the large puzzle of improving the techniques used in laboratories worldwide when expanding cells for cartilage repair.
Certain questions were raised as aims for the different research projects resulting in the papers in this thesis. Some of them could be answered while others require further research.

Important findings in the thesis are as follows:

- An addition of stem cells derived from adipose to a culture with chondrocytes could reduce the number of chondrocytes needed for a stable cartilage repair. This finding opens the possibility to use such a combination as a one stage procedure without a need of cell expansion.

- To obtain a secure cartilage production in a chitosan scaffold, 12-25 x 10^6 chondrocytes/cm^3 are needed. This is equal to 2-4 x 10^6 chondrocytes/cm^2 and is many more cells than have traditionally been used for clinical implants. However, these results are related to only one type of scaffold.

- A certain degree of de-differentiation is needed for a strong cell expansion. The culture time in scaffolds affects the grade of re-differentiation and the subsequent *in vivo* cartilage production.

- *In vitro* chondrogenesis in scaffolds induces a signaling pattern similar to that in fetal development. The expression of HES5 and Notch1 in the monolayer culture in the current study further shows that the chondrocytes possess a dedifferentiated primitive phenotype at this stage.

- Chondrogenesis and integration of chondrocyte seeded scaffolds *in vivo* can be enhanced by pre-culturing the chondrocytes in media formulations containing TGF-β1 and dexamethasone.

- Different compositions of the culture media influence the handling properties and the degree of integration to adjacent cartilage.

- A self-assembling peptide using a gel coating on cell seeded scaffolds showed evidence of better eliciting the migration and differentiation capacity in pig chondrocytes.

- When the use of cartilage fragments for a one stage cartilage repair procedure is desired, the use of peptide gel coating may enhance the cartilage repair.

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