Production of neocartilage tissues using primary chondrocytes

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Dedicated to my family

"It's a long way to the top if you wanna rock'n'roll."
-Ronald "Bon" Scott

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Abstract

Hyaline cartilage is a highly specialized tissue, which plays an important role in the articulating joints of an individual. It provides the joints with a nearly frictionless, impact resisting surface to protect the ends of the articulating bones. Articular cartilage has a poor self-repair capacity and, therefore, it rarely heals back to normal after an injury. Overweight, injuries, overloading and genetic factors may initiate a degenerative disease of the joint called osteoarthritis.

Osteoarthiritis is a major global public health issue. Currently, the most used treatment for large articular cartilage defects is joint replacement surgery. However, possibilities to replace this highly invasive operation with strategies based on tissue engineering are currently investigated. The idea of the tissue engineering is to optimize the use of the cells, biomaterials and culture conditions to regenerate a new functional tissue for the defect site.

The goal of this thesis was to manufacture cartilage tissue in cell culture conditions *in vitro*. Bovine primary chondrocytes isolated from the femoral condyles were used in all the experiments for neocartilage production. The samples were collected for histology, gene expression level quantifications, and analyses of proteoglycan (PG) content and quality. The histological sections were stained for type II collagen and PGs, the quantitative RT-PCR was used to observe the relative expressions of aggrecan, Sox9, procollagen α_2(I) and procollagen α_1(II) genes. The PGs were quantified using a spectrophotometric method, and agarose gel electrophoresis was used to separate the PGs according to their size.

In the two first studies, we optimized the culture conditions of *in vitro* scaffold-free culture technique to produce the native-type hyaline cartilage of a good quality. We found out that high glucose concentration and hypertonic medium at 20% oxygen tension promoted the best hyaline-like neocartilage tissue production. Glucosamine sulfate supplementation, low oxygen tension, 5 mM glucose concentration and a transient TGF-β_3 supplementation were not beneficial for the neocartilage formation in the scaffold-free cell culture system.

In the third study, we used these newly defined, optimized culture conditions to produce the neocartilage tissues in the HyStem™ and the HydroMatrix™ scaffold materials and we compared these tissues to the ones grown as scaffold-free control cultures. We noticed that there was no difference between the controls and the scaffolds, and occasionally the scaffold-free

controls had produced better quality cartilage than the ones with the scaffolds. Overall, the neocartilage tissues were of good hyaline-like quality in the third study. Their extracellular matrix contents were close to the native cartilage, although the neotissues lacked the zonal organization typical to the normal articular cartilage. The tissues had the right components, but their ultrastructure differed from the native cartilage.

In conclusion, we were able to optimize our *in vitro* neocartilage culture method further, and discovered a good combination of the culture conditions to produce hyaline-like cartilage of good quality. Surprisingly, the scaffold materials were not beneficial for the cartilage formation.

Tiivistelmä

Lasi- eli hyaliinirusto on pitkälle erikoistunutta kudosta, jolla on erittäin tärkeä rooli yksilön nivelten toiminnassa. Kudos suojaa ruston alapuolista luuta muodostamalla lähes kitkattoman ja joustavan liikkumista helpottavan pinnan. Lasiruston oma uusiutumiskyky on hyvin heikko, ja näin ollen kudos vain harvoin paranee alkuperäisen kaltaiseksi vaurion jälkeen. Ylipaino, vammat, liiallinen kuormitus tai geneettiset tekijät voivat käynnistää rustokudoksen rappeutumisen. Tätä tilaa kutsutaan nivelrikoksi.

Nivelrikko on valtava kansanterveydellinen ongelma. Keinonivelleikkaus on nykyisellään ainoa hoitokeino pinta-alaltaan laajojen nivelruston vaurioiden hoitoon. Vaihtoehtoja tämän suuren ja invasiivisen kirurgisen operaation korvaamiseksi tutkitaan kuitenkin koko ajan ympäri maailmaa. Kudosteknologian ajatuksena on optimoida solujen, biomateriaalien ja erilaisten kasvatusolosuhteiden käyttö uuden, alkuperäisen kaltaisen toiminnallisen kudoksen luomiseksi vauriokohtaan.

Väitöskirjan kaikissa kolmessa osatutkimuksessa uudisrustokudoksia tuotettiin käyttäen naudan polven rustosta eristettyjä primäärisiä rustosoluja. Näytteet kerättiin histologisia analyysejä, geenin ilmentymistutkimuksia ja proteoglykaanisisällön ja -jakauman (PG) analyyseja varten. Histologisista leikkeistä värjättiin tyypin II kollageeni ja PG:t, ja kvantitatiivista RT-PCR -menetelmää käytettiin aggrekaani-, Sox9-, prokollageeni α_2(I)- ja prokollageeni α_1(II)-geenien suhteellisten ilmentymistasojen määrittämiseen. Proteoglykaanisisältö analysoitiin käyttäen spektrofotometristä menetelmää, ja PG:t eroteltiin kokonsa perusteella agaroosigeelielektroforeesia käyttäen.

Kahdessa ensimmäisessä osatutkimuksessa optimoitiin tukirakenteetta kasvattujen uudisrustojen kasvatusolosuhteita natiivin kaltaisen lasiruston tuottamiseksi. Havaitsimme, että korkea glukoosipitoisuus ja hypertoninen elatusaine yhdistettynä 20 % happiosapaineeseen tuotti parhaimman laatuista uudisrustokudosta tutkituista yhdistelmistä. Glukosamiinisulfaatin lisäys, matala happiosapaine, 5 mM glukoosi konsentraatio tai TGF-β_3:n lisääminen alkuvaiheessa eivät edesauttaneet uudisrustokudosten muodostumisessa.

Kolmannessa osatutkimuksessa otettiin käyttöön uudet, hyväksi havaitut kasvatusolosuhteet yhdistettynä HyStem™ and HydroMatrix™ -tukimateriaaleihin, ja niitä verrattiin tukirakenteettomaan kasvatusmenetelmään. Tutkimuksessa havaittiin, ettei tukirakenteettoman

kontrollin tai tukimateriaalien välillä ollut mitään eroa, ja että kontrollikasvatukset tuottivat ajoittain jopa parempaa rustoa kuin tukimateriaalein kasvatetut. Kaiken kaikkiaan kaikki tuotetut uudiskudokset muistuttivat laadullisesti lasiruston kaltaista kudosta. Molekyylisisältö lähenteli natiivia rustoa, vaikkakin uudiskudoksista puuttui normaalille nivelrustolle tyypillinen vyöhykkeinen järjestäytyminen. Kudoksissa oli parhaimmillaan oikea määrä oikeita komponentteja, mutta ne eivät vain olleet järjestäytyneet oikealla tavalla.

Onnistuimme optimoimaan uudisrustokudosten kasvatusmenetelmäämme. Löysimme hyvän kasvatusolosuhteiden yhdistelmän, jonka avulla kykenimme tuottamaan lasiruston kaltaista uudisrustokudosta. Hivenen yllättäenkin, tukimateriaalit eivät olleet avuksi tutkimuksessamme uudisrustokudoksia muodostettaessa.

Abbreviations

4αPDD	4-α-phobol-12,13-didecanoate
ACI	Autologous chondrocyte implantation
BMP	Bone morphogenetic protein
CZ	Calcified zone
DZ	Deep zone
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FGF-2	Fibroblast growth factor 2
GAG	Glycosaminoglycan
HA	Hyaluronan, hyaluronic acid
hAMSCs	Human adipose tissue-derived mesenchymal stem cells
IGF-1	Insulin-like growth factor 1
iPSCs	Induced pluripotent stem cells
LOX	Lysyl oxidase
MACI	Matrix-induced autologous chondrocyte implantation
MZ	Middle zone
MMPs	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
PBS	Phosphate buffered saline

PCM	Pericellular matrix
PEGDA	Polyethylene glycol diacrylate
PG	Proteoglycan
PGA	Polyglycolic acid
PLA	Polylactic acid
PRG4	Proteoglycan 4
RHAMM	Receptor for hyaluronan-mediated motility
SB	Subchondral bone
SZP	Superficial zone protein
SZ	Superficial zone
Sox9	Sex determining region Y –box 9
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
TRPV4	Transient receptor potential vanilloid 4

List of original publications

This thesis is based on the following studies, which are referred to in the text by their Roman numerals

I. Qu CJ, Lindeberg H, Ylärinne JH and Lammi MJ (2012) Five percent oxygen tension is not beneficial for neocartilage formation in scaffold-free cell cultures. *Cell Tissue Res* 348 (1):109-117

II. Ylärinne JH, Qu CJ and Lammi MJ (2014) Hypertonic conditions enhance cartilage formation in scaffold-free primary chondrocyte cultures. *Cell Tissue Res* 358 (2):541-550

III. Ylärinne JH, Qu CJ and Lammi MJ (2015) Comparison of the neocartilage generated in scaffolds and scaffold-free agarose gel supported primary chondrocyte culture. *Submitted manuscript*

Introduction

Articular cartilage is an avascular, aneural and alymphatic tissue. Its purpose in the body is to ease the movement of an individual by reducing the friction in the joint surfaces, and also to provide the joints with elasticity to protect the subchondral bone. The tissue consists of chondrocytes, extracellular matrix (ECM) and water. The chondrocytes keep up the homeostasis in the tissue by degrading the existing one and producing new molecules to the ECM. The ECM is responsible for the functional properties of the tissue. Type II collagen is the main collagen type present in the tissue, and a major macromolecule of the cartilage. It is highly cross-linked and, thus, forms a mesh-like structure providing the tissue with its mechanically strong support. Proteoglycans (PGs) are dispersed in this collagen network and provide the tissue with its resilience by attracting water molecules. When pressure is applied on the tissue, the water molecules detach from the PGs and the cartilage gives in, thus, giving a suspension to the joint.

If this tissue homeostasis is disturbed by, for example, an injury, the whole system may collapse. A joint dislocation may cause damage to the cartilage, and due to tissue's poor self-repair capacity it may not heal properly. This often leads into a condition called osteoarthritis (OA). In OA, the articular cartilage begins to degenerate and eventually it can lead to a loss of practically the whole cartilage tissue in the joint. This causes pain and difficulty to move for the individual, and eventually the individual medical costs are also reflected on the society. Total annual costs caused by OA are estimated to be billions of dollars in USA only.

At the moment, there is no good cure for OA. There are only methods to make the life of the patient easier with certain non-invasive and invasive methods. Some surgical treatments may help when the lesion in the cartilage is relatively small in size. However, when large areas of the cartilage tissue have degenerated, the only solution is joint replacement surgery. In best case scenario, the artificial joint can last for couple of decades but eventually it will wear out. This makes it obvious that the surgery is not suitable for young persons. Something more permanent is required.

The solution may be found from tissue engineering, which combines the cells, the signaling molecules and the scaffold materials to regenerate the lost tissue of the defect site in the joint. It is a challenge, which takes a lot of optimization regarding the scaffolds, the signaling molecules and even the cell types. There is a huge variety of both synthetic and natural scaffolds available at the moment. The roles of the suitable growth factors have not

been fully discovered, and it is yet to be decided whether to use stem cells or primary cells.

In this study, primary chondrocytes were used to investigate which culture conditions would improve the cartilage formation in the scaffold-free tissue cultures. These optimized conditions were then applied in the last study to compare the tissues formed by the chondrocytes cultured in the scaffold-free system and those embedded within two commercially available scaffolds. It is of the utmost importance to reveal the optimal conditions with the fully differentiated primary cells before moving into the use of the stem cells.

In general, solving the challenges of the regenerative medicine is like a huge puzzle, where the pieces are scattered around the world for the different groups to find and apply in their attempts for better outcomes of the manufactured tissues. This thesis project is one aiming to locate a couple of those pieces, and trying to bring them at a disposal of all the research groups pondering around the same questions.

Review of the literature

Articular cartilage

Cartilage types in human body

The human body consists of several different types of tissues with different functions, which eventually complete the structure of a fully functional human being. The different tissues are formed by different types of cells, which all produce definite molecules to ensure the tissue's functionality. Connective tissues do not only serve a connecting purpose in an organism, but they also include tissues with supportive or separating function. For example, bones, muscles, ligaments, skin and even blood are considered as connective tissues. Cartilage is a one type of connective tissue with certain specific and unique features.

Cartilage can be found in many sites in the human body, such as the nose, the ears, the spine and the joints. Depending on the tissue's location, it has a different function and, therefore, a set of different molecular components. Based on the fine structure and function, the cartilage tissues can be divided into three different types: elastic cartilage, fibrocartilage and hyaline cartilage. The elastic cartilage has a high concentration of elastic fibers, which give the tissue strength and elasticity. Outer ear is an example, which consists of the elastic cartilage. That type of the tissue can easily be bent and twisted, and still it returns easily to its original shape, mostly thanks to these elastic fibers (Athanasiou, 2013).

Ligaments and tendons are made of fibrocartilage, which is an immensely strong and durable tissue. Large amounts of type I collagen fibers with parallel orientation provide those tissues with their required strength. The tendons are the links between muscles and bones and, thus, they play a very important role in the movement of the individual. The ligaments secure the joints in place and allow their movement only to certain directions (Athanasiou, 2013).

The hyaline cartilage is present on the articulating joint surfaces all around the body. Its main function is to protect the joints' bony ends by reducing the friction and providing the joint with a suspension system. It has also a very special molecular structure, which makes it possible for the tissue to fulfill its purpose. Type II collagens provide the tissue with the strength, and PGs are

the source of its resilience, or cushion, against the impacts and the loading (Athanasiou, 2013).

Synovial joint

Three different joint types can be found in the human body: synarthroses, amphiarthroses and diarthroses. Synarthroses (or fibrous joints) are immovable joints which can be located for example in the sutures of the skull or in the ligaments connecting fibula and tibia. Amphiarthroses or the cartilaginous joints are slightly movable. They can be located, for example, at the vertebra (symphyses) or where the ribs connect the sternum (synchondroses). The intervertebral discs are made of the fibrous cartilage, and they attach the bony vertebra bodies together.

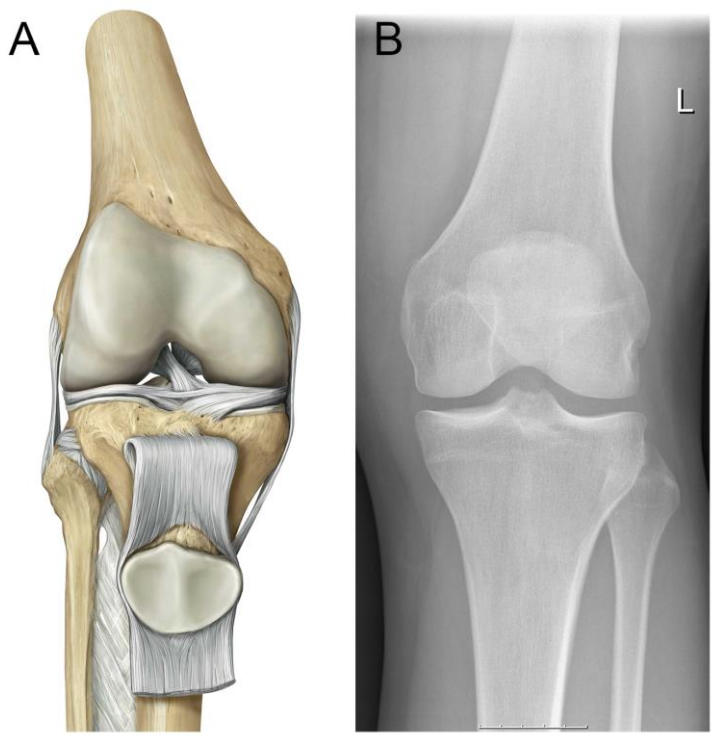

Figure 1. A: Drawing of a human knee joint. Knee is a synovial joint, which means that it has a synovial capsule around the articulating area of the joint. The capsule contains synovial fluid, which both nourishes the hyaline cartilage tissues and lubricates the joint surfaces. The joint is held together and supported by the ligaments and the muscles (Illustrator: Karl Wesker, Atlas of Anatomy, 2nd edition). B: Radiographic image of a human knee joint (24-year-old male, author's own knee). Cartilage can not be seen in X-ray images except as an empty space between the articulating bones. In this case, there is no narrowing in the joint-space which indicates that there is not notable change in cartilage thickness in the joint. L = Left.

However, the diarthroses or the synovial joints are probably the most studied ones in the field of the cartilage tissue engineering. This is the most mobile joint type. In the synovial joints, the articulating ends of the bones are covered with the hyaline cartilage (**Figure 1**), and a synovial capsule surrounds the whole joint. The capsule contains synovial cavity, which is filled with synovial fluid. The joint itself has a different anatomy depending on the site of the body. Knee, hip, shoulder, ankle and wrist are some examples of the synovial joints.

The synovial fluid is essential for the proper function of the synovial joint. The cells of the synovial membrane surrounding the joint secrete the fluid into the capsule. The synovial fluid reduces the friction and, thus, makes the movement of the articulating surfaces easier (Schmidt et al., 2007). It contains large amounts of hyaluronan (HA) and lubricin [also known as the superficial zone protein (SZP) and proteoglycan 4 (PRG4)], which enhance the lubricant properties of the fluid. Hyaluronan has a high viscosity, and it works as a lubricant reducing shear stress (Ogston and Stanier, 1953). The lubricin/SZP/PRG4 is responsible for the boundary lubrication in the synovial joint. If there is a deficiency in their production, the cartilage surface is not properly protected against mechanical tear and wear. This in turn can cause further problems in the cartilage tissue leading to severe consequences (Marcelino et al., 1999; Flannery et al., 1999; Rhee et al., 2005b; Rhee et al., 2005a; Jay and Waller, 2014).

The synovial fluid is also responsible for the proper nutrient and oxygen flow to the hyaline cartilage tissue, as well as transporting the waste products away from the tissue. This is due to a lack of the blood vessels in the cartilage tissues. Thus, the synovial fluid is the only source of these vital substances to the chondrocytes, keeping up the homeostasis in the tissue. The previous studies have shown that in the mature cartilage, no nutrient flow occurs through the subchondral bone (Honner and Thompson, 1971; Maroudas et al., 1968). The joint movement also partially contributes to the nutrition of the tissue. The motion causes pressure, which pumps the molecules in and out of the tissue, thus, improving the nutrient flow. However, this does not significantly affect the small molecules, like glucose and oxygen, but more the larger ones such as the enzymes, the growth factors and the cytokines. The movement helps in the distribution and mixing of the fluid, and indirectly affects the nutrient flow into the tissue (O'Hara et al., 1990).

Structure and function of articular cartilage

By naked eye, the articular cartilage appears shiny and white tissue, which has very smooth, even glassy-like surface. When the surface is touched by hand, it feels very slippery, almost frictionless. That is partly due to the smoothness of the surface, but mostly it is caused by the synovial fluid, which contains the lubricin/SZP/PRG4 and HA. These molecules help to reduce the friction and ease up the movement of the joint surfaces against each other.

When we take a look at the inside of the hyaline cartilage tissue, we may observe by microscopy of stained histological sections the composition of the tissue. The main constituents of the cartilage tissue are the cells, the ECM and water. The cells are called chondrocytes (Athanasiou, 2013). Usually they are referred to as sphere-shaped, but in fact their morphology depends on the area of the cartilage. A pericellular matrix (PCM), surrounds the chondrocytes, and the combination of the cell and the PCM is often referred to as the chondron. The PCM can be distinguished from ECM by its type IV collagen content, which has been estimated to play a role in attaching the chondrocyte into the PCM. It is estimated that the PCM has a rather significant biomechanical role for the chondrocytes regarding especially the stress-strain and the water flow (Guilak et al., 2006).

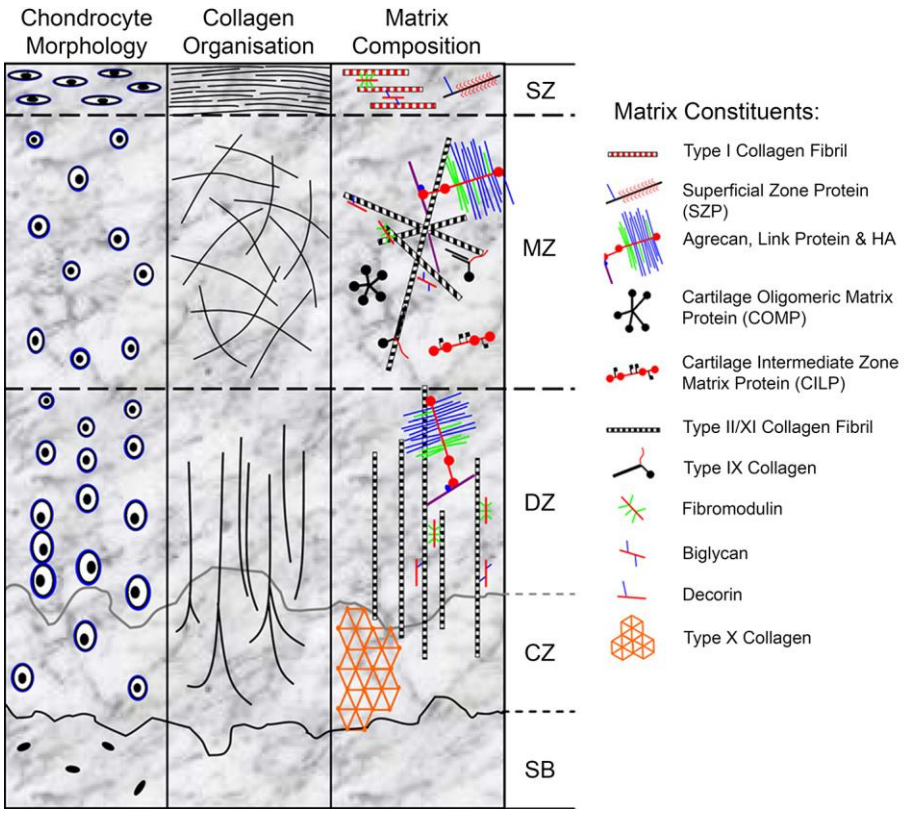

Figure 2. A schematic drawing of the microscopical structure of the hyaline cartilage. The panel on the left shows the organization and the shape of the chondrocytes, and the segment in the middle enlightens the organization of the collagens in the tissue. The chondrocytes in the superficial zone are rather flat and become more round in the middle zone. Deep zone chondrocytes organize themselves in column-like structures. Similarly, the collagens in the deep zone are aligned perpendicularly to the surface. In the middle zone, they are organized like a mesh, and in the surface zone the fibers are parallel to the surface of the tissue. The panel on the right gives overall information about the ECM composition. SZ: superficial zone, MZ: middle zone, DZ: deep zone, CZ: calcified zone and SB: subchondral bone. Figure adapted from (Hayes et al., 2007).

The ECM is produced and normally degraded by the chondrocytes, which keep up the homeostasis of the tissue. The ECM consists mainly of the collagens (approximately 60% of the dry weight), the PGs (20-40% of the dry weight) and water (even 80% of the wet weight), which all play a crucial role in the function and structure of the articular cartilage. The collagens are triple-helical proteins, which have a specific G-X-Y amino acid sequence to allow the formation of the helix. The triple-helix may assemble from one, two or three gene products, depending on the collagen type. For example, type II collagen has three α_1 protein chains, while type I collagen has two α_1 and one α_2 chains (Jackson, 1978).

Type II collagen is one of the most important collagen molecules in hyaline cartilage, representing approximately 90-95 % of all the collagens in the tissue (Sophia Fox et al., 2009; Buckwalter et al., 2005). It is not produced in this amount anywhere else in the body. The collagens, especially type II, in combination with types IX and XI, provide the cartilage with its strong and durable "skeleton" and the shear-resisting smooth surface (**Figure 3**). Depending on the zone of the tissue, the collagen fibers are organized differently. On the surface, they are parallel to the surface to resist abrasion, but deeper in the tissue they are perpendicular to the surface and create mesh-like structures, which strengthen the tissue against loads and pressure (**Figure 2**). This tight mesh also keeps the large aggregating PGs and cells immobilized, when the motion and the pressure is applied on the tissue in the joint (Jeffery et al., 1991; Sophia Fox et al., 2009; Buckwalter et al., 2005).

While the collagens provide the cartilage with its tensile stiffness and strength, the PGs are responsible for the resilience of the tissue. The PGs consist of a protein core into which one or several glycosaminoglycan (GAG) carbohydrate chains are attached. The cartilage PGs can be divided into large (aggrecans) and small ones (biglycan, fibromodulin and decorin) according to their size. The aggrecan is a large PG, which is very important for the proper function of the cartilage tissue. Keratan, dermatan and chondroitin sulfates, and HA are the most important GAGs in the cartilage. The GAGs are polysaccharides, which consist of repeating, usually sulfated, disaccharide units. This disaccharide is composed of hexuronic acid (glucuronic or iduronic acid) and hexosamine (glucosamine or galactosamine) molecules (Buckwalter et al., 2005). Keratan sulfate is an exception from the rule, since instead of hexuronic acid it contains neutral galactose. Hyaluronan is the only non-sulfated GAG.

Aggrecans form large macromolecular aggregates in the tissue by attaching to a central HA core (**Figure 3**) (Hardingham and Muir, 1972). Furthermore, multiple GAGs (approximately 50 keratan and 100 chondroitin sulfate chains) bind covalently to the aggrecan core protein covering these macromolecules with negative charges provided by their sulfate groups. The binding of multiple aggrecans to HA builds up a huge macromolecule (Buckwalter et al., 2005). The negative charge brought by the GAGs attract positively charged ions, which in turn attract large amounts of water molecules into the proximity of these macromolecules. This phenomenon provides the articular cartilage tissue with its resilience. Once pressure is applied on the tissue, some water is squeezed out of the tissue causing also tissue compression (Buckwalter et al., 2005).

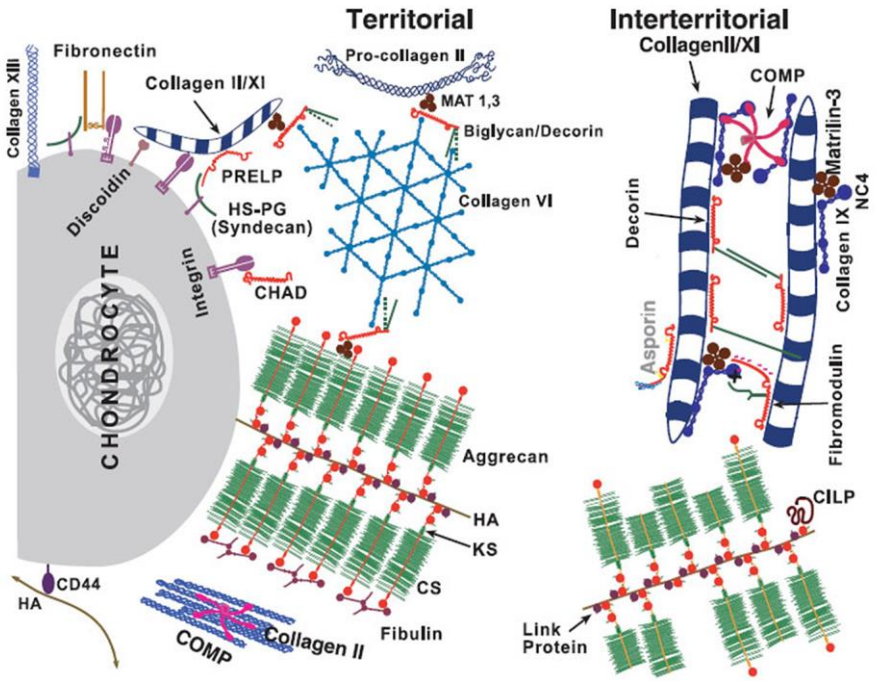

Figure 3. Molecular constituents of articular cartilage tissue. The ECM consists of various different molecules, which play a specific important role in the proper function of the tissue. The collagens are responsible for the strong structure and the PGs, such as aggrecan, provide the tissue with its elasticity. Figure adapted from (Heinegård, 2009).

One of the most interesting aspects of articular cartilage is the zonal organization of the tissue (**Figure 2**). It can be divided into four different zones, in which the shapes of the cells and the organization and quantities of the ECM molecules vary. The topmost zone is called the superficial or tangential zone (SZ). The collagen fibers there are parallel to the surface. The PG content is lower than in the deeper tissue, since the primary function of the superficial zone is to resist the erosive mechanical forces. The superficial zone chondrocytes are flattened in their morphology. The SZP is also secreted by the superficial chondrocytes, and the protein is specific only to this zone. It has friction-reducing properties on the cartilage surface and the synovial fluid (Sophia Fox et al., 2009; Buckwalter et al., 2005).

The middle (or transitional) zone lies just beneath the superficial one (**Figure 2**). The collagen fibers form a randomly-oriented mesh in the

middle zone, while the PG content increases compared to the SZ. The morphology of the chondrocytes change also to a more sphere-like one. The transitional zone possesses higher PG content and lower collagen content than the SZ, and, therefore has a bigger role in the resilience of the tissue (Sophia Fox et al., 2009; Buckwalter et al., 2005).

The deep zone (also known as the radial zone) is between the middle and the calcified zones (**Figure 2**). In this zone, the chondrocytes form column-like arrangements. These columns are perpendicular to the surface in the same way as the collagen fibers are. The PG content is the highest, and the collagen fibers are thick in this zone. A line called tidemark, from which the calcified zone begins, borders this zone. The calcified zone anchors the cartilage tissue to the subchondral bone (Sophia Fox et al., 2009; Buckwalter et al., 2005).

The chondrocytes

Chondrocytes are the cells dwelling in the cartilage tissue. They, like all cells, have a very specific phenotype in order to produce the appropriate ECM components for the tissue to fulfill its demanding purpose. Collagens and PGs are the major, and the most important, components of the cartilage ECM secreted by the chondrocytes. The cells are spread sparsely all over the tissue with a different morphology and slightly different phenotype depending on the zone they inhabit (**Figure 2**). They have a quite slow proliferation rate.

Chondrocytes arise from mesenchymal stem cells (MSCs). During chondrogenic differentiation, the MSCs go through condensation phase where they aggregate together in high density cellular spheroids, where the differentiation to chondrocytes begins (Archer and Francis-West, 2003). The transcription factor Sox9 plays a very important role in the differentiation. It activates the expression of procollagen α_1(II) gene and, thus, acts as an important transcription factor for determining the cells' chondrogenic commitment (Lefebvre et al., 1997). There are several different growth factors that are considered to be important in the chondrogenic differentiation and the chondrocyte maturation. Most of these belong to the transforming growth factor-β (TGF-β) superfamily, but there are also others, including fibroblast growth factors (FGF) and insulin-like growth factor (IGF-1) (Lin et al., 2006; Freyria and Mallein-Gerin, 2012). Previous studies have shown that the three TGF-β isoforms 1, 2 and 3 stimulate the chondrocyte proliferation and the ECM production (Zimber et al., 1995; de Haart et al., 1999; Richmon et al., 2005). The TGF-β_1 induced chondrogenic differentiation in the MSCs (Johnstone et al., 1998), and similar results were

shown for the human MSCs in pellet cultures using TGF-β_3 supplementation (Mackay et al., 1998). Bone morphogenetic proteins (BMP) also belong to the same superfamily, and there are indications that BMP-2 can promote N-cadherin expression, which in turn allows the MSC aggregation in the early stage of the chondrogenesis (Haas and Tuan, 1999). The BMPs also appear to upregulate the Sox9 expression, affecting also the chondrogenic differentiation of chondroprogenitor cells (Hatakeyama et al., 2003).

Depending on the area of the cartilage the cells live in, they have different fates. Those that dwell in the growth plate area differentiate to hypertrophic state, and eventually go through apoptosis, or turn into osteoblasts. The cells that inhabit the areas above the growth plate survive, live on and keep up the homeostasis of the tissue (Archer and Francis-West, 2003).

It is well known that chondrocytes lose their normal phenotype when cultured in monolayers *in vitro* (Benya and Shaffer, 1982; Schnabel et al., 2002). The expression of procollagen α_1(II) drops after a couple of passages, meanwhile the expression procollagen α_2(I) is highly upregulated. Procollagen α_2(I) as a transcript for α_2 chain of type I collagen is usually expressed in chondrocytes only at very low levels. In *in vitro* cell culture, these kind of changes act as indicators of the changed phenotype of the cells.

The environment in articular cartilage

The environment surrounding the chondrocytes could be described as rather harsh, and it may be even fatal to some other cell types. Due to the avascularity of the tissue, the cells face certain challenges concerning the nutrient and the oxygen flow and the removal of the metabolic waste materials. The only way for chondrocytes to get sufficient amounts of oxygen and glucose is via diffusion from synovial fluid, which itself is an ultrafiltrate of the blood. It is obvious that concentrations of the oxygen and the glucose that reach the deep zone chondrocytes are quite low (Archer and Francis-West, 2003).

The accumulation of the ECM macromolecules, the PGs in particular, into the tissue causes that parts of the tissue reach very hypertonic conditions. It has been shown that the osmolarity in the superficial areas of the tissue can be around 350 mOsm, whereas in the deep zone it may reach even 450 mOsm (Urban et al., 1993; Hopewell and Urban, 2003; Oswald et al., 2008). In the normal tissues with vascularization, the levels are usually isotonic (around 300-320 mOsm), and in standard cell culture conditions this is the molarity most often used. However, that is not normal for the chondrocytes and, therefore, it obviously may have an effect on their behavior and

metabolism. It has been shown that the cartilage tissue properties improve when chondrocytes or adipose tissue-derived stem cells were used in combination with the hypertonic culture medium (Oswald et al., 2011; Jurgens et al., 2012).

Opposite to the osmolarity, the oxygen tension in the tissue drops dramatically the deeper we go due to the absence of the vascularization down to approximately 5% or even lower in the deeper areas of the tissue (Zhou et al., 2004). Yet, chondrocytes can endure this; they are capable of withstanding days under very hypoxic, almost anoxic, conditions (< 0.1% O_2) (Grimshaw and Mason, 2000). The ambient 20% oxygen tension is the normal standard used for the cell culture. Obviously this is not normal for the chondrocytes. For example, our group's previous study has shown that the low oxygen tension has beneficial effects on the chondrocytes' phenotype, increasing the PG synthesis and procollagen α_1(II) gene expression in monolayers (Qu et al., 2009). It has also been shown that the low oxygen tension improves chondrogenic potential helping the stem cells to differentiate into chondrocytes (Bornes et al., 2015; Cao et al., 2015).

Similarly to the oxygen tension, the glucose concentration in the tissue gets gradually lower in the deeper parts of the cartilage (Mobasheri et al., 2002; Zhou et al., 2008). Chondrocytes may be able to survive days without oxygen, but a sufficient glucose supply is vital for them (Grimshaw and Mason, 2000; Bibby and Urban, 2004). It is not only a vital energy source for them, but also an important structural precursor, a building block, for the production of the GAGs (Mobasheri et al., 2002; Qu et al., 2007; Cigan et al., 2013). It has been shown that glucose concentration has an effect on the cell viability and the GAG synthesis (Heywood et al., 2006a). A diabetic glucose concentration of 4.5 g/l (high glucose) is quite often used in the normal cell culture media to ensure a proper amount of glucose to maintain the viability of the cells. For the cells, a low glucose (1.0 g/l) concentration could be more natural, which may also improve the phenotype of the cells during *in vitro* culture.

As described above, the environment in the hyaline cartilage is very demanding for the cells, but the chondrocytes can manage with very little resources. They can adjust their metabolism and proliferation at low levels, thus minimizing their energy requirements. The chondrocytes are very well adapted to their harsh environment. If the balance in the tissue is disturbed, it may confuse the cells causing also change in their phenotype.

Osteoarthritis

There are several different pathologies concerning the cartilage tissue, but in this thesis I focus on OA, which is the most common type of the arthritis among people. In OA, the cartilage starts to degenerate, which in turn may cause pain, swelling of the joints and difficulty to use the joint. It can affect basically almost any joint in the human body, still the hips and knees are the most commonly affected ones. OA is a disease, which progresses itself over time.

OA is more common among the elderly people than in the young populations, yet, it affects practically every age group. It is estimated that the disease affects approximately 27 million Americans (Lawrence et al., 2008). In the year 2012 in Sweden, 26.6% of all the people age at 45 years old or older had been diagnosed with OA. It has also been estimated that its percentage will rise to 29.5% by the year 2032 (Turkiewicz et al., 2014). Thus, there is no doubt that OA causes the societies huge burden also economically. For example, in the USA it is estimated that the direct and indirect cost of OA are billions of dollars (Bitton, 2009).

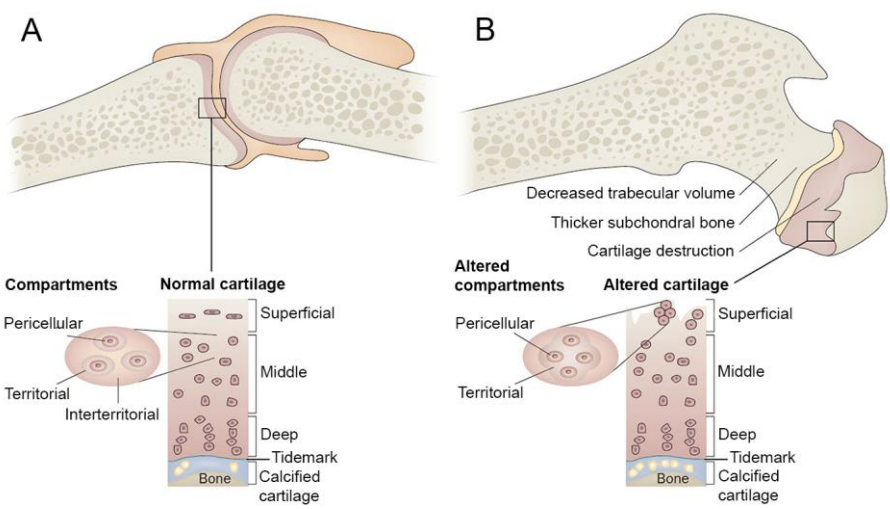

Figure 4. Comparison of normal (A) and osteoarthritic joints (B). In the late osteoarthritis (B) we can see distinctive changes in the tissue both in the macroscopical and the microscopical levels. The cartilage fibrillates at the surface, clefts within the tissue will appear, the gradually the cartilage wears off from the joint surface. The chondrocyte morphology also changes, and clustering of the chondrocytes occurs in the degenerating tissue. Figure adapted and modified from (Heinegård and Saxne, 2011).

Symptoms of osteoarthritis

Osteoarthritis causes pain and stiffness, and usually the swelling can also be observed in the joint area. The disease does not begin suddenly; it develops slowly over years and the pain and stiffness increase over time. Normally, the cartilage starts to degenerate slowly from certain focal area, which increases friction in the area causing pain, swelling and eventually inflammatory response, which drives the disease forward. Also, the subchondral bone is remodeled; the bone stiffens in order to try to respond to the changed biomechanics, the vascularization reaches the tidemark area of the cartilage, and osteophytes are formed (Felson, 1988; Buckwalter, 1995; Buckwalter et al., 2005).

The whole process can be divided into three stages. First, the ECM is damaged and its structure is disrupted. The PG contents start to decrease, while the water content increases simultaneously, which eventually weakens the mechanical properties of the tissue making it more prone to further damage. Fibrillation and erosion of the tissue can be visible on the surface (Buckwalter et al., 2005). In the second stage, the chondrocytes respond to the alterations and start to proliferate and form clusters, which are surrounded by the newly formed ECM molecules (**Figure 4**). This is the repair response of the cells against the catabolic proteases and this stage may last for years. The third stage follows when the cells fail to maintain the ECM composition. Then the anabolic response of the chondrocytes is overweighed by the catabolic forces leading to a progressive loss of the cartilage (Buckwalter et al., 2005).

Inflammation plays also a part in OA progression. The most accepted theory nowadays is that the loose cartilage pieces or the ECM fragments detached, for example, as a result of injury cause the synovial cells to secrete certain inflammatory mediators. These molecules include interleukins, tumor necrosis factor-α (TNF–α) and fibroblast growth factor 2 (FGF-2), among others. The inflammatory mediators affect the superficial cartilage chondrocytes by inducing the production of matrix metalloproteinases (MMPs) and apoptosis of the cells. The MMPs in turn promote the cartilage degradation causing a vicious cycle of degeneration (Berenbaum, 2013; Lee et al., 2013).

After several years or even decades, the end stage of OA is just two bones scraping against each other in the joint with highly increased friction, causing high pain and a highly reduced movement of the joint.

Causes of osteoarthritis

There are several reasons that may initiate OA. It has been shown that some genetic factors lie behind the disease, but usually factors more physical in nature initiate the slow progression of OA. Basically, it is considered that the alterations in the loading or in the molecular structure of the tissue have a major impact on the initiation of OA. The most common factors associated with OA are ageing, obesity, joint overuse, injuries, malalignment of joints, a weak physical condition (weak muscles) and genetics. Usually, there is not only a single predisposing factor, but they are combined.

Ageing is commonly associated with OA, since the older the population gets the higher the prevalence of OA is. Over 25% of all people over 45-year-old have OA in the knees or the hip. It is also estimated around 80% of the population will have (radiographically detectable) knee OA at the age of 65 (Lawrence et al., 2008). Half of the people over 65 are estimated to suffer from the symptomatic OA in their life (Murphy et al., 2008). Although it cannot be concluded that OA is directly caused by the old age, the ageing certainly has a cumulative effect on the disease. Accumulative effect of tear and wear over the years obviously plays its own role, but also it may be that the senescence of the ageing chondrocytes has an effect on the tissue structure and homeostasis (Loeser, 2009).

Obesity causes extra load on the cartilage in the joints, which means excessive stress on the tissue. The research has shown that there is definitely a connection between obesity and OA (Felson et al., 1988; Hart et al., 1999; Blagojevic et al., 2010). An abnormal loading increases the chance of degeneration of cartilage and OA. Apparently, too little exercise is also bad for the joints (Jortikka et al., 1997; Helminen et al., 2000). When an individual has a poor physical fitness, meaning their muscles are weak, the muscles are not so capable of bearing the weight of the individual. The weakness in quadriceps has been associated with OA (Slemenda et al., 1997; Brandt et al., 1999). Besides the muscle weakness, also the ligament injuries can cause abnormal contact angles in the joints. This increased load in particular areas of the articulating joint is likely to increase the chance of OA. Similarly, also the disuse of the joints has been associated with degeneration of cartilage and OA (Buckwalter, 1995).

Injuries of the joint are associated with highly increased chance of OA. When the articular cartilage is damaged, it disturbs the homeostasis of the tissue. It is a well-known fact that the self-repair capacities of the cartilage are low. When cartilage is not capable of repairing itself, its capability to endure loads get lower and this in turn causes a snowball effect leading to OA. For

example, many athletes suffer several injuries to their joints possibly already at a very young age, which increases the chance they develop OA (Buckwalter and Lane, 1997; Lohmander et al., 2004; Bekkers et al., 2012). All in all, the sports appear to have both good and bad aspects for the joints. Exercise strengthens the muscles and loads the cartilage tissues, which both are good for the joints. Also, it keeps the weight at control. At the same time, the sports injuries to the joint may initiate the cartilage degeneration. So, it would be best to choose the kind of sports, that have a lower risk of joint injury (Buckwalter and Martin, 2004).

The genetics definitely play a role in OA progression. Usually, the genes connected with OA are important for the ECM. These include genes for, for instance, type II collagen (Sahlman et al., 2004) and aggrecan. There are also some other well defined genetic disorders, like alkaptonuria, which are more connected with one gene, and significantly increase the changes of OA. In alkaptonuria, there is a problem with homogentisate 1,2-dioxygenase enzyme. This causes homogentisic acid accumulation to the articular cartilage, which eventually causes black colorization and degeneration of the tissues (Cetinus et al., 2005).

Diagnosis and treatments of osteoarthritis

At the moment, there is no single test to confirm that the patient has OA. The diagnosis has to be made by assessing the patient's symptoms and medical history, physically examining the joints and using either X-ray or magnetic resonance imaging techniques. The medical history helps the doctor to evaluate the previous condition of the patient. OA does not just appear suddenly, so there can be hints spread through the patient's medical history. Still, it is usually the pain, which brings the patient to the physician, and at this stage OA is usually advanced.

Physical examination gives the doctor a further understanding of the location and the amount of pain in the patient's joints, as well as to assess their movement. Data from magnetic resonance imaging or X-ray supports the information achieved by the other two methods. The imaging techniques help the doctor to see inside the knee and see, whether there are changes in the structure of the joint or in the amount of the cartilage tissue itself. For example, in X-ray images the physician can observe joint space narrowing, which is a sign of the advanced OA. The cartilage itself cannot be seen from the radiographs, so the doctor has to assess the positions and distances of bones (Falah et al., 2010). At the moment with the current techniques, it is almost impossible to diagnose OA in its early stage because the changes are subtle. More fine-tuned techniques are under investigation all the time.

For example, biomarkers relating to OA progression are being studied. Synovial fluid can be used to detect certain markers relating to cartilage injury, which in turn give us further information about the biochemical changes happening in the joint. Furthermore, understanding of these degradative pathways of OA can give information about how these vicious processes can be interrupted and halted. It has been shown that, for example, an injury to the knee promotes aggrecan degradation which can be detected in elevated levels of N-terminal Alanine-Arginine-Glycine-Serine neoepitope (ARGS-aggrecan) in both synovial fluid and blood immediately after injury (Swärd et al., 2012; Larsson et al., 2014). ARGS-aggrecan is present in synovial fluid for several years after injury and it can act as a marker for distinguishing in between injured and healthy joints (Struglics et al., 2011). Furthermore, Larsson et al investigated levels of certain proinflammatory cytokines in the synovial fluid and noticed that increased levels of interleukin 6 and TNF-α are associated with increased risk of OA. The patients who had increased levels of these cytokines in their synovial fluid were, for example, 5-times more likely to have a progressed joint space narrowing than those, whose interleukin-6 and TNF-α were not elevated (Larsson et al., 2015). In 2015 Kumahashi et al published results showing that acute knee injuries can be associated with degradation of type II collagen. They observed higher levels of type II collagen epitope C2C in synovial fluid after knee injury when compared to uninjured controls. The levels were higher for 7 years (Kumahashi et al., 2015).

Although there is no simple test for OA at present, this kind of biomarker research can provide valuable tools for the diagnostics and the development of new kinds of treatments, which can interfere the molecular pathways involved with cartilage degradation in OA. In the future it might be possible to detect subtle, radiographically non-detectable OA changes in the joint just by taking a synovial fluid or even a blood sample.

Several invasive and non-invasive treatments have been developed for OA. Common for all of these treatments is that none of them cure or heal the disease properly. They just help the patient to endure the pain and postpone the inevitable joint replacement surgery. They give more years for the old joint and improve the quality of life. There is no cure for OA. At this moment there are only ways to improve the quality of life of the patient.

Loss of weight, exercise, dietary glucosamine or chondroitin sulfate supplementation and HA-injections are some to list the non-surgical methods. The loss of weight is especially helpful for the obese patients. When the patient weights less, there is also less loading on the joints, which makes the symptoms less painful. It has been shown that the loss of weight and

exercise help the patients to reduce the pain (Christensen et al., 2007; Jenkinson et al., 2009; Bliddal et al., 2014). When one is physically fit and has a decent muscular strength, the load on joint is lower because the muscles can support and take more load.

The effects of glucosamine and chondroitin sulfates are rather interesting. In 2003 European League against Rheumatism (EULAR) concluded that both chondroitin and glucosamine sulfates would be helpful for the pain and might have even effect on structure modification in knee OA (Jordan et al., 2003). More recently, in 2014 Osteoarthritis Research Society International's (OARSI) guidelines for non-surgical management of knee OA recommended that glucosamine is not appropriate for the disease modification, but that it is uncertain whether it has some effect on relieving the symptoms. Chondroitin sulfate had the similar status (McAlindon et al., 2014). The present consensus is that the research results are controversial, and that no true effect has been proven. The data is heterogenous, and in many studies biased (Zhang et al., 2010). In 2012, Henrotin reviewed that it is still an open question whether glucosamine treatment has any benefit for treating OA. (Henrotin et al., 2012). Anyhow, the products are cheap and have very little to no side-effects and therefore, it is valuable to investigate their effects more.

Hyaluronan injections straight to the synovial capsule have also been shown to help the symptoms. This is thought to be caused by the enhanced lubrication effect of extra HA, but HA seems to also have some chondroprotective properties via CD44 binding (Altman et al., 2015). Hyaluronan injections can still give only a temporary relief, helping the patient for maximum of some months. However, these injection improve the quality of life of the patients and show a real benefit for example in knee OA (George, 1998; Richette et al., 2015).

Cartilage repair

Hyaline cartilage has very limited self-repair capacity. This is partly due to the absence of vascularization, and partly due to the tight ECM of the cartilage, which hinders the migration of the chondrocytes (Hunziker, 1999). Therefore, there is a great interest in developing better methods to repair the damaged or the lost cartilage. Many different surgical methods have been developed with varying results.

Cartilage self-repair

Although the self-repair capacity of the articular cartilage is generally poor, a limited repair is still possible. Mostly this depends on the size and the depth of the lesion. If the lesion is shallow, the blood flow of the subchondral bone is not able to reach the defect site and the MSCs are not able to migrate there and aid in the repair. The repair is also almost impossible when the lesion is large in size, exceeding so-called critical-size lesion.

If the lesion is deep and there is blood clotting in the site, the MSCs entering the cartilage from the blood flow can attempt the repair. Sadly however, this often results in an inferior quality, fibrous cartilage tissue, which is not able to function in a similar way as the original hyaline cartilage. The fibrous cartilage has a different ECM composition and organization and, therefore, this self-repaired tissue is not likely to last for a long time under the loading that normal healthy hyaline cartilage easily bears (Huey et al., 2012).

Because of the poor self-repair capacity, and usually the poor quality tissue it produces, there is a great interest around the world to be able to repair the cartilage defects in some other ways. Surgical and cell-based therapies have been vastly developed during the last decades, and they continue to develop even further.

Current surgical methods in use

Several kind of surgical methods for the cartilage repair have been developed throughout the years. Basically, the methods depend on surgically affecting the joint to induce repair. Next, I briefly go through some of the most used techniques. Along with these, debridement and lavage are also used, but they do not exactly aim for the repair. They are used more to remove loose pieces of the tissue and to smoothen the surfaces to improve the functionality of the joint and relief the symptoms.

Microfracturing or drilling (**Figure 5A**) is one way to induce the self-repair in the cartilage. It is based on the ability of the MSCs to enter through blood flow to the defect site. The idea is to drill holes into the subchondral bone and, in this way, to allow blood to flow and to clot in the lesion. The MSCs in the clot are then hoped to generate cartilaginous tissue to the damaged area. This, however, very rarely (if ever) produces hyaline-like tissue, and does not provide much help with the larger lesions. Usually, the new tissue is fibrocartilage in nature, which is not mechanically as durable as the hyaline cartilage. Positive in this technique is that it requires only one surgery to initiate the healing. The surgery is quick to do, and the fibrocartilage

provides temporary relief. The results suggest that microfracturing has a positive effect when the lesions are small in size, meaning less than 2-3 cm^2 (Falah et al., 2010; Oussedik et al., 2015). The effects are normally positive for the first couple of years, after which the fibrous neotissue starts to degenerate (Mithoefer et al., 2009).

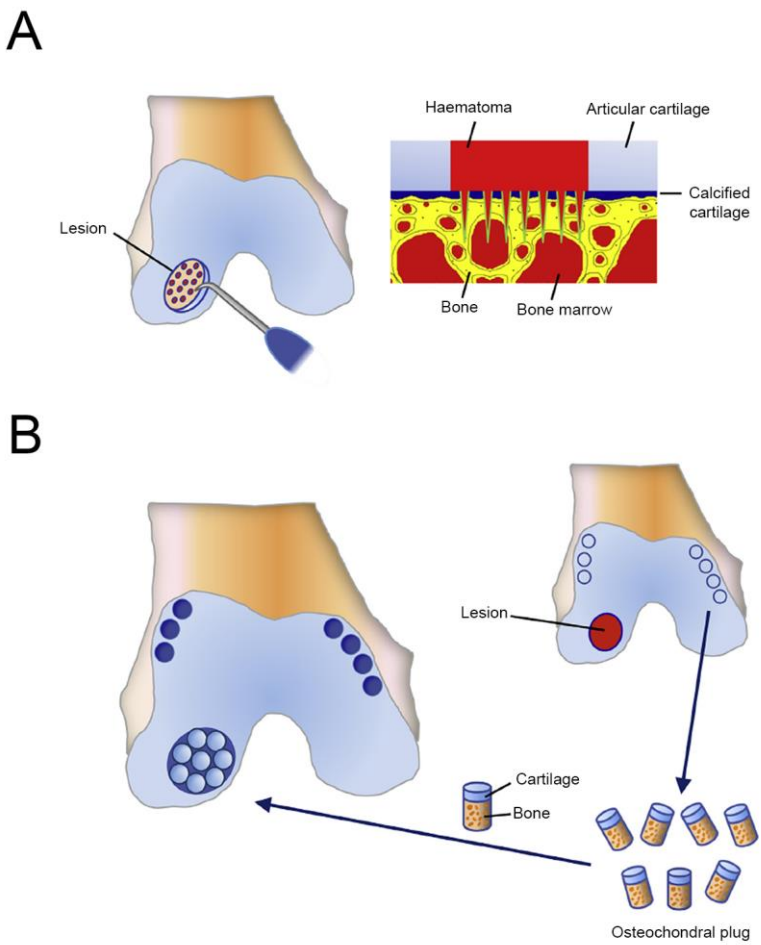

Figure 5. A drawing showing the principal idea of microfracturing (A) and mosaicplasty (B). A: In microfracturing, holes are made into the subchondral bone which allows blood flow into the defect site. The idea is that, eventually, a haematoma forms and the MSCs in the clot regenerate the tissue. B: In mosaicplasty, osteochondral plugs are harvested from a non-weight-bearing area of the joint and they are used to fill up the lesion site in the more critical area of the joint. Figures adapted from (Hunziker et al., 2015).

Another possibility is to use autologous tissue implantation in the repair of cartilage using a technique known as mosaicplasty (**Figure 5B**). In this method, cylindrical osteochondral blocks are drilled out of non-weight bearing areas of the articulating joint, which are then implanted to the defect area. The hope is to stop degeneration of the tissue at the lesion site with these implanted tissues (Hangody et al., 2004). The transplants help to bear the load, but the attachment takes time and the positive result is far from certain. The spaces between the cylinders are clotted by the blood flow from the subchondral bone, which again is likely to produce the fibrous cartilage (Burks et al., 2006). Similarly, the donor site repair tissues become fibrous cartilage. The new lesion in the non-weight-bearing area, caused by the collection of these transplants, is another problem to consider. The fibrocartilage has different collagen organization than hyaline cartilage and, therefore, is not capable to bear the loads the native tissue is. However, this repair method also requires only one surgery, which reduces the surgery-related risks.

Autologous chondrocyte implantation (ACI)

Autologous chondrocyte implantation (**Figure 6**) is a more sophisticated method to repair cartilage. It is also one of our best options at the moment. In the first generation of this technique, the primary chondrocytes are harvested from non-weight bearing area of the joint and are then expanded in cell culture laboratory. Once the adequate number of the chondrocytes have been acquired, the cells are seeded to the defect site under a periosteal flap, under which they are allowed to produce their ECM and fill the lesion (Brittberg et al., 1994). In the second generation of ACI, the periosteal flap was changed into a collagen membrane to overcome issues associated with the graft hypertrophy (Peterson et al., 2000; Micheli et al., 2001). This was an improvement for the method, and seemed to reduce the hypertrophy of the repair site's surface. Later, the researchers wanted to improve the chondrocyte spreading, as well as to get rid of the sutures and the annoying possibility of cells leaking out from the membrane-covered repair site (Sohn et al., 2002). The third generation of this repair was introduced under the name matrix-induced ACI (MACI). In the MACI, the cells are seeded onto a type I/III collagen membrane, and this graft is then planted into the defect site without sutures using fibrin glue (Cherubino et al., 2003; Brittberg, 2010).

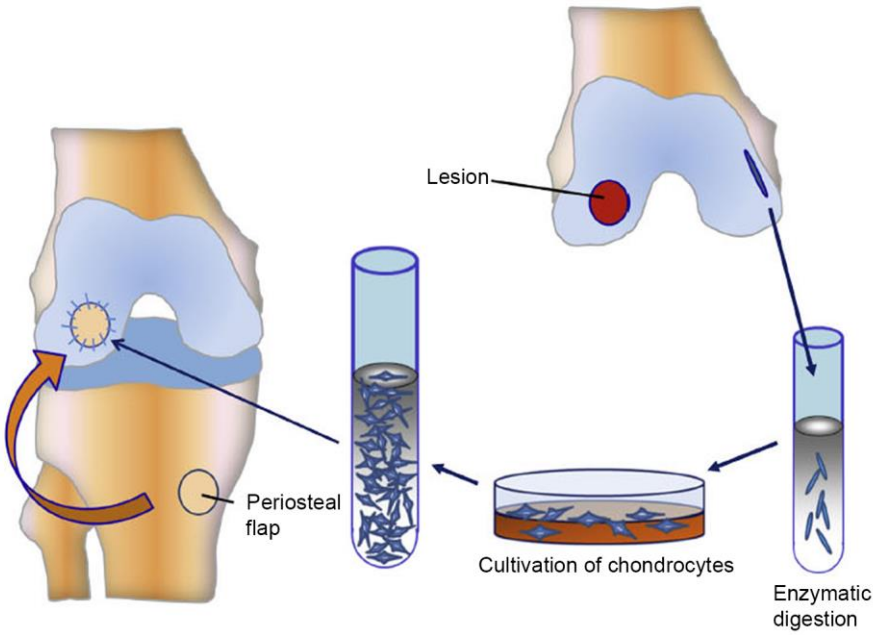

Figure 6. Autologous chondrocyte implantation (ACI). In this repair technique, cartilage is harvested from a non-weight-bearing area of the joint. Chondrocytes are isolated by enzymatic digestion and multiplied *in vitro*. When adequate number is reached, the cells are seeded into the defect site. In the first generation of this technique, a periosteal flap was sutured over the lesion to keep the cells in place. Later a matrix induced technique was developed where cells are seeded onto a collagen membrane, and this graft is secured in place using fibrin glue. Figure adapted from (Hunziker et al., 2015).

The ACI method has shown some encouraging and promising results, but there are also some drawbacks. Firstly, the procedure requires two operations. In the first one, the surgeon harvests the cartilage and then the second one, the actual implantation, follows after the expansion of the cell material. This can be stressful time for the patient. One surgery would be more comfortable for the individual and, furthermore, carry less risks. In addition, the surgeon also causes a new lesion to the tissue when he/she harvests the cartilage. Even though it is located on a non-weight-bearing area, the repair tissue is likely fibrocartilage, which is not optimal to fulfill hyaline cartilage's duties. The two surgeries and the *in vitro* expansion of chondrocytes take also time and are costly.

Another issue is the tendency of the chondrocytes to dedifferentiate towards fibroblast-like cells when cultured and expanded in monolayer. The cells lose their chondrocytic phenotype and express less and less the articular

chondrocyte-specific genes [e.g., aggrecan and procollagen α_1(II)] (Schnabel et al., 2002). The cells begin to resemble much more their progenitors (Tallheden et al., 2006). However, the dedifferentiation problem could be solvable using three-dimensional culture method and defined culture environment. Also, decreasing the procollagen α_1(I) gene expression by small intefering RNA duplexes could be one way to improve the chondrocytic phenotype.

The reported outcome of the ACI method has shown that 75% of the patients were satisfied with the results after 4 years (McNickle et al., 2009). The resulting repair tissue was sometimes hyaline cartilage, but most of the times only the fibrocartilage (Horas et al., 2003; Roberts et al., 2009). Still, the outcome seems to be better than with microfracturing, resulting more often in the hyaline-like tissue, and also being more effective for the larger lesions (Oussedik et al., 2015). Furthermore, the ACI tissues are not as stiff as the surrounding original tissue. Peterson et al reported 90% stiffness (mean follow-up 54.3 months)(Peterson et al., 2002), while Vasara et al reported only 62% stiffness after 1 year of ACI (Vasara et al., 2005) when compared to the surrounding cartilage. Despite this, even the first generation ACI seems to give a durable solution with good long-term outcomes. Even though the repair tissue would be fibrous, instead of hyaline, the patients seem to get relief, and the treatment really improves their quality of life (Brittberg et al., 2003; Peterson et al., 2010). It seems that if the graft endures the first two years, it will also last succesfully much longer (Brittberg et al., 2003). Repair with the newer techniques may have even better outcomes, since the tendency of the chondrocytes overgrowth may be better controllable, and the three-dimensional dispersion of the cells can be ensured.

All in all, the ACI method holds a great promise, and is one of the best options for treatment of articular cartilage lesions at the moment. The major problems at the moment are the dedifferentiation of the cells alongside with time, the expenses and the requirement of two operations.

Regenerative medicine and tissue engineering

Regenerative medicine is relatively new branch of science, in which scientists try to use cells to repair or replace the lost or damaged tissue. The organ transplant rejection and shortage of organ donors is a real issue costing patients their lives. The researchers in regenerative medicine try to bypass these problems using tissue engineering as their tool. Nowadays, growing fully functional organs *in vitro* is not that farfetched or "science fiction" idea,

as it used to be some decades back. The scientists around the world are taking steps towards that goal each day.

Why then focus on doing regenerative medicine research on articular cartilage, which is not actually that vital for maintaining the life of an individual? Cartilage does not pump our blood, it does not regulate our hormonal levels or take care of our breathing. Actually, if there is nothing wrong with it in your body, you scarcely spare a thought for it. That is in fact the secret of that tissue. It makes you happy by simply not giving you any idea of its existence. The articular cartilage is at its best, when we do not even know its existence. However, if something goes wrong with it, it messes up our everyday life. Every movement of the limb with that damaged joint is painful. Sometimes even staying still is agony. Good, healthy, articular cartilage improves individual's quality of life beyond measure. To move freely is what we are made for. That is also what articular cartilage tissue engineering and repair ultimately aims for. A well-repaired articular cartilage also diminishes the health-care costs via fewer sick-leave days of the OA patients.

Principle of tissue engineering

Basically, the assumption for all the repair methods for the articular cartilage defects at the moment is that they are not long-term solutions. They usually produce the fibrous cartilage, which does not fill the purpose the hyaline cartilage is for. The fibrous cartilage is not built to endure constant loading the joints go through. Furthermore, the only current therapy for large articular cartilage defects, the total joint replacement surgery, is not that long-term, either. The artificial joint wears out in use and eventually it has to be replaced. It may last over 20 years, but still the eventual replacement makes it very inconvenient for relatively young patients who, for example, are under their sixties. Total knee replacement surgery at young age would mean that the operation would have to be repeated during their life. The operation is large, and it always carries the risks of a big surgical operation, as well as the quite long recovery times. The joint replacement is aimed for the older patients with the more progressed OA, leaving the younger people with freshly injured cartilage tissues practically without a solution.

This is where the tissue engineering steps in. The idea is to minimize the invasiveness of the surgery, and bring a long-lasting solution to the cartilage and the joint repair. Tissue engineering is quite simple in principle. First of all, we need an adequate quantity and quality of the cells to produce the tissue we need. Some kind of biocompatible and biodegradable scaffold

material can be combined with the cells to give them support and the initial three-dimensional structure for the tissue. It is also possible to use only cells without the supporting material to overcome some problems that the scaffold can cause (**Figure 7**) (Langer and Vacanti, 1993; Hunziker, 2002; Vinatier et al., 2009).

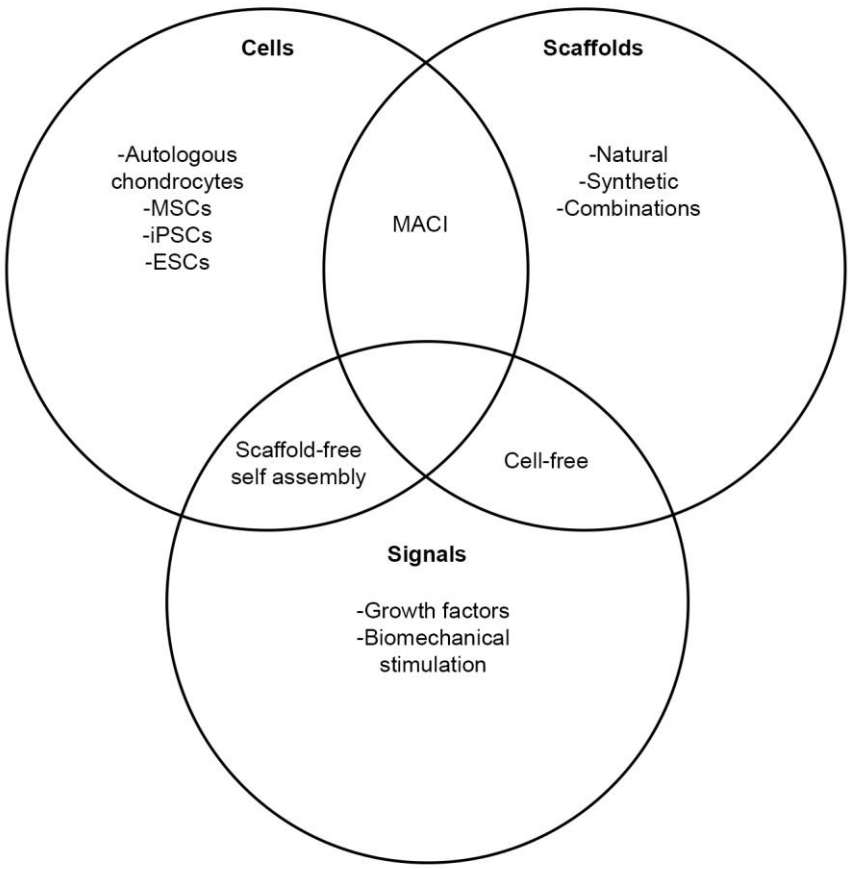

Figure 7. The principal idea of the tissue engineering. The image visualizes the three different aspects of the tissue engineering, and the overlapping area give information about some of the techniques the combinations have given birth to. Figure adapted and modified from (Makris et al., 2015).

Lastly, some physical/chemical signals may be required to guide the cells to their correct pathway to produce a functionally perfect tissue for regeneration of the lost or the damaged one. The signals can be certain growth factors or mechanical stimuli, but the general idea is to give the cells the hints they need to produce the functional tissue that meets the physical

challenges of the body (**Figure 7**) (Langer and Vacanti, 1993; Hunziker, 2002; Vinatier et al., 2009).

Primary chondrocytes or stem cells?

Primary chondrocytes are obtained from articular cartilage tissue. In basic research, they are usually harvested from animal joints, bovine tissue being very popular for its large yield of the chondrocytes from the big joints. For clinical applications, the autologous chondrocytes attract a greater popularity. The best asset of the primary chondrocytes is that they express the chondrocyte-specific genes straight after isolation, and that they are ready to produce the functional ECM. The negative side is that the availability of the donor tissues is usually low, and they have to be expanded *in vitro*. When expanded, the primary chondrocytes tend to dedifferentiate and lose their phenotype.

Stem cells have been considered to overcome the issue of the limited number of the primary chondrocytes available. MSCs have been under focus in the recent years, showing a great promise for the regenerative medicine purposes. Chondrocytes can be differentiated from the MSCs, so they appear to be an ideal candidate for the cartilage tissue engineering. The MSCs can be isolated from many different areas of the body, the most popular options being bone marrow and adipose tissue (Beane and Darling, 2012). They have excellent proliferative potential, which basically allows the researchers to expand the cells *in vitro* as much they would need for their purposes. In order to differentiate the MSCs, they are usually cultured in three-dimensional pellets or scaffolds (Freyria and Mallein-Gerin, 2012).

Using stimulation by certain growth factors, MSCs can be differentiated to chondrocytes. For example, the members of TGF-β -superfamily and FGF-2 have given good results in differentiating the MSC to chondrocytic cells (Heng et al., 2004; Grassel and Ahmed, 2007; Freyria and Mallein-Gerin, 2012). However, a major problem with the MSCs is that they tend easily to progress by terminal differentiation into hypertrophic chondrocytes, which is unwanted. Normally, the hypertrophic chondrocytes are present at the calcified area of the cartilage, where tissue turns to bone. These chondrocytes express different genes than the articular pre-hypertrophic chondrocytes specific to their zone and, therefore, produce also different kind of the ECM and the tissue (Freyria and Mallein-Gerin, 2012). The consensus, however, seems to be that the best possible chondrogenic differentiation at the moment is achieved by supplementing serum-free culture medium with dexamethasone, ascorbate and some growth factors of the TGF-β-superfamily.

Induced pluripotent stem cells (iPSCs) are obviously one other very intriguing option for the cartilage tissue engineering. They are pluripotent stem cells, which can be induced from the patient's own somatic cells by introduction of certain growth factors (usually Oct4, Sox2, Klf4 and c-Myc). They overcome the ethical and technical difficulties of the embryonic stem cells (ESC) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). However, the techniques are so young that there are not many publications about the use of the iPSCs in articular cartilage tissue engineering. In 2014 Lee et al were able to induce fibroblast-like synoviocytes to pluripotent stem cells using a lentiviral vector containing all required factors for the induction. The cells were isolated from the patients with OA or rheumatoid arthritis (Lee et al., 2014a). Developing this kind of techniques is valuable for developing patient and disease specific diagnosis methods and treatments. Recently, the iPSCs induced by RNA molecules was successfully used to manufacture the footprint-free human iPSCs (Boreström et al., 2014). Furthermore, in 2015 it was shown that chondrogenically differentiated human iPSCs could be used to repair cartilage defects in immunodeficient rats and pigs (Yamashita et al., 2015). These results might prove to be very useful in the cartilage tissue engineering in the future.

Signals: Growth factors and biomechanical stimulation

There is a lot of evidence which suggests that the cells alone or with a scaffold are not enough. Several different studies have been performed using different molecular or biomechanical stimuli to evaluate their effects on the cartilage ECM production and the neotissue quality *in vitro*. The stem cells need the growth factors to guide them to differentiate into the desired direction, while the dedifferentiation of the primary chondrocytes should be halted, when they are expanded, using some other signals.

The growth factors play an important part in the chondrogenic differentiation of the stem cells and the maturation of the chondrocytes, and supplementation of the cell and tissue cultures with the appropriate ones has been studied a lot. The members of TGF-β-superfamily are very important for the cartilage maturation along with FGFs and IGFs. Especially TGF-βs 1 and 3, and BMP-2 have given positive results in MSC or chondrocyte cultures (Blunk et al., 2002; Elder and Athanasiou, 2009; Freyria and Mallein-Gerin, 2012). For example, TGF-β_1 improved chondrogenesis and proliferation, and increased collagen formation after 4 weeks culturing of bovine calf articular chondrocytes on biodegradable polyglycolic acid scaffolds (Blunk et al., 2002). A transient supplementation of TGF-β_3 in combination with dynamic compressive loading improved the mechanical characteristics of the neotissue produced by the primary bovine

chondrocytes (Lima et al., 2007). In the culture conditions used in our group, TGF-β_3 induced the best chondrogenic differentiation of human bone marrow-derived MSCs (Ylärinne et al., 2014). However, contradictory results have also been reported, for example, for TGF-β_1, which decreased the PG synthesis of the OA chondrocytes (Verschure et al., 1994).

The supplementation of the cultures with some other presumably important molecules for cartilage, such as glucosamine sulfate, has been suggested to be helpful for the cartilage tissue engineering. Glucosamine is an important molecule for the GAG production, since its UDP-sugar precursor is needed as a building block in the GAG biosynthesis (Qu et al., 2007). Previously, glucosamine supplementation has been reported to stimulate chondroitin sulfate synthesis in a rat model and in the human osteoarthritic chondrocytes (Bassleer et al., 1988; Setnikar and Rovati, 2001), as well as to upregulate aggrecan gene expression (Dodge and Jimenez, 2003). However, contradictory results of glucosamine concentrations that can be achieved after an oral administration of glucosamine have also been published (Mroz and Silbert, 2004; Qu et al., 2006; Qu et al., 2007; Qu et al., 2009).

Normally, the articular cartilage has to endure various mechanical factors, including hydrostatic pressure, shear stress and compressive strain. Even during the fetal stage, there is movement of the joints, which causes the mechanical loading to transduce intracellular signals, which are important for the developing tissue. Dynamic compressive loading has been shown to have better effects on the tissue formation and the ECM secretion than static loading (Bonassar et al., 2001). This effect might be partially due to the mechanical signals, but also due to the improved nutrient and waste transfer (Kim et al., 1994). Similarly, the constant “static” hydrostatic pressure was found to have negative effect on the ECM production (Lammi et al., 1994). The results with dynamic compressive loading look really promising. In 2001, it was demonstrated to increase both the PG and the collagen contents when loading was combined with IGF-1 supplementation (Bonassar et al., 2001). Similarly, in combination with TGF-β_3 the Young’s modulus and the GAG-levels of the neotissues were similar to native cartilage after bovine primary chondrocytes were cultured for 28 weeks in agarose hydrogel (Lima et al., 2007). Addition of a sliding motion to the dynamic compression induced further positive effects also in the friction coefficient of the neotissue surface, as well as in the stiffness and the molecular composition of the engineered tissue (Grad et al., 2012).

Scaffolds for tissue engineering

The purpose of the scaffold usage in tissue engineering is to provide the used cells with a three-dimensional, strong and supportive matrix straight from the beginning of the culture. Besides, the chondrocytes lose the phenotype they normally exhibit when cultured at two-dimensional environment. The scaffolds provide the cells with a more natural environment for the growth and the proper ECM production. Furthermore, they ensure more even distribution of cells in the neotissue (Hunziker, 2002; Vinatier et al., 2009).

The scaffolds can be manufactured as gels, sponges, foams or meshes to mention a few. Whatever the structure of the scaffold may be, there are some important requirements for them. The materials should biocompatible and biodegradable, meaning that they should not be toxic or harmful for cells and that the cells should be able to degrade them. Eventually, it is desirable that the cells would replace the scaffold material with the fully functional ECM of their own production (Hunziker, 2002; Vinatier et al., 2009).

The scaffolds can basically be divided into two different groups: natural and synthetic. For example, collagens and many polysaccharides, such as agarose, chitosan and alginate, are among the natural scaffold materials. Polylactic (PLA) and polyglycolic acids (PGA) are a couple of very well-known and widely used synthetic materials. Even though their monomers are natural, the polymer is synthetically produced (Campoccia et al., 1998). The natural materials are usually considered more biocompatible and biodegradable than the synthetic ones. Yet, they often lack, for example, the strength or the stability required for the scaffold. Therefore, more modifiable and designable synthetic scaffold materials have also raised a scientific interest.

In cartilage tissue engineering, especially hydrogel-based materials are found interesting due to their high water content, which is also present in the native tissue. Therefore, the collagen- and HA-based biomaterials have been very much investigated. They both are natural components of the cartilage, and can be easily used to prepare hydrogel scaffolds for the cartilage tissue engineering. Both also play a very crucial role in the function of the cartilage tissue. These molecules are known to be biocompatible, and the cells are able to integrate their own ECM with the scaffold, and also degrade them during the assembly of the neotissue (Hunziker, 2002; Vinatier et al., 2009).

The use of HA as a scaffold material has been investigated a lot. It is a very important GAG in the body, and particularly in the cartilage. It is a polymer of two sugars: D-glucuronic acid and N-acetyl-D-glucosamine (Laurent and

Fraser, 1992). In articular cartilage, for example, it provides aggrecans with a central thread, into which multiple of aggrecans can attach creating a huge water-absorbing macromolecular complex providing the tissue with its elasticity. Hyaluronan can be solubilized to water where it forms a viscous hydrogel-like structure. Furthermore, the cells can recognize it via their CD44 (Knudson and Knudson, 1991; Akmal et al., 2005) or receptor for hyaluronan-mediated motility (RHAMM) (Hardwick et al., 1992) receptors. Basically, it would seem an ideal candidate for the cartilage tissue engineering, since it is so important for its function. However, the consensus now is that HA alone in its purified form is not good enough biomaterial for the tissue engineering due to its rapid resorption and short residence time in the tissue (Campoccia et al., 1998). Non-modified HA is also a rather soft biomaterial.

Therefore, the focus has moved to modified HA-based materials. HyStem™ is one commercial product among them. It consists of normal HA modified with thiol groups (carboxymethyl HA-thiopropanoylhydrazide). These thiol groups give the molecule the ability to form cross-links, when suitable agent is present. The cross-links make it more stable, and the forming hydrogel is stronger and more rigid. The gelation process is initiated by the addition of a polyethylene glycol diacrylate (PEGDA). This ingenious idea would hypothetically allow injection of the cells with the modified HA and the cross-linker separately to the lesion site in the joint. Then gelation would occur when the two components would be mixed with the cells. There are some previous studies concerning thiol-modified HA-scaffolds. The thiol-HA was cross-linked using air oxidization and hydrogen peroxide (Liu et al., 2005). This resulted in disulfide cross-links between the molecules. Films, which had good biocompatibility, were prepared using this method, however, the fibroblasts were not able to attach on the film when cultured on top of it (Liu et al., 2005).

The commercial HyStem™ product has at least been used in one published work so far (Heo et al., 2015). In that study it is shown that periostin stimulates the proliferation of transplanted human adipose tissue-derived mesenchymal stem cells (hAMSCs). However, the HyStem™ scaffold material had only a minor side role in the research. Previously, a basically similar scaffold (HA-thiopropanoyl hydrazide cross-linked using PEGDA) was shown to have good *in vivo* biocompatibility and cytocompatibility (Zheng Shu et al., 2004).

Self-assembling peptides can be considered as a kind of a hybrid between natural and synthetic scaffolds. The amino acids of the peptides are natural, but they are synthetically arranged to peptides, so in a sequences that have

self-assembling structures. For example, peptide amphiphiles have a hydrophobic tail attached to a short peptide sequence. When β-sheet region is attached to this, the peptide assembles into a cylindrical nanofiber. Further additions to the structure help the peptides to form cross-links and gelate (Cui et al., 2010; Matson and Stupp, 2012).

HydroMatrix™ is a commercial scaffold material, which forms highly cross-linked hydrogels from peptide nanofibers, according to the product description. The peptides in the product go through self-assembly, and form cross-links when temperature in the product rises and the ionic strength changes. Hypothetically, this means that, similarly to HyStem™, HydroMatrix™ could be injected to the lesion-site with the cells embedded within it. When the temperature of the product rises in the body, the peptides would self-assemble encapsulating the cells inside the newly formed hydrogel. HydroMatrix™ has been used in at least two previously published peer-reviewed studies (Asthana and Kisaalita, 2012; Stoppoloni et al., 2013), whereas the self-assembling peptides have been studied more extensively (Cui et al., 2010; Hartgerink et al., 2002; Matson and Stupp, 2012). Stoppoloni et al showed the positive effects of L-carnitine supplementation on the GAG production and cell proliferation in the human primary chondrocyte cultures (Stoppoloni et al., 2013). HydroMatrix™ was used for creating the three-dimensional scaffold, and the results showed that it is cytocompatible (Stoppoloni et al., 2013). However, neither of these studies using HydroMatrix™ directly evaluated its effects on the cells. Galler et al showed that another multidomain amphiphilic self-assembling peptide was cytocompatible and biodegradable, and, furthermore, it increased the cell viability and allowed the cell migration (Galler et al., 2010). Finally, Ni et al showed that self-assembling peptide nanofiber scaffold was able to improve dopaminergic differentiation and maturation of ESCs compared to monolayer culture or Matrigel scaffold (Ni et al., 2013).

The scaffolds bring many benefits to tissue engineering. They provide the cells with three-dimensional environment and the initial rigid support for the production of their own ECM. They help the cells right from the beginning, so that they have native-like environment. Often one can also incorporate some signal molecules or attachment sites to the scaffolding structures, so that the cells can be guided even better to the desired direction. The negative sides to the scaffolds sometimes are the poor biocompatibility and biodegradability. Biologically-derived materials often are well biocompatible, but they might lack the strength for the support or cause immunologic responses. If the scaffolds are too much modified, it can cause problems with biodegradability or viability of the cells. The cells might

not be able to get rid of the scaffold, which in turn may have unpredictable consequences.

Scaffold-free self-assembly of cartilage

To overcome the problems the scaffolds may cause, a lot of effort has also been put into the development of scaffold-free culture techniques. The main idea of creating cartilaginous tissue via self-assembly is to trust the chondrocytes' capability to assemble correct type of tissue by themselves, producing the good quality and quantity of the ECM straight from the beginning. There are several different ways to initiate the self-assembly process with the cells (DuRaine et al., 2015).

The cell-sheet technique is one way to produce scaffold-free neotissue. In this method, the cells are first cultured in a monolayer. Then the cell sheets are detached, and they can be either rolled or layered, depending on the purpose and the desired tissue type (DuRaine et al., 2015). Some promising results have been achieved by using this technique, especially to produce cylindrically-shaped cartilage for creating a functional trachea (Tani et al., 2010).

Rotational forces can also be utilized in the scaffold-free tissue engineering. Slow centrifugation of the cells makes them aggregate with each other, then the aggregates can be used to make a larger three-dimensional neotissue (DuRaine et al., 2015). These can then be used to regenerate the lost tissue in, for example, a cartilage lesion.

It is also possible to manufacture the cartilage tissue without any external forces involved in the process. Hayes et al. published results in 2007 that they had been able to produce zonally organized cartilage tissue using the bovine primary chondrocytes and insert-based scaffold-free culture method (Hayes et al., 2007). Kyriacos Athanasious's group has also been very successful in the utilization of this method, having produced several very promising and interesting results. A scaffold-free self-assembly method in agarose gel wells has also resulted in a good quality hyaline-like cartilage neotissue (Elder and Athanasiou, 2008; Hu and Athanasiou, 2006a; Hu and Athanasiou, 2006b; Ofek et al., 2008). Addition of several defined soluble factors appears to improve the tissue quality even further. Mechanical loading (MacBarb et al., 2014) and hyperosmolarity in combination with 4α-phorbol-12,13-didecanoate (4αPDD) could positively enhance the neotissue properties by increasing collagen content and the neotissue stiffness (Eleswarapu and Athanasiou, 2013; Lee et al., 2014b). Recently, a Japanese group published results, in which rather successful repair of the

osteochondral defect in rabbits was achieved using the MSCs and a scaffold-free culture method of the graft (Ishihara et al., 2014).

As reviewed above, the scaffold-free techniques show a great promise for the tissue engineering purposes. The development of these techniques could make it possible to avoid the problems caused by the scaffold materials, still giving a chance to manufacture almost native-like neotissues. However, these techniques need further optimization, especially regarding the culture environment. Once all the signals and cues in the environment are correct, we should be able to produce the kind of tissue, which is exactly similar to the native hyaline cartilage.

Aims of the study

The ultimate goal of this research was to optimize the culture conditions for the growth of the native-like articular cartilage neotissue by the primary chondrocytes *in vitro*. To reach this goal, the project was divided into sections, in which the different factors in the culture media and the environment were systematically optimized. Lastly, the found optimal conditions were applied to test whether two different scaffold materials would be able to improve the quality of the tissue produced *in vitro* in comparison to scaffold-free cultures.

This thesis study was aimed to:

1) Clarify whether the low oxygen tension (5% O_2) or glucosamine sulfate can improve the cartilage formation by the primary chondrocytes in scaffold-free culture system,

2) Study the effects of hypertonia, glucose concentration and the presence of TGF-β_3 on the formation cartilage, and

3) Compare the scaffold-free system with two commercially available scaffolds in the manufacture of cartilage under the culture conditions optimized in the previous studies.

These studies aimed to improve our tissue culture methods to manufacture the functional and native-like articular cartilage tissue *in vitro*. The achieved finding can help in the efforts to take the next step to try and repair articular cartilage defects in animal models.

Materials and methods

Production of the cartilage neotissues

Chondrocytes and their isolation (Studies I, II and III)

The primary chondrocytes were obtained from the bovine knees provided by three different abattoirs (Atria, Kuopio Finland; HK Scan, Outokumpu Finland and Strömdahla, Nordmaling, Sweden). The animals were approximately from 12 to 24 months old. An overnight collagenase (*Clostridium histolyticum*, Sigma) digestion protocol was used to isolate the cells. First, the cartilage was cut off from the femoral condyles of the knees and sliced into smaller tissue pieces. Next, the cartilage pieces were placed into digestion medium (100 U/ml collagenase, 1% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 50 μg/ml 2-Phospho-L-ascorbic acid trisodium salt in high glucose DMEM), and the digestion was initiated with the following program:

10 h stirring

2 h without stirring

1 h stirring

2 h without stirring

1 h stirring

End without stirring.

After the digestion, the isolated chondrocytes were filtered through a nylon filter with a pore size of 150 μm. The cells were then centrifuged, washed with PBS and counted, and finally seeded to the inserts or cell culture plates.

Samples cultured in inserts (Studies I and II)

In the studies I and II, the samples were cultured in Transwell™ or Transwell-COL™ inserts (Corning, Lowell, MA, USA; **Figure 8**). First, the inserts were equilibrated in the cell culture medium for at least 1 h before the cell seeding. The inserts were placed into the wells of a 24 well plate and 600 µl of the cell culture medium was added to each well. Subsequently, 100 µl of the same liquid was poured into each insert.

Figure 8 The Transwell™ inserts used in studies I and II. The inserts are cell culture wells, which have a water-permeable membrane as a bottom. The membrane allows nutrient flow also for the lower part of the neotissue cultivated inside the insert. The inserts were placed in a standard 24-well plate well for the culture period. The culture medium was poured in both the well and on the top of the tissue maturing in the insert. Scale bar = 6 mm.

After the equilibrium of the inserts, the medium was removed and 6 million freshly isolated bovine primary chondrocytes were seeded into each insert.

In the study I, the low-glucose DMEM (1 g/l glucose) medium was used. It was supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine and 50 µg/ml 2-Phospho-L-ascorbic acid trisodium salt with or without 100 µM glucosamine sulfate. The samples were cultured in a humidified incubator (5% CO_2, 37°C) at 20% or 5% O_2 atmosphere.

In the study II, the samples were cultured in the low glucose (1 g/l glucose) DMEM (low glucose), the high glucose (4.5 g/l glucose) DMEM (high glucose) or the hypertonic (4.5 g/l glucose, 390 mOsm) high glucose DMEM (hypertonic high glucose) supplemented with 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine and 50 µg/ml 2-Phospho-L-ascorbic acid trisodium salt with or without 10 ng/ml TGF-β_3. The supplementation of TGF-β_3 was transient for the first 3 weeks of culture. The samples were cultured in a humidified incubator (5% CO_2, 37°C) at 20% or 5% O_2 atmosphere.

Samples cultured in agarose gel wells (Study III)

For the study III, the agarose wells were prepared for the tissue culture. An agarose gel (1 %) was prepared in PBS. A round mold (d = 1 cm) was placed on a 6 cm plate, then 7 ml of hot gel in liquid phase was pipetted around the mold. The plate was allowed to gelate for 30 minutes in room temperature (**Figure 9**). The samples were cultured in the wells in the hypertonic high glucose DMEM in a humidified incubator (5% CO_2, 37°C) at 20% O_2 atmosphere.

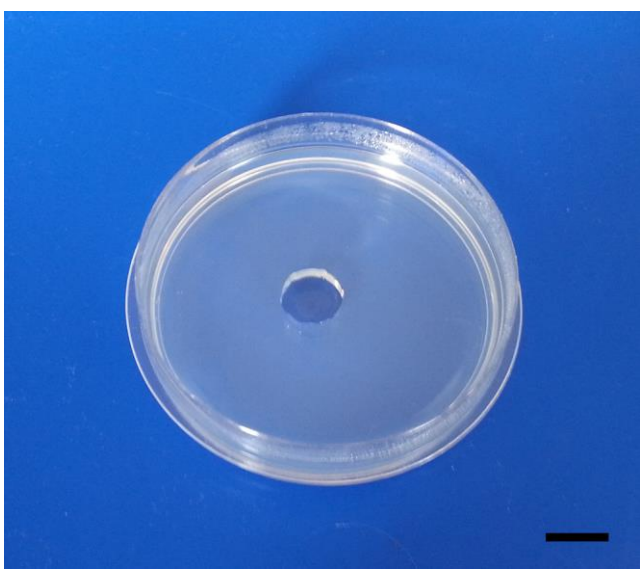

Figure 9 Agarose gel well used in the study III. Molten 1% agarose gel was poured around a mold placed in a 6 cm cell culture plate. After gelation, the mold was removed and the cells were pipetted into the well for neotissue formation with or without the scaffolds. Scale bar = 1 cm.

Sample collection and analyses

In the Study I, the samples were collected after 2, 4 or 6 weeks of culture, while culture period was 6 weeks for all the samples in the study II. In the study III, culture periods of 1, 3 and 6 weeks were used for the sample collections.

Histological stainings

The paraformaldehyde fixed tissues were dehydrated with ethanol and embedded in paraffin. Five μm thick sections were cut from the middle part of the tissues, and they were stained with toluidine blue or immunohistochemically for type II (studies I, II and III) and type I (Study I) collagen. The anti-type II collagen mouse monoclonal antibody (Holmdahl et al., 1986) was used for the staining of type II collagen and anti-type I collagen rabbit polyclonal antibody (Abcam) for collagen type I.

Proteoglycan extraction, quantitation and structure analysis

The PGs of the tissue were extracted in 50 mM sodium acetate buffer (pH 5.8) containing 4 M GuHCl, 10 mM sodium EDTA and 5 mM benzamidine-HCl at 4°C for 48 h. The PGs were then precipitated by addition of three-fold volume of 100 % ethanol, centrifuged and the pellets were washed with 75% ethanol. The dried pellet was then dissolved into sterile water. The GAG contents were analyzed from the solutions using dimethylmethylene blue analysis method (Farndale et al., 1986). The standards used in the analysis were made from shark chondroitin sulfate (Sigma). The absorbances were measured using a spectrophotometer at wavelength of 535 nm.

The PG subpopulations were separated using agarose gel electrophoresis. The precipitated PGs (5 μg) from each sample was dissolved into sample buffer (1% SDS in 1xTRIS, acetic acid, EDTA buffer) and boiled for 5 minutes. Dye solution (5 μl of 60% sucrose and 0.5% bromophenol blue in water) was added into the tubes, and the mixtures was pipetted in 1.2% agarose gel. Electrophoresis was performed for 3 h (50 mA, 35 V). The gels were fixed in methanol and stained with toluidine blue solution (1% toluidine blue in 3% acetic acid). The excess dye was removed using 3% acetic acid and the gels were scanned.

Gene expression analysis by qRT-PCR

Total RNA was extracted with EUROzol (Study I) or TRI-reagent (Studies II and III) according to manufacturers' instructions, and the RNA was

quantified using spectrophotometry (Nanodrop, Wilmington, DE, USA). The total RNAs were reverse transcribed into cDNAs using Absolute Max QRTase kit (ABgene, Epsom, Surrey, UK) (Study I) or Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) (Studies II and III). The reverse transcription was performed using MJ Research PTC-200 device (Waltham, MA, USA).

The RT-PCR was performed using 4 µl of cDNA and 11 µl of master mixture including Maxima SYBR Green/ROX Master Mix (Thermo Fisher Scientific, Surrey, UK), and forward and reverse primers for the house-keeping gene and the genes of interest (**Table 1**). MX3000P Real Time PCR system (Stratagene, La Jolla, CA, USA) was used for the analyses.

Table 1 The sequences and the concentrations of the primers used in the RT-qPCR analyses of the studies

Gene	**Primer sequence**	**Conc.**
RPLP0	*Forward*: CAACCCTGAAGTGCTTGACAT	300 nM
	Reverse: AGGCAGATGGATCAGCCA	
GAPDH	*Forward*: TTCAACGGCACAGTCAAGG	300 nM
	Reverse:ACATACTCAGCACCAGCATCAC	
Aggrecan	*Forward*: CACTGTTACCGCCACTTCCC	100 nM
	Reverse: GACATCGTTCCACTCGCCCT	
Procollagen α_1(II)	*Forward*: CATCCCACCCTCTCACAGTT	100 nM
	Reverse: GTCTCTGCCTTGACCCAAAG	
Procollagen α_2(I)	*Forward*: TGAGAGAGGGGTTGTTGGAC	300 nM
	Reverse: AGGTTCACCCTTCACACCTG	
Sox9	*Forward*: CATGAAGATGACCGACGAG	300 nM
	Reverse: GTTCTTGCTCGAGCCGTTGAC	

Tissue height

In the studies II and III, the tissue heights were calculated from the histological sections using a microscope (Carl Zeiss Axioimager M2, Thornwood, NY, USA). The heights were measured from three different sections from the same sample from three different sites. The averages of these were calculated and presented in column chart.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance and Bonferroni post hoc tests. The software used for the calculations was IBM SPSS Statistics.

Results

Low oxygen tension and glucosamine sulfate supplementation (Study I)

The oxygen tension is very low in the articular cartilage tissue *in vivo*. Previous study from our research group had shown that it could upregulate cartilage-specific gene expression responsible for the ECM production in the primary chondrocytes cultured in monolayers (Qu et al. 2009). Therefore, we wanted to investigate the effect of 5% O_2 on scaffold-free tissue cultures.

Glucosamine sulfate is widely used as a dietary supplement for the treatment of OA. Although our research group's previous studies with primary chondrocytes cultured in monolayers had shown no beneficial effects of glucosamine sulfate supplementation on the cartilage-specific gene expression (Qu et al., 2007; Qu et al., 2009), we still wanted to find out whether glucosamine sulfate supplementation in the long-term scaffold-free tissue cultures would improve the ECM composition and the quality of the manufactured tissue. Furthermore, we wanted to observe its effects on cartilage-specific genes and type I collagen.

Macroscopical appearance

All the manufactured tissues had a white, glassy, hyaline cartilage-like appearance (Study I, Fig. 1), yet they were quite unevenly shaped. Usually, the samples cultured at 20% O_2 were somewhat larger in size than the samples cultured at 5% O_2 atmosphere. There were no obvious differences in the macroscopical appearance between the glucosamine sulfate-treated and the control samples.

Histology

The histological sections showed that the thicknesses of the neotissues increased throughout the culture period, regardless of the O_2 tension (Study I, Fig. 2 a,b). The toluidine blue staining was more intense in the samples grown at 20% O_2 than at 5% O_2 (Study I, Fig. 2 c-f). Under 20% O_2 atmosphere, the staining intensity appeared stronger in the samples cultured without glucosamine sulfate supplementation (Study I, Fig. 2 c,e), while at 5% O_2 atmosphere, the difference was not that clear (Study I, Fig. 2 d,f)

Similarly, the staining for type II collagen was more intense in the samples grown at 20% O_2. Glucosamine sulfate did not produce any obvious differences between the samples. The staining for type I collagen was weak for all of the samples (Study I, Fig. 2 c-f). It was noticeable that all the samples, especially when grown at 5% O_2, had a weaker stainings in the middle part of the tissues (Study I, Fig 2).

GAG quantification and PG separation

The GAG contents appeared to be higher in the samples grown at 20% O_2 than at 5% O_2. However, the difference was not statistically significant (Study I, Fig. 3a). The agarose gel electrophoresis showed only slowly migrating bands, similar to those in native articular cartilage, in every sample analysed. There were no visible fast moving bands, representing the small PGs, such as decorin, biglycan and fibromodulin, in any sample (Study I, Fig. 3b).

Gene expression

The gene expression of procollagen α_1(II) in the samples grown at 20% O_2 was significantly higher than in the samples grown at 5% O_2 (Study I, Fig. 4b). There were no other statistically significant differences between the treatments in any other genes of interest, although there was a tendency to the higher gene expressions in the samples cultured at 20% O_2 than at 5% O_2 (Study I, Fig. 4).

TGF-β_3 supplementation, low oxygen tension, high glucose concentration and hypertonic culture medium (Study II)

In the study II, we continued our optimization further and took additional factors in to account. TGF-β_3 is a chondrogenic growth factor, which has an important role in the maturation process of the articular cartilage tissue. In the previous studies, it has been shown that especially a transient supplementation of this growth factor may have beneficial outcomes regarding the tissue quality (Byers et al., 2008; Lima et al., 2007). Similarly, the hypertonic culture medium has given some promising results concerning the cell numbers and the neotissue qualities (Oswald et al., 2008; Oswald et al., 2011; Jurgens et al., 2012).

Furthermore, we investigated the hypoxic conditions in the neotissue culture, since after all the low oxygen tension is native for the chondrocytes. Since the chondrocytes depend mainly on glycolysis in their metabolism, the high glucose medium was taken under investigation upon a hypothesis that the higher glucose concentration could ensure a proper diffusion of nutrients to the inside of the tissues and, thereby, improve the formation of the neotissue.

Macroscopical appearance and thickness of the tissues

After 6-week-culture periods, all the tissues resembled the hyaline cartilage in their macroscopical appearance. The tissues were white, smooth and glossy on the surface, yet they could be unevenly and irregularly shaped. Also, the samples grown at 20% O_2 and in the high glucose or the hypertonic high glucose mediums were often more harder and resilient, when cut during the sample collection.

Overall, the tissues grown in the high glucose and the hypertonic high glucose media were the thickest. The thicknesses of the neotissues were able to reach even the native tissue level. The samples cultured in the hypertonic high glucose at 20% O_2 without the growth factor supplementation were significantly thicker than those cultured in the low glucose (Study II, Fig. 2).

Histology

The neocartilage tissues generated in the high glucose and the hypertonic high glucose media at 20% O_2 without the TGF-β_3 supplementation showed the strongest toluidine blue staining, with some samples reaching almost the levels of native cartilage. At 5% O_2, as well as with low glucose medium at

20% O_2, the staining was rather weak in all of the samples, and there were more holes and empty areas in the histological sections. The staining was most evenly distributed in the samples cultured with the high glucose and the hypertonic high glucose media at 20% O_2 (Study II, Fig. 3a). The staining for type II collagen was strong in all the samples, while it was most evenly distributed in the samples cultured in the hypertonic high glucose at 20% O_2 (Study II, Fig. 3b).

TGF-β_3-supplemented samples showed also the strongest toluidine blue staining when cultured at 20% O_2 in the presence of the high glucose and the hypertonic high glucose medium (Study II, Fig. 4a). The staining was often as intense as in the native tissue. The staining was weaker for the samples cultured at 5% O_2, as well as for the samples cultured at 20% O_2 and the low glucose medium (Study II, Fig. 4a). Type II collagen staining in the samples cultured without TGF-β3 supplementation was similar to the samples cultured with TGF-β_3 supplementation. Hypertonic high glucose and high glucose samples at 20 % O_2 had the most even staining (Study II, Fig. 4b). In general, the samples cultured at 5% O_2 or with the low glucose medium at 20% O_2 stained weakest and showed more empty unstained areas inside the neotissues.

GAG quantification and PG separation

The DMMB analysis for the extracted PGs provided us with information of the total GAG-contents of the neotissues. The GAG content was highest in the samples, which were cultured in hypertonic high glucose media without supplementation of TGF-β_3. Furthermore, the lowest GAG levels were observed in the samples cultured in low glucose with the supplementation of TGF-β_3 at 5% O_2. The variation between the samples was relatively high, and no statistically significant differences were found (Study II, Fig 5a). Similarly to study I, only the slowly migrating bands corresponding to large proteoglycans were observed at the agarose gel electrophoresis (Study II, Fig 5b).

Gene expression

Aggrecan

Amongst the TGF-β_3-untreated samples the aggrecan gene expression was significantly lower in the high glucose medium at 20% O_2 when compared to the freshly isolated chondrocyte control samples. With the TGF-β_3 treatment, the samples cultured at 5% O_2 with the low glucose medium showed significantly higher expression of aggrecan than the high glucose or

the hypertonic high glucose samples cultured at the same oxygen tension. Furthermore, the aggrecan expression was significantly lower in the high glucose samples cultured at 20% O_2, and in the high glucose and the hypertonic high glucose samples cultured at 5% O_2 when compared to control (Study II, Fig. 6a).

Sox9

The low glucose sample without the TGF-β_3 supplementation at 5% O_2 had significantly higher expression of the Sox9 in comparison of the high glucose sample cultured at 5% O_2. Furthermore, all the samples had significantly lower expression of Sox9 compared to freshly isolated control chondrocytes except for the TGF-β_3-supplemented low glucose samples cultured at both 20% and 5% O_2 (Study II, Fig. 6b).

Procollagen α_1(II)

The samples cultured at 20% O_2 with the hypertonic high glucose medium without TGF-β_3 supplementation had significantly higher expression of the procollagen α_1(II) than the samples cultured with the low glucose or the high glucose media under same oxygen tension. Also, the hypertonic high glucose 20% O_2 sample with the TGF-β_3 supplementation showed significantly higher expression than the high glucose 20% O_2 sample with the TGF-β_3 supplementation. Furthermore, the hypertonic high glucose 20% O_2 samples with or without TGF-β_3 supplementation were the only ones that did not have significantly lower expression of the procollagen α_1 (II) when compared to the control (Study II, Fig. 7a).

Procollagen α_2(I)

Without the TGF-β_3 supplementation, the hypertonic high glucose sample cultured at 20% O_2 expressed the procollagen α_2(I) in a significantly higher level than the control or the low glucose samples at 20% O_2. Within the TGF-β_3-supplemented samples, the high glucose sample at 20% O_2 had significantly higher expression than low glucose at 20% O_2. Also, the high glucose and the hypertonic high glucose samples at 20% O_2 had significantly higher expression of the procollagen α_2(I) when compared to control (Study II, Fig. 7b).

Comparison of scaffold-free system to HyStem™ and HydroMatrix™ scaffolds (Study III)

Researchers in the field of regenerative medicine have shown a great interest towards various scaffolding biomaterials for tissue engineering purposes. High water content of articular cartilage makes especially hydrogel-based scaffolds interesting for the cartilage tissue engineering. In the study III, we compared two different commercial hydrogel scaffold materials, HyStem™ and HydroMatrix™, with the scaffold-free culture method.

We hypothesized that the scaffold materials, especially the HA-based HyStem™, would provide the tissues with a better support already from the beginning of the culture, thus providing the tissues with better quality and more regularly shaped neotissues. We also wanted to improve our culture method from commercial inserts to agarose gel wells, which would provide a better nutrient and oxygen flow for the neotissues.

Macroscopical appearance and thickness of the neotissues

The tissues generated scaffold-free and in the HyStem™ scaffold were most often regularly shaped, whereas with the HydroMatrix™ scaffold grown neotissues had more often irregular shapes. The scaffold-free-generated tissues were usually the largest in size (Study III, Fig. 2a). Overall, the tissues generated in the study III, had a more regular, disc-like shape when compared to previous two studies.

Thicknesses of the neotissues were comparable to the thickness of bovine native femoropatellar articular cartilage tissue. The chondrocytes embedded in the HydroMatrix™ seemed usually to produce a significantly thinner tissue than the scaffold-free or HyStem™ scaffold grown equivalents (Study III, Fig. 2b).

Histology

Toluidine blue staining revealed no distinct differences on the tissues generated between the scaffold-free controls and the HyStem™ or the HydroMatrix™ scaffolds. Intensity of the staining was high even after one week of culture, and it became even more intense as the culture went on. The stain was evenly distributed even though there still were some empty and unstained areas. The staining levels were comparable to native cartilage tissues (Study III, Fig. 3a).

The staining for type II collagen was similar to toluidine blue staining. There were no distinctive differences between the samples grown as scaffold-free constructs and the two different scaffold tissues. The type II collagen staining intensities were also comparable to native articular cartilage (Study III, Fig. 3b).

GAG quantification and separation of PGs

The GAG content rose in all of the samples throughout the culture period. There were no statistically significant differences between the tissues generated scaffold-free and in the scaffolds, but after 6 weeks of culture the GAG -contents reached the level of native articular cartilage (Study III, Fig. 4a). Agarose gel electrophoresis visualized the same results as in the previous studies: all the samples showed only slowly migrating bands (Study III, Fig 4b).

Gene expression

There were no statistically significant differences in the expressions of the cartilage specific genes [aggrecan, procollagen α_1(II) and Sox9] between the tissues generated in scaffold-free and scaffolds even though their expressions were slightly down-regulated over time (Study III, Fig. 5a-c).

The expression of procollagen α_2(I) was highly upregulated after 1 week of culture, and the expression was the highest at 3 week time point compared to the other time points. At 3-week culture time point, the HyStem™ sample had significantly higher expression level of the procollagen α_2(I) than control, the HyStem™ or the HydroMatrix™ samples at 1 week time point (Study III, Fig. 5d).

Discussion

Low oxygen tension was not beneficial for the cartilage formation by the primary chondrocytes

The chondrocytes in the articular cartilage live under specific hypoxic conditions in tissue, which also has a high turgor pressure in a hypertonic environment. Therefore, it was hypothesized that the low oxygen atmosphere would be beneficial for the production of ECM in the manufactured tissues. However, it was noticeable in the first two studies (Studies I and II) that the histological sections cut from the neotissues manufactured at 5% oxygen tension showed much lighter staining for the PGs and the type II collagen, especially in the middle parts of the tissues. These neotissues had also more and larger necrotic areas than their equivalents grown at 20% O_2.

The culture medium with the low glucose concentration often had similar effects on the neotissues' microscopical appearance as did the 5% O_2. The tissues formed in the low glucose medium were thinner than the tissues produced with the high glucose and the hypertonic high glucose media, which reached nearly the height of native tissue (Study II). Thus, it seems that the low glucose concentration and the 5% oxygen tension restricted the ECM production and the growth of the tissues. Heywood et al showed that the oxygen consumption of isolated chondrocytes is dependant on glucose concentration of the culture medium. They noticed that when 3D cell-agarose constructs were cultured in low glucose medium, the oxygen consumption increased dramatically (Heywood et al., 2006b). In our studies, the cells were cultured in both low glucose culture medium and low oxygen tension which means the chondrocytes had very low concentrations of both of the very important molecules needed in their metabolism. When the low glucose apparfently increased their oxygen consumption rate, they were not able to get sufficient amount of oxygen for their metabolism because the oxygen tension was so low in the culture environment. This has definitely affected the ECM production of the chondrocytes. If the cells lack the molecules for keeping up their normal metabolism they definitely have trouble producing the massive hyaline cartilage ECM.

It was also interesting that even though the 5% O_2 and the low glucose medium produced poorer looking neotissue than those cultivated at the higher glucose concentration and the 20% oxygen tension, their expressions of the cartilage specific genes (Sox9, aggrecan) was often higher, while the expression of procollagen α_2(I) was much lower (Study II). This information

could be useful in the future studies. In Study III, when the hypertonic high glucose medium was used in a combination with 20% O_2 the expression of the cartilage-specific genes dropped during 6-week-culture period, and the procollagen α_2(I) was highly upregulated after 1 week of culture.

Our results on the use of low oxygen tension did not improve the formation of neocartilage, which was against the hypothesis based on the conclusions made from 2D-culture experiments, which induced the aggrecan and procollagen α_1(II) gene transcription (Qu et al 2009). When the chondrocytes are seeded on 3D-culture systems, which do not have the proper ECM, they can be assumed to have a need for rather fast metabolism and oxygen consumption at the beginning of the culture, which should balance upon further cultivation. Therefore, it would be very interesting to test what would happen to the cartilage-specific gene and procollagen α_2(I) expression levels, if the cells were first cultured in the glucose- and oxygen-rich hypertonic environment for few days or up to one week, and then the oxygen tension and the glucose concentration would gradually be dropped to the native tissue level. Our results suggest that this kind of set up might improve the gene expression profiles in the neotissues.

Glucosamine sulfate and TGF-β_3-supplementations did not improve the cartilage tissue quality

In the study I, the glucosamine sulfate supplementation seemed to have neither harmful nor beneficial effect on the neotissue formation and the ECM production. One could not observe any difference in the histological sections cut from the neotissues, neither was there any difference in the staining for the PGs or the type II collagen. The gene expression data showed that supplementation of the tissue cultures with glucosamine sulfate rather had negative effects on the cartilage formation. This also raises a question how useful can dietary supplementation of glucosamine sulfate be if this high concentration given directly to neotissue cultures has no effect. The previous research done with monolayers showed no more encouraging results(Qu et al., 2007; Qu et al., 2009).

TGF-β_3-supplementation had similarly no effect on the cartilage tissue quality in the study II. Previously, it has been shown that the TGFs may increase the ECM production and stimulate the chondrocyte proliferation (Zimber et al., 1995; de Haart et al., 1999; Richmon et al., 2005). Previous studies have also shown that especially transient supplementation of TGF-β_3 can improve the mechanical properties and the PG content of the neotissues

(Byers et al., 2008). These results were achieved using primary chondrocytes from young calves, though. Our results did not show any distinctive benefit in the ECM production in the TGF-β_3-supplemented samples versus the non-supplemented equivalents. This is rather surprising compared to the previous results by other groups. The cells used in our studies were obtained from older animals, which may have affected the outcome.

Hypertonic high glucose medium provided the best ECM production

The results of the second study (Study II) suggest that the hypertonic high glucose culture medium used under 20% O_2 helps the primary chondrocytes to produce the best possible neotissue out of the tested environmental combinations. The histological sections showed the most intensive staining for the PGs and the type II collagen under these conditions regardless of TGF-β_3 supplementation. Furthermore, the presence of 20% O_2 and the hypertonic high glucose medium significantly upregulated the expression of procollagen α_1(II). Thus, it seems that higher glucose concentration and higher oxygen tension improve the cellular viability inside the tissue and boost their metabolism.

This information is supported by the results of some previous researches. It has been shown that hypertonic culture medium can have significant effects on the ECM production of the cells and, therefore, on the tissue quality of the manufactured tissue (Negoro et al., 2008; Xu et al., 2010; Lee et al., 2014b). For example, when the chondrocytes were cultured in alginate beads under 380 or 370 mOsm, they produced the highest amount of the GAGs (Negoro et al., 2008; Xu et al., 2010).

Scaffold-free culture method produced similar neotissues as the methods using HyStem™and HydroMatrix™ scaffolds

In study III, we found out that there were basically no significant differences between the neotissues produced as scaffold-free and scaffold-containing constructs. The chondrocytes cultured as scaffold-free, or embedded in the HyStem™ or the HydroMatrix™ scaffolds, all produced high amounts of the ECM with good PG content and intensive stainings for the type II collagen. The PG content in each sample increased over time, reaching almost the native level after 6 weeks of culture.

To our knowledge, this is the first study where these two scaffold materials have been compared with the scaffold-free culture method. Based on these results, it looks like the scaffold may not give any distinctive advantage over the scaffold-free method when neotissues are produced from the primary chondrocytes *in vitro*. It would have been difficult to distinguish the three different types of tissue constructs from each other macroscopically or microscopically. However, the HydroMatrix™ produced most often the most irregularly shaped tissues compared to the scaffold-free control or the HyStem™. This is likely due to the scaffolds gelation procedure, which required very careful addition of culture medium on top of the cell-HydroMatrix™ suspension. This was quite hard to do in practice, and oftentimes it resulted in distorted shape of the scaffolds.

Although the scaffold materials did not provide clear advantage for the cartilage tissue formation, the scaffold materials could be helpful *in vivo*, if lower cell densities must be applied. In this study, we used a rather high cell density of 6 million chondrocytes, which may be difficult to achieve in real life applications at the moment. When there are less cells to spare, the scaffold material properties would probably have a higher impact on the tissue strength. This is especially important during the mechanical loading aimed on the repaired cartilage during a patient's recovery. Furthermore, the HyStem™ could help the neotissues to keep their shape. Increasing the amount of the cross linking agent results in higher amount of cross-links between the hyaluronan molecules. This would result in more rigid hydrogel which in turn could help the neotissues to retain their initial shape.

Agarose gel well system seemed to improve neotissue quality

In this project, we used both plastic inserts and agarose wells for the chondrocyte seeding and further cultivation. When histological sections and macroscopical shape of the neotissues manufactured in these two different systems are compared, the agarose gel wells appeared to improve the control on the tissue shape and viability. This can be partially explained as an outcome of the improved nutrient flow from the lateral sides of the agarose wells' walls. The staining for both the PGs and the type II collagen were more evenly distributed throughout the tissues there. In the studies I and II, the plastic inserts as a culture vessel were obviously not able to provide the forming tissues with a proper nutrient flow. The agarose gel wells increased the surface area in contact with the culture medium and, seemed to improve

the ECM production of the chondrocytes, when compared to the plastic inserts.

The agarose gel wells have been successfully used also before our study. Professor Athanasious's group has used this type of the culture method in a number of studies, and they have also been able to produce good quality hyaline tissues (Hu and Athanasiou, 2006a; Hu and Athanasiou, 2006b; Ofek et al., 2008; Eleswarapu and Athanasiou, 2013). An advantage in the agarose gel-based method is also its modifiability. Basically, it is possible to prepare wells of any size and shape. Eventually this means that even though the lesion in the joint would be irregularly shaped, the agarose well could be adjusted accordingly, and one could grow a patch of neotissue *in vitro* exactly fitting the defect.

This culture method may also be further improved in the future by chancing the cell culture plates into tubes. The agarose gel lifts the surface of culture medium quite high, which in turn might increase the risk of contamination during the culture. If a tube would be used it would not only lower the risk of contamination, but also increase the nutrient and oxygen flow when medium could access the tissue from below. Also by increasing the height of the medium path, the hydrostatic pressure could be increased, which as a mechanical load factor could be advantageous for the formation of the cartilage tissue.

Conclusions

In this project, we aimed at developing better methods for the neocartilage production using the primary chondrocytes. In studies I and II, the culture environments were optimized, and in study III these optimized culture conditions were used to investigate whether the use of the hydrogel scaffolds and the agarose gel wells could further improve the quality of the tissues produced by tissue engineering.

The main findings in this thesis research were:

1. The low oxygen tension and the low glucose concentration in the culture medium did not improve the quality of the cartilage neotissue produced by the primary chondrocytes in insert culture system.

2. The glucosamine sulfate and the transient TGF-β_3 supplementations were not helpful nor harmful for the cartilage neotissue formation by the primary chondrocytes in insert culture system.

3. The hypertonic culture medium with the high glucose concentration proved to be beneficial for the neocartilage formation.

4. The scaffold-free chondrocyte culture method produced similar neocartilage as cultivation of the chondrocytes embedded within the HyStem™ or the HydroMatrix™ scaffolds.

5. The use of the agarose gel wells seemed to improve the neotissue quality

This thesis project provided useful information for the production of the hyaline cartilage-like neotissues using the primary bovine chondrocytes. We were able to improve our scaffold-free culture method, and we proved that the tissue quality was not inferior to those tissues taking advantage of the chondrocytes embedded within the hydrogel scaffolds.

The future challenges are how to improve the tissue quality even more, and how to make them to structurally resemble more the native hyaline cartilage. Now the neotissues had good PG and collagen contents. However, the organization of these molecules was not similar to the native cartilage. The neotissues lacked the zonal organization typical to the hyaline cartilage.

One possible solution to this could be the addition of several different signals to guide the cell behavior better. Introductions of mechanical stimuli and some chemical signals [lysyl oxidase (LOX), 4αPDD] have already proven to be helpful. Addition of 4αPDD can improve the tensile stiffness of the neotissues via activation of transient receptor potential vanilloid 4 (TRPV4) channels (Ca^{2+} -permeable cell membrane protein) (Eleswarapu and Athanasiou, 2013; Lee et al., 2014b). It has been shown that hypoxia upregulates LOX expression which in turn increases the amount of pyridinoline crosslinks between the collagen fibers enhancing the neotissues mechanical properties (Makris et al., 2013). Also the culture vessel can be improved, or even better, a tailored bioreactor could be introduced. Furthermore, the proper timing of introducing lower oxygen tension, lower glucose concentration and other signaling factor can also prove to be the key in guiding the chondrocytes to a more favorable direction.

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