Healing Cartilage
-Aspects on Regenerative Methods

Lars Enochson

UNIVERSITY OF GOTHENBURG

Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg

2013

Cover illustration: *The Road Goes Ever On*

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lars.enochson@gu.se, lars.enochson@gmail.com

Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Sahlgrenska Academy
University of Gothenburg

Correspondence:
Lars Enochson
Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Bruna Stråket 16
SE-413 45 Göteborg
Sweden

ISBN 978-91-628-8850-3

Printed in Gothenburg, Sweden 2013
Ineko AB, Gothenburg

“It's a dangerous business, Frodo, going out your door.
You step onto the road, and if you don't keep your feet,
there's no knowing where you might be swept off to.”

J. R. R. Tolkien, The Lord of the Rings

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ABSTRACT

Articular cartilage has poor intrinsic capacity to heal and defects can cause severe pain for the patient. If the healing process is not assisted the damage might deteriorate and lead to the onset of osteoarthritis. Autologous chondrocyte implantation is a successful method in treating focal cartilage defects, with good clinical outcome. Low cellularity of the tissue and low proliferative capacity of the chondrocytes are limitations to the treatment.

The aim of the present thesis was to improve assisted articular cartilage healing and to evaluate how an eventual osteoarthritis progression could be halted. In particular, we investigated how anabolic chondrogenic processes in chondrocytes and chondrocyte derived induced pluripotent stem cells can be improved, thereby optimising the use of autologous cells in articular cartilage regenerative therapies and methods. Further, we studied if the application of plasma-mediated ablation can induce an anabolic response in the chondrocytes. Finally, we investigated how GDF5 signalling, a pathway implemented in the development of osteoarthritis, affects cartilage homeostasis.

The results indicated that plasma-mediated ablation induces an anabolic response in chondrocytes. ECM production by the chondrocytes was improved by optimizing the standard chondrogenic medium through the use of factorial design of experiments. We were able to demonstrate that GDF5 can contribute to the redifferentiation process, and has potential in inhibiting degenerative processes in the cells. Finally, the reprogramming of chondrocytes into induced pluripotent stem cells showed that these cells could be useful tools in the determination of cell signalling pathways in tissue regeneration and disease.

In conclusion, the methods investigated in this thesis can be used to improve the regenerative capacity of the articular chondrocytes and the thesis sheds further light on the intricate problems of healing cartilage.

Keywords: Cartilage, regeneration, osteoarthritis, induced pluripotent stem cells, factorial design, growth factors

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Det artikulära brosket är en bindväv som täcker benens ledytor och fungerar som en stötdämpare när vi rör oss. Den förser dessutom leden med en glatt yta som underlättar själva rörelsen. Broskvävnaden saknar blodkärl och om den skadas läker den inte automatiskt som andra vävnader. På grund av detta måste läkeprocessen stödjas och görs inte det är risken stor att skadan förvärras och broskytan nöts sönder. Ignoreras skadan är risken också stor att ledsjukdomen osteoartros initieras, vilket är en degenerativ sjukdom mot vilken det saknas botemedel. Autolog broskcellstransplantation (eng. ACI) är en av de få funktionella metoder med vilken man kan restaurera avgränsade skador i broskytan. I metoden används patientens egna broskceller för att läka skadan. Cellerna tas ut från en lågt belastad del av patientens brosk, odlas i en laboratoriemiljö och transplanteras därefter åter in i patienten i skadan. Cellerna bygger då upp ny vävnad och fyller ut skadan. Metoden har visat på goda kliniska resultat med fler än 35 000 patienter behandlade över hela världen. Nackdelarna med metoden innefattar låg tillgång på celler och att dessa celler får en förändrad broskbildningsförmåga under odlingsprocessen.

Syftet med denna avhandling har varit utveckling av metoder för att läka broskskador, samt att studera hur en osteoartrosprocess kan motverkas. Vi undersökte hur broskbildningen av kondrocyter och kondrocyter som omprogrammerats till stamceller (s.k. iPS-celler) kunde påverkas för att förbättra deras användning i en eventuell klinisk tillämpning. Statistisk försöksplanering användes för att optimera det odlingsmedium som används för att inducera broskbildning, och cellerna stimulerades med olika tillväxtfaktorer och ett elektriskt plasma i samma ändamål. Vi undersökte vidare hur en specifik faktor, Growth and Differentiation Factor 5 (GDF5) kan påverka en osteoartrosprocess i kondrocyterna.

Med de metoder som användes kunde vi inducera en förbättrad broskbildning med en ökad produktion av extracellulär matris hos både kondrocyter och iPS-celler. Vi kunde dessutom med hjälp av GDF5-stimulering inhibera en cellsignaleringsväg som är aktiverad i osteoartros och som bidrar till nedbrytningen av vävnad. Det sistnämnda är en indikation på att GDF5 skulle kunna vara en möjlig kandidat för en medicin för att bota, eller åtminstone bromsa, ett osteoartrosförlopp.

Sammantaget har vi i denna avhandling visat på hur broskcellernas regenerativa förmåga kan förbättras och den belyser vidare de intrikata problem man stöter på när man försöker läka brosk.

LIST OF PAPERS

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

I. Enochson L, Sönnergren H, Mandalia V, Lindahl A. (2012) Bipolar radiofrequency plasma ablation induces proliferation and alters cytokine expression in human articular cartilage chondrocytes. *Arthroscopy*. 28(9), 1275-1282

II. Enochson L, Brittberg M, Lindahl A. (2012) Optimization of a chondrogenic medium through the use of factorial design of experiments. *Biores Open Access*. 1(6), 306-313

III. Enochson L, Stenberg J, Brittberg M, Lindahl A. GDF5 reduces MMP13 expression in human chondrocytes via DKK1 mediated canonical Wnt signalling inhibition. (*Submitted, Osteoarthritis and Cartilage*)

IV. Boreström C, Simonsson S, Enochson L, Bigdeli N, Brantsing C, Ellerström C, Hyllner J, Lindahl A. Footprint free human iPSCs from articular cartilage with redifferentiation capacity – a step towards a clinical grade cell source (*Submitted, Stem Cells Transl Med*)

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ABBREVIATIONS

3D	Three-dimensional
ACAN	Aggrecan
ACI	Autologous chondrocyte implantation
ACT	Autologous chondrocyte transplantation
ADAMTS	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif
ADAMTS-4	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif 4
ADAMTS-5	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif 5
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APMA	*p*-aminophenylmercuric acetate
ASC	Ascorbic acid
AT	Adenine-Thymine
BDM	Basic differentiation medium
BMP	Bone morphogenetic protein
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BMP14	Bone morphogenetic protein 14
BMPRI	Bone morphogenetic protein receptor 1
BMPRII	Bone morphogenetic protein receptor 2
BSA	Bovine serum albumin
CDH1	Cadherin 1
CDMP1	Cartilage derived matrix protein 1
cDNA	Complementary deoxyribonucleic acid
c-iPSC	Chondrocyte-derived induced pluripotent stem cell
C-MYC	Avian myelocytomatosis viral oncogene homolog
COL1	Collagen, type 1
COL1A1	Collagen, type 1, alpha 1
COL2	Collagen, type 2
COL2A1	Collagen, type 2, alpha 1
COL2A1, type A	Collagen, type 2, alpha 1, type A
COL2A1, type B	Collagen, type 2, alpha 1, type B
COL10A1	Collagen, type 10, alpha 1
COMP	Cartilage oligomeric matrix protein
CREBBP	CREB binding protein
CS	Chondroitin sulphate

Ct	Cycle threshold
DAPI	4’,6-diamidino-2-phenyldole
DEX	Dexamethasone
DKK1	Dickkopf Wnt signalling pathway inhibitor 1
DMEM	Dulbecco’s modified eagle medium
DMEM-HG	High-glucose Dulbecco’s modified eagle medium
DMMB	1,9-dimethylmethylene blue
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DoE	Design of experiments
DTT	Dithiothreitol
EB	Embryoid body
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESCs	Embryonic stem cells
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Fluorescein diacetate
FGF2	Basic fibroblast growth factor
f-iPSC	Fibroblast-derived induced pluripotent stem cell
FOSL1	FOS-like antigen 1
FRZB	Frizzled related protein
FZD	Frizzled
GAG	Glycosaminoglycan
GDF5	Growth and differentiation factor 5
GLU	Glucose
GSC	Goosecoid homeobox
GSK3β	Glycogen synthase kinase 3β
HA	Hyaluronic acid
HMGB1	High mobility group box 1
HNF3β	Hepatocyte nuclear factor, forkhead box A2
HSA	Human serum albumin
hEL	Human embryonic lung fibroblast
hESC	Human embryonic stem cells
hrbFGF	Human recombinant basic fibroblast growth factor
HRP	Horseradish peroxidase
HuWIL	Human foreskin fibroblast
IHC	Immunohistochemistry
IL1β	Interleukin 1beta
IL6	Interleukin 6
IL8	Interleukin 8

ITS	Insulin-transferrin-selenium
iPSCs	Induced pluripotent stem cells
JNK	c-Jun NH(2)-terminal kinase
kDa	Kilo Dalton
KLF4	Kruppel-like factor 4
KS	Keratan sulphate
LEF	Lymphoid enhancer factor
LIN	Linoleic acid
LIN28	Lin-28 homolog
LP	Link protein
LRP 5/6	Low-density lipoprotein receptor related protein 5 and 6
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
MIXL1	Mix paired-like homeobox
MMP	Matrix metalloprotease
MMP13	Matrix metalloprotease 13
MSC	Mesenchymal stem cells
MSD	Mechanical shaver debridement
mRNA	Messenger ribonucleic acid
NANOG	Nanog homeobox
NEAA	Non-essential amino acids
NuFF	Neonatal human foreskin fibroblasts
NT4	Neurotrophin4
OA	Osteoarthritis
OCT4	Octamer-binding transcription factor 4
OSKM	OCT4, SOX2, KLF4, cMYC transfection mix
OSKML	OCT4, SOX2, KLF4, cMYC, LIN28 transfection mix
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	Population doublings
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide
PE/ST	Penicillin-Streptomycin
PG	Proteoglycan
PI	Propidium iodide
PPARD	Peroxisome proliferator-activated receptor delta
PPIA	Peptidylprolyl isomerase A/Cyclophilin A
PTOA	Post-traumatic osteoarthritis
qRT-PCR	Quantitaive real-time polymerase chain reaction
RS	Response surface
RSM	Response surface modelling
RIPA	Radio-immunoprecipitation assay

RNA	Ribonucleic acid
ROCK	Rho-associated kinase
SEM	Scanning electron microscope
SMAD	Mothers against decapentaplegic homolog
SNP	Single nucleotide polymorphism
SOX2	Sex determining region Y-box 2
SOX6	Sex determining region Y-box 6
SOX9	Sex determining region Y-box 9
SRY	Sex-determining region on the Y chromosome
TCF	T cell factor
TGFβ1	Transforming growth factor β1
TGFβ3	Transforming growth factor β3
TIMP1	Tissue inhibitor of metalloproteinase 1
TIMP3	Tissue inhibitor of metalloproteinase 3
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	Tumour necrosis factor α
TSA	Tyramide signalling amplification
UTR	Untranslated region
UV	Ultraviolet
VCAN	Versican
WNT3A	Wingless type 3a
WNT9A	Wingless type 9a

1 INTRODUCTION

Joint pain is a troublesome thing. It is one of the most common causes of disability around the world[1] and the disability and dependence it implies is a huge burden on society, raising healthcare expenditure and causing loss of work[1]. In the middle of this, at the centre of attention, is the most intricate and exciting tissue of the joint, or to some of us, the whole body: the articular cartilage.

1.1 Role of articular cartilage

Cartilage is a connective tissue, which together with bone forms the skeleton. There are three different types of cartilage in the human body; hyaline cartilage, fibrous cartilage and elastic cartilage and out of these, hyaline cartilage is the most abundant[2]. The name hyaline comes from the Greek word hyalinos, meaning in glass, and it is not hard to understand why as the hyaline cartilage has a glossy, almost translucent pearly-white colour. When cut with a scalpel, the texture resembles that of water chestnut, although harder. The composition of the tissue appears simple at a first glance as it lacks both vascularisation and innervation, and only one single cell type harbours in it[3]. The opposite is, however, soon revealed. When found at the interface between the bony surfaces in the articulating synovial joints, such as the knee, hyaline cartilage is referred to as articular cartilage. There, it covers the ends of long bones and acts as a shock absorber and provides a wear-proof surface for the articulating motion. Due to high water content and a complex interconnected network of collagen fibres in the extracellular matrix (ECM), it has a unique ability to withstand high physical forces and still be very elastic[4,5].

1.2 The chondrocyte

The cells in cartilage are called chondrocytes, and they are solely responsible for the maintenance of the tissue. The number and size of chondrocytes vary with the different regions in the cartilage. The main population are rounded or polygonal in shape, and the cells diverging from this are located at the surface of the tissue and at the tissue boundaries, where they instead are elongated and flattened[2]. The chondrocytes exist singly or in groups within small cavities in the ECM called lacunae. These lacunae are fluid filled basket-like structures that are believed to dampen mechanical, osmotic and physicochemical changes during dynamic loading, thereby protecting the cells[5]. There are long distances between the lacunae, and the chondrocytes only comprise 2-10% of the tissue volume, making the tissue relatively

acellular[5,6]. The cells express several integrins, including annexin V and anchorin CII, through which they attach to the surrounding ECM. An important key receptor on the ell surface is the CD44 receptor, which binds to hyaluronic acid, an essential structural feature of the tissue [7,8]. As there is no vascularization in the tissue, the chondrocytes rely on diffusion for the access to nutrients and for the removal of waste[6]. This lack of blood flow also results in an environment with low oxygen tension, where the partial pressure ranges from ~10% at the surface to levels below ~1% near the subchondral bone[6,9].

1.3 The extracellular matrix

The ECM upholds the structural integrity and mechanical strength of the articular cartilage and is composed of two major building blocks: collagen fibres and proteoglycan (PG) molecules (Fig. 1). A number of collagens are present in the matrix, including collagens I, II, VI, IX, X and XI[7]. The collagens are formed from three polypeptide-chains that coil around each other forming twisted super-helix conformations. Some of these further assemble into rigid fibrillar structures with high tensile strengths. Of all the collagens in the tissue, collagen II is the most abundant, forming the bulk of the extensive collagen fibril network[7]. Collagen II fibrils can be found in two splicing variants that are differentially expressed during development: type IIA and type IIB. Type IIA is mainly expressed in pre-chondrocytes, and the expression later changes into mainly type IIB as the chondrocytes mature[10]. Collagen types IX (fibril associated collagen that does not form fibres) and XI are present to a minor extent, having supportive and growth-limiting function to the collagen II fibrils[11,12]. The role of collagen VI is largely unknown, although it is believed to be an anchoring point for the chondrocyte to its surrounding ECM[13]. Collagen X is produced in the deep hypertrophic zone of the articular cartilage, supporting and regulating the calcification procedure, and collagen I is mainly found in the uppermost parts of the tissue[7,11,14]. The cartilage oligomeric matrix protein (COMP) supports these collagen structures. It is a five-armed bouquet-shaped molecule which has binding regions to both collagen I, II and IX and is believed to play a role in fibril formation and maintenance of the cartilage ECM[15].

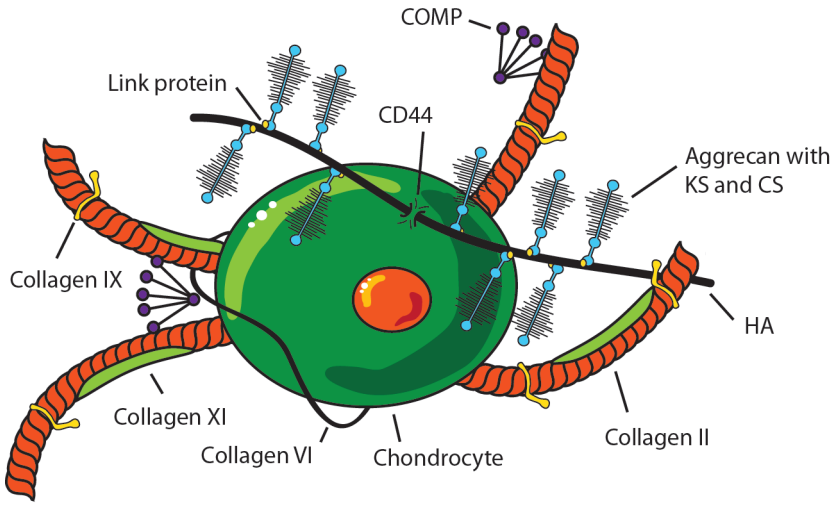

Figure 1. An illustration of a chondrocyte and the surrounding ECM with collagens, proteoglycans and support molecules[16].

The second major building block of the articular cartilage is the proteoglycans, which consist of a protein core with one or more glycosaminoglycan (GAG) polysaccharides or oligosaccharides attached. A variety of these molecules exist in the tissue, and aggrecan is the most abundant. It has a protein backbone with three globular domains with an outstretched region between domain 2 and 3. This region has binding sites for the covalent attachment of the highly negatively charged GAGs keratan sulphate (KS) and chondroitin sulphate (CS)[17]. Together, these molecules form a bottlebrush structure with high water retention abilities. The first globular domain of the aggrecan backbone also has an attachment region for hyaluronic acid (HA) and the small glycoprotein link protein (LP), and large aggregated proteoglycan complexes are formed through the binding of several aggrecan molecules to one large HA molecule. HA is thereby a key component of the ECM, as it provides a backbone for these large complexes, as well as providing attachment regions for the chondrocytes through the cells CD44 receptors[5]. LP binds both aggrecan and HA, stabilizing the interaction between the two, contributing to the stability of the cartilage[5]. The tissue also contains several small leucine-rich repeat PGs, including decorin, biglycan, fibromodulin and lumican[2]. These PGs have several roles in the tissue. They regulate collagen fibril diameter and fibril-fibril interactions, and they protect the collagens from proteolytic degradation. They also bind to growth factors, and it is believed that they have a role of retaining growth factors in the cartilage tissue[2,18].

The excellent load bearing properties of hyaline cartilage comes from the combination of properties of PGs and the collagens. The negative charges of KS and CS make the molecules strongly hydrophilic and in combination with their non-ideal nature, they can reach high swelling pressures[19]. The collagens have very high tensile strengths and the cross-linked network of collagens restrict swelling of the PGs[7]. The high swelling pressure resulting from the combination of the PGs and collagens is thereby the reason for the special compressive properties of the cartilage, which are essential for joint articulation and toughness[7,19].

1.4 Structure of articular cartilage

Articular cartilage can be divided into four layers (Fig. 2). The topmost layer is called the superficial zone, and comprises 10-20% of the tissue thickness. This zone is responsible for most of the tensile properties of the cartilage. The cell density is at its highest, and the cells have a flattened and elongated morphology[7]. They produce an oily fluid called lubricin that lubricates the cartilage surface, allowing for near frictionless motion between the cartilage surfaces in the joint[4]. The cells are surrounded by a close knit web of ordered thin collagen I fibrils that run parallel to the surface and to each other, thereby providing a mesh with high tensile properties[5,7]. The combination of these properties allows for good resistance against the shear forces imposed by the articulating motion of the joint[20].

Below the superficial zone, representing 40%-60% of the tissue volume, the middle zone has an increasing amount of proteoglycans. The collagen fibres have an isotropic distribution, are thicker and mainly consist of collagen II. This matrix composition has intermediate properties between resisting shear forces and compressive forces. Cell density is low and the cells have a spherical shape, considered typical for articular cartilage[7,21].

Further down, approaching the subchondral bone, the cell density is even lower and the collagen fibrils are thicker and arranged perpendicular from the bone. The amount of proteoglycans is at the highest percentage of the whole tissue, and the compressive resistance is also the highest. This zone is referred to as the deep zone, and comprises approximately 30% of the cartilage volume[7,20,21].

Closest to the bone a partly calcified layer contains larger chondrocytes that have entered a hypertrophic state, in which the collagen expression profile switches from collagen II to collagen X. The tissue has intermediate mechanical properties between those of the uncalcified cartilage and those of the subchondral bone[7,14,22].

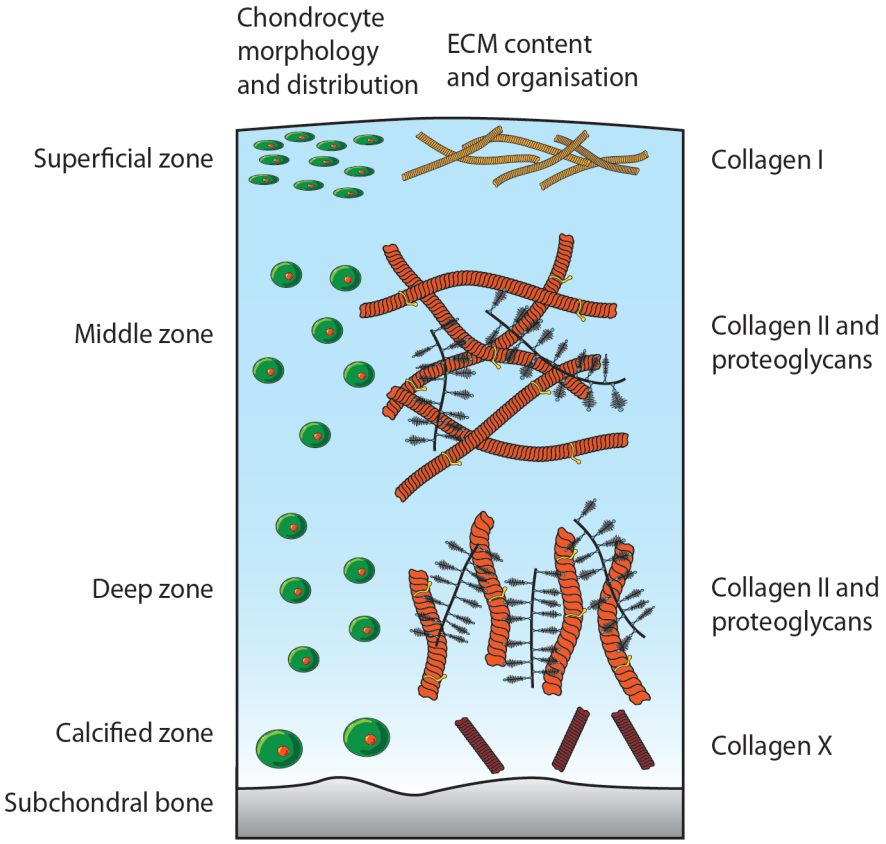

Figure 2. Representation of the general structure of articular cartilage, from the articular surface down to the subchondral bone. (Modified and reprinted with permission from A R Poole[7].)

1.5 Synovial joint development

The articular cartilage is one part of the complex synovial joint structure, which also includes ligaments that support the structure, synovial fluid that supplies nutrients and lubrication, and a fibrous capsule that surrounds the cavity[23]. Close knowledge of how the joint develops is useful when addressing regenerative questions, such as the in vitro differentiation of stem cells. Furthermore, several of the pathways implemented in limb development are dysregulated in the progression of osteoarthritis (OA) disease and close knowledge of developmental processes allows for deeper understanding of the disease[24].

The first steps in forming the musculoskeletal tissues of the limb are the formation of the primitive streak at gastrulation and the following formation

of a bi-potent mesendoderm cell population, which is driven by an intricate balance between canonical Wnt pathway signalling and BMP pathway signalling. During formation of the three germ layers, some of the mesendoderm cells further differentiate into a mesoderm population that is highly involved in the formation of the joint[25-28].

The formation of the joint can thereafter be divided into two main phases: the formation of cartilaginous anlagen and the following formation of the joint space between these anlagen[29]. The anlagen are formed in a process where undifferentiated mesenchymal cells, originating from the mesoderm germ layer, migrate to areas destined to become bone. Together with increased proliferation this results in high cell density at specific sites. This process is also known as the mesenchymal condensation. The high cell density improves intercellular communication and is essential for chondrogenesis to occur[30]. The cells change expression patterns from high expressions of HA and collagen I to hyaluronidase and the SRY (sex-determining region on the Y chromosome)-box containing gene 9 (SOX9) protein. This further increases cell-cell contact via induced expression of cell adhesion molecules N-cadherin and N-CAM, which stimulates increased chondrocyte maturation[30,31]. The SOX9 protein is one of the most critical transcription factors upregulated in this process, as it, apart from the cell adhesion molecules, regulates the expression of collagen II and aggrecan[32,33]. The SOX9 expression has in turn been suggested to be regulated by Transforming Growth Factor β (TGFβ), a key player for the chondrogenic differentiation [30,34]. At the end of this stage, COMP, together with tenascins and thrombospondins initiate transition from chondroprogenitor cells to fully committed chondrocytes. In this transition, the cells begin expressing mature ECM, with collagens II, IX and XI, as well as aggrecan[30].

During the interzone formation phase, the joint structures and the synovial cavity begin to form. At this point, the joint is composed of two layers, or anlagen, of densely packed cells which will later on form the cartilage surfaces of the long bones. Between the anlagen an intermediate layer of loosely packed cells will continue to form synovium, ligaments and joint capsule[35]. Members of the TGFβ superfamily, such as bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) are fundamental players in this process, controlling the chondrogenic differentiation of the cells[36]. A strict control of canonical Wnt signalling is also essential for a correct development[23,37].

The cavitation process, where the intermediate layer separates from the cartilage layer, is still not fully understood. Mechanical stimuli from muscle-driven motion appear to be important, together with cell death and an increased production of HA and lubricin. This causes reduced cell-cell contacts and allows for the formation of a fluid filled cavity between the

cartilage surfaces[23,29,35]. After the separation of the anlagen, the tissues continue to mature into a fully functional joint.

Continuously, the formation of long bones takes place in an endochondral ossification process. The chondrocytes in the cartilage are divided into two zones, the upper contains resting chondrocytes, and the lower contains proliferative chondrocytes that produce ECM. As the cartilage grows, the lower chondrocytes enter a hypertrophic state where they increase in size and the collagen II expression is replaced by collagen X. This matrix is then gradually invaded by blood vessels, and osteoblasts follow this invasion and replace the hypertrophic cartilage with mineralized bone[38,39]. The resting chondrocytes in the upper zone are then activated and replace the cartilage in a process of appositional growth[40].

1.6 Important signalling pathways

Several of the pathways that are implemented in the formation of the synovial joint and the articular cartilage tissue can be used and manipulated to induce cartilage formation *in vitro*. They can also be used to activate or inhibit tissue degradation. Three major signalling pathways that are important in these processes are the TGFβ1 signalling pathway, the GDF5 signalling pathway and the canonical Wnt signalling pathway.

1.6.1 TGFβ signalling

Members of the transforming growth factor beta superfamily play vital roles in the development and homeostasis of various tissues, including cartilage, and they are among the earliest signals in the cartilage development[31]. These proteins, over 35 different are included, regulate cell fate and control ECM synthesis and degradation[41,42]. They are divided into two subfamilies, the TGFβ/Activin/Nodal subfamily and the BMP/GDF/MIS (Muellerian Inhibiting Substance) subfamily. The subgroups are based on sequence homology and what pathways the proteins activate. TGFβ1, the first member of the family, was discovered 25 years ago, and it is a potent stimulator of cartilage ECM production[43,44]. It is, together with an isoform, TGFβ3, a key growth factor for *in vitro* chondrogenesis[45-48].

The TGFβ1 signal is activated in the cell when the protein binds to a serine-threonine kinase receptor complex consisting of the cell surface receptors TGF receptor type I and II. When no substrate is present, the receptors exist as homodimers on the cell surface. Upon binding of substrate, the receptors associate in a complex consisting of a type I receptor dimer and a type II receptor dimer, leading to phosphorylation and activation of the type I receptor by the type II receptor[34,44,49]. As of today, seven different type I receptors have been discovered and five different type II receptors, all of

which can be mixed and matched, resulting in a huge span of possible interactions[50].

The signal is after activation mediated via SMAD proteins. These proteins are divided into three classes: 5 receptor-regulated SMADs (numbers 1, 2, 3, 5 and 8), one co-SMAD (number 4) and two inhibitory SMADS (numbers 6 and 7). Phosphorylation of the receptor-regulated SMADs by the type I receptor can lead to several events, including release of the receptor complex, recruitment of more SMADs to the membrane and the formation of SMAD complexes with the co-SMAD. It is this complex that activates downstream genes by translocating to the nucleus where it binds to various transcription factors, including the for chondrogenesis essential SOX9 factor, activating downstream targets such as collagen II and aggrecan[44,49,50]. The TGFβ signalling is then further complicated through SMAD interactions with mitogen-activated protein kinases (MAPKs), and signalling via SMAD-independent pathways such as the Erk, JNK and p38 MAPK kinase pathways. This signalling is however poorly characterized[44,49,50].

1.6.2 GDF5 signalling

GDF5 is a 290 amino acid protein that belongs to the BMP family and the TGFβ superfamily. The protein, also known as BMP14 and cartilage derived matrix protein 1 (CDMP1), was originally characterized as a protein from cartilage extracts, that could induce cartilage and bone formation in subcutaneous implants[51-53]. It is a key regulator of mesenchyme condensation and chondrogenic differentiation during joint formation, and mutations in the gene can lead severe to defects in the developing skeleton, including brachypodism (shortening of limbs) [36,54], and have been implemented in the progression of OA[55,56].

Belonging to the same superfamily as TGFβ, it signals via the same family of receptors as other BMPs. GDF5 preferentially binds to BMP receptor IB (BMPRIB)[57], and the signals are mediated via SMAD-1, -5 and -8, and via the p38 MAPK pathway[57,58]. Despite the importance of GDF5 for the development of cartilage and joints, the downstream signalling of the gene is largely unknown.

1.6.3 Canonical Wnt signalling

One of the fundamental molecular mechanisms during embryonic development and tissue homeostasis is signalling by Wnt glycoproteins. 19 variants of these secreted ligands have been discovered this far, and they can signal via three Wnt pathways: The canonical pathway, also called the β-catenin pathway as β-catenin is the transducer of signal, the planar cell polarity (PCP) pathway and the Ca^{2+}/CamMKII pathway[59]. All pathways are important for proliferation, cell specification and differentiation within the

skeleton. The canonical pathway is required for embryonic joint specification and formation and for chondrogenesis. One of its roles is to keep chondroprogenitor cells in a proliferative state, preventing maturation into chondrocytes[24,31,60].

The canonical Wnt signalling pathway ligands, such as Wnt3a, signal through the interaction with two types of receptors at the cell surface, the serpentine seven transmembrane Frizzled (FZD) family receptors and the single-pass transmembrane low density lipoprotein receptor related proteins 5 and 6 (LRP5/6). In the absence of Wnt ligands, glycogen synthase kinase 3β (GSK3β) phosphorylates the canonical Wnt signal mediator β-catenin in a destruction complex with a set of co-proteins[24]. The phosphorylation leads to an ubiquitin-mediated degradation of β-catenin in the proteasomes. When Wnt ligands bind their receptors at the cell surface, the formed ligand-receptor complex will bind the destruction complex, which effectively reduces the activity of GSK3β. This results in reduced phosphorylation of β-catenin, and an accumulation of the protein in the cell cytosol, leading to the subsequent translocation of the protein into the cell nucleus[24,61]. Nuclear β-catenin interacts with DNA bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins which induces expression of Wnt downstream target genes such as peroxisome proliferator-activated receptor delta (PPARD) and FOS-like antigen 1 (FOSL1) [59,62].

Important inhibitors of the canonical Wnt signalling pathway are the secreted Wnt antagonists frizzled related protein (FRZB) and dickkopf 1 (DKK1)[59].

1.7 Cartilage damage

1.7.1 Focal damages

Despite its resilient design, articular cartilage in the knee is often damaged. Mechanical injuries and lesions in the cartilage are common and can be caused by everything from compromised joint protection, such as muscle weakness and mal-alignment of the joint, to excessive load, including physical activities with abnormal pressure to the joint, obesity and acute trauma[63,64]. Even gentle motion such as walking results in a pressure of 40-50 atmospheres to the cartilage surface[5]. The injuries include everything from slight fibrillation of the surface to cracks and tears in the surface and loosening pieces of cartilage. The irregularities in the cartilage surface that these focal defects cause interrupt the smooth articulating motion, which reduces wear resistance[65]. Typical symptoms of injuries include local pain, swelling of the joint and even locking of the joint due to loose debris that obstruct the motion[63].

In the assessment of focal damage, different classifications are used. Damages can be either chondral, when they only affect the cartilage, or osteochondral, when the subchondral bone is included. Chondral defects are usually further divided into sub-classes: full thickness, partial thickness, fibrillation or loose flap of cartilage. Several different scoring systems exist for this assessment, such as the International Cartilage Repair Society (ICRS) scoring system (Table 1)[63,66,67].

Damages that do not penetrate to the subchondral bone often fail to heal spontaneously, and the main reason for this is believed to be the lack of vascularisation. Upon damage, there is no bleeding or formation of a blood clot and no access to repair cells or growth factors. If the damage is left untreated, the disruption of the smooth lubricated surface will remain and the tissue is likely to degenerate over time under mechanical wear[63].

Grade of injury	**Description**
Grade 0	Normal
Grade I	Superficial fissuring
Grade II	<1/2 of cartilage depth
Grade III	>1/2 of cartilage depth to subchondral plate
Grade IV	Osteochondral lesion through subchondral plate

Table 1. ICRS scoring system for cartilage defects[66].

1.7.2 Osteoarthritis

Osteoarthritis (OA) is a progressive joint disease that causes destruction of the cartilage ECM, which leads to loss of function and pain in the joint. It is one of the most common musculoskeletal diseases in the world, where more than 30% of the population above the age of 45 are affected, and over the age of 65 as many as 75% of the population have developed OA to some degree[68,69]. For at least 12% of patients with OA in the lower extremities, the development is a secondary effect to joint trauma, also called post-traumatic OA (PTOA)[70]. The time course from the initial damage to the point where OA can be clinically determined is long, ranging from 2 to 5 years for larger traumas and lesions, to decades for less severe injuries, making it difficult to find the precise cause of initiation[65]. The traumatic event is believed to cause a series of biological events that lead to disease initiation[63,71].

In the remainder of the cases – idiopathic OA – the cause of disease is often unknown, and it is likely that there is not one but several different events that combined cause the onset and progression. With age, cartilage becomes more mineralized and looses its flexibility to applied pressure, increasing risk of damages to the articular cartilage from excessive load. Other mechanisms, all connected to the aging process, which impose on the functionality of articular cartilage are cumulative oxidative damage, shortening of telomeres and an accumulation of mutations[72]. As chondrocytes

age, the synthesis of proteoglycans is decreased and the ability to respond to certain growth factors is reduced. Further, mechanical stress, obesity and inheritance are predisposing factors for its development[73-75]. Predisposing factors can also initiate local inflammatory responses, causing upregulations of interleukin 1β (IL1β) and tumour necrosis factor α (TNFα), in either chondrocytes or other cells in the synovial compartment. These factors are a common part of the disease and they increase expression of matrix degrading enzymes[76].

The progression of OA is a result of mechanical wear and an imbalance between anabolic and catabolic factors in the tissue. In healthy tissue, matrix-modulating enzymes are strictly controlled. In diseased tissue, this control is disrupted with an upregulation of matrix degrading enzymes, mainly matrix metalloproteinases (MMPs) and aggrecanases (mainly A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, ADAMTS), where MMPs mainly degrade collagens and ADAMTS mainly degrade proteoglycans. MMP13 is believed to be the most important MMP, as it degrades collagen II and is highly upregulated in OA cartilage[77]. For aggrecanases, ADAMTS4 and ADAMTS5 are the main actors in OA. Of the ADAMTS proteins, they have the highest specific activity for aggrecan cleavage and are expressed in areas of aggrecan depletion in OA tissue[78], although they have a broad spectrum of targets, including the decorin proteoglycan and COMP[79,80]. The collagen proteins, that have a slow turnover[19], resist degradation in the early stages of the disease, but crosslinking of the network is loosened. This leads to release of proteoglycans and a reduced resistance to mechanical stress as a result. In later stages collagens are also degraded[73]. The naturally occurring defence against ECM degradation consist mainly of Tissue Inhibitors of Metalloproteinases (TIMPs). In healthy tissue, these inhibitors control activity of degenerative enzymes. In diseased tissue, the delicate balance tips over in favour of the matrix degrading enzymes, and the reason for this is largely unknown. There are four different known TIMPs, and TIMP1 is the most potent inhibitor of MMP13 and TIMP3 is the most potent inhibitor of ADAMTS4 and ADAMTS5[81,82].

Due to its complexity and multifactorial nature, there are few safe and effective therapeutic options for the treatment of OA, and most of those are palliative[83].

GDF5 and canonical Wnt signalling in osteoarthritis

Some of the features in OA resemble events during synovial joint development, and both the canonical Wnt pathway and the GDF5 signalling pathway, which are essential during development, have implications in the disease.

Wnt ligands, such as Wnt3a show increased expressions after cartilage damage and in tissues affected by OA, which results in the subsequent activation of the Wnt pathways in the cells. This activation disturbs cartilage tissue maintenance, with increased expressions of matrix degrading enzymes. It also initiates calcification of the tissue[24,62,71,84,85]. The canonical Wnt pathway is further implemented in the disease as mutations in the FRZB gene has shown associations with increased OA susceptibility. The mutation leads to reduced capacity to inhibit the Wnt signal[86,87]. Further, dysregulation of DKK1 expression is also associated with increased OA progression[88-92].

For the GDF5 signalling pathway, mutations in the GDF5 gene have been shown to cause increased susceptibility to develop OA[55,56,93]. A single nucleotide polymorphism (SNP, rs143383 T/C) located in the 5'-UTR of the GDF5 gene has been shown to have a connection to hip and knee OA in a range of ethnic groups. The result of this mutation is a slight reduction in the activity of the GDF5 promoter, indicating that even a minor imbalance in the GDF5 expression can lead to OA, although the exact role of the GDF5 for this development has not yet been elucidated[55,56].

1.8 Articular cartilage repair strategies

1.8.1 Marrow stimulation techniques

Over the years, different approaches have been utilized in attempts to induce a repair response in focal defects in articular cartilage. The first and still most commonly used methods are generally called marrow stimulation techniques[94]. Examples are Pridie drilling, abrasion chondroplasty and microfracture. These methods involve creation of a full defect and an induced bleeding from the subchondral bone. A blood clot is thereby formed in the damaged area, acting as a natural scaffolding structure for various types of blood-borne repair cells. Nutrients and growth factors can also access the damaged area[94]. The blood clot adheres badly to cartilage, and better to the bony surface, and seemingly through this, the healing tissue closest to the bone is ossified[95]. Further up in the lesion, ossification ends and cartilage is formed. This cartilage, however, has fibrocartilage structure, and it is a poor substitute for hyaline cartilage. It integrates poorly with the native tissue and as it has inferior mechanical properties compared to the articular cartilage it eventually starts to degrade[94-96].

1.8.2 Autologous chondrocyte implantation

Twenty six years ago, in 1987, Brittberg *et al*[97] used for the first time a new method for regeneration of articular cartilage called Autologous Chondrocyte Transplantation (ACT), also termed Autologous Chondrocyte Implantation(ACI). It involves two surgical procedures, and in the first, a small biopsy is arthroscopically harvested from a minor load-bearing area of the articular cartilage in the injured knee (Figure 3). In a tissue culture laboratory the biopsy is mechanically minced with a scalpel and digested with a collagenase, resulting in a single cell suspension of chondrocytes. The chondrocytes are expanded *in vitro* and the few hundred thousand cells retrieved from the biopsy become several millions. In the classical version of the procedure, the cells are thereafter loaded into a syringe in suspension and injected into the defect in a second surgical procedure. A periosteal flap is sutured over the defect to keep the cells confined, and the cells will then produce a hyaline-like repair tissue in the defect[97].

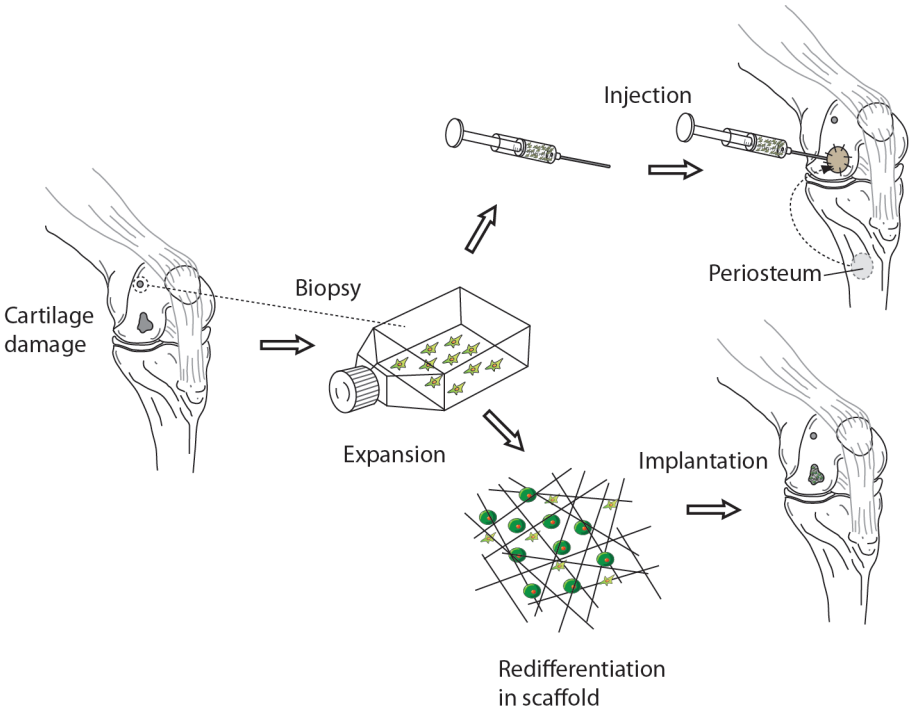

Figure 3. Schematic view of the ACI procedure. The cells can be implanted in suspension (top) or in a biomaterial construct after redifferentiation (bottom).

In developments of the procedure, the periosteum is replaced by resorbable collagen I/III membrane[98] and biomaterial scaffolds have ben introduced as

cell containers[99-101]. In the scaffold-assisted developments, after expansion, cells are seeded into a biomaterial scaffold and cultured for two to three weeks *in vitro*. During this period, the cells attach to the material and are induced to produce cartilaginous tissue. This biomaterial/cartilage construct is then, in the second surgical procedure, glued or sutured in place, effectively filling the defect void. Several advantages exist with these methods, including reduced leakage of cells after implantation and improved cell distribution. Patient rehabilitation times are often reduced as these procedures can be performed arthroscopically, compared to the open knee surgeries used with the previous methods. The constructs also have some load bearing qualities, supporting the tissue[63,99-101].

More than 35 000 patients have been treated worldwide, and over 80% with good recovery in a 2-10 year follow-up[102]. In a 10-20 year follow-up, 74% of the patients reported that their status was the same or better than before the treatment, and 92% were satisfied and would do the procedure again[103].

1.9 Tissue engineering

1.9.1 The tissue engineering triad

Tissue engineering is an interdisciplinary field of research that combines engineering and the life sciences to develop functional biological substitutes that can restore, maintain or improve tissue function[104]. It is closely related to regenerative medicine, and these terms are interchangeably used in this thesis. Tissue engineering can be described as a triad, combining cells, scaffolds and different culture conditions for the formation of viable and functional constructs[105]. The ACI procedure can be considered to be one of the few tissue engineering methods applied in the clinic.

The cells are the viable part of the tissue engineering construct. They produce ECM and supply the biological function of the constructs. The scaffolds are used as container for the delivery of cells to the patient. They should support cell attachment and differentiation, and promote growth of the cells and tissue in three dimensions. Often they also have mechanical properties that support the tissue while it regenerates[104,106]. The final part of the triad is the environmental and culture conditions which ranges from stimulatory cues that provide optimal physicochemical conditions for the cells to growth factors that induce differentiation of the cells and bioreactors for the addition of fluid flow, adjustment of oxygen tension and the application of pressure to the cultures[107,108].

1.9.2 Cell sources in cartilage tissue engineering

Several cell sources can be considered for the formation of cartilage *in vitro*, ranging from adult cells such as the chondrocytes used in the ACI procedure and mesenchymal stem cells (MSCs) to embryonic stem cells (ESCs) and the relatively new induced pluripotent stem cells (iPSCs) [97,105,109-112], and there are advantages and disadvantages with all of them. Several studies have used MSCs in cartilage tissue engineering applications, as they are relatively easy to access and can be induced to form cartilage[45,109,113,114]. These cells do, however, have a predisposition to form hypertrophic cartilage and bone, and are not optimal for formation of hyaline cartilage[115,116]. Furthermore, the MSCs also have a limited proliferative capacity and their ability to form functional tissues is reduced with time in expansion and increased donor age[117,118].

Embryonic stem cells (ESCs) show promise, as they have an unlimited proliferative capacity and are pluripotent, meaning that they can differentiate to all cell types of the body, including chondrocytes[119,120]. Since these cells are not autologous, they can, however, elicit an immune response after implantation and the high genetic expression of markers of pluripotency or oncogenes implies that they also have the ability to form teratomas (tumours that include cells of all three germ layers). ESCs also face an ethical dilemma in the use of human embryos as a cell source[121]. The iPSCs show the same promise as the ES cells, but have the advantage that as they can be autologous the ethical concerns are avoided. The main issue with these cells is the formation of teratomas[122,123]. In this thesis, the cell sources have been chondrocytes and iPSCs.

Chondrocytes

Chondrocytes are a well-documented cell source that forms a functional tissue *in vitro* and *in vivo*[97,124-126]. As a result of the low cellularity of the donor tissue, the chondrocytes need to be expanded in order to obtain sufficient numbers for the treatment. During this expansion, the cells undergo a process called dedifferentiation, in which comprehensive changes in morphology, as well as a change in gene expression profile takes place. Genetic markers expressed in the mature cartilage are downregulated, including collagen II, aggrecan, COMP and SOX9, and genes expressed in more immature cells are upregulated, including collagen I and the proteoglycan versican[127,128]. In the developed ACI protocols, the cells are due to these events redifferentiated, i.e. induced to express the correct genes and take on the rounded morphology again, before they are implanted. After sufficient numbers have been reached in expansion procedure, the cells are seeded into the biomaterial scaffold as described above and induced to redifferentiate by a defined culture medium that includes TGFβ1. During this

redifferentiation process, the cells begin to produce the correct ECM again. During this redifferentiation phase, the expressed ECM provides the cell/biomaterial construct with proper mechanical properties required for the implantation. The expansion stage is critical, as a to long expansion of the cells results in an irreversible dedifferentiation of the cells[129]. As an effect of this, size of defects that can be treated might be limited. The taking of the biopsy means a trauma to the cartilage surface, and there might be risk of donor site morbidity[130]. Further more, there is also a patient dependent variation in the ability of the chondrocytes to form cartilage *in vitro*.

Induced pluripotent stem cells

In 2006 Yamanaka *et al*.[131] showed that an adult differentiated cell could be reprogrammed into a pluripotent stem cell, through targeted gene modifications with viral transduction techniques. Transfecting four factors (OCT4, SOX2, KLF4, and c-MYC, also called the OSKM factors) into the adult cells, and thereby overexpressing these factors, the cells were transformed in to cells with similar morphology as ESCs, growing not as a cell mat but in colonies, and these cells were named induced pluripotent stem cells (iPSCs). The iPSCs further had the proliferative capacity of ESCs, showed similar gene expression patterns and had the ability to form teratomas and tissues from all three germ layers in defined protocols[131,132]. During these differentiation processes, expressions of pluripotency markers are often downregulated, opening up the use of the cells in clinical applications. Since the first publication, several different cell types have been reprogrammed, including fibroblasts, keratinocytes, hepatocytes and pancreatic β cells[133-136].

The viral integration of the factors into the genome has been an impediment to use the iPSCs clinically, as reactivation can occur, which could lead to tumorigenesis[137]. Viral-free methods have therefore also been developed for the same procedure, where the OSKM factors are transfected as synthetic mRNA molecules[138], generating transgene free iPS cells. mRNA coding for LIN28 is also usually added in these protocols as this facilitates the reprograming[139]. As mRNA molecules quickly degrade, transfections of the cells are performed daily for an extended period of time (Fig. 4)[138,140]. Once reprogrammed, no further transfection is needed as the cells produce the pluripotency markers themselves.

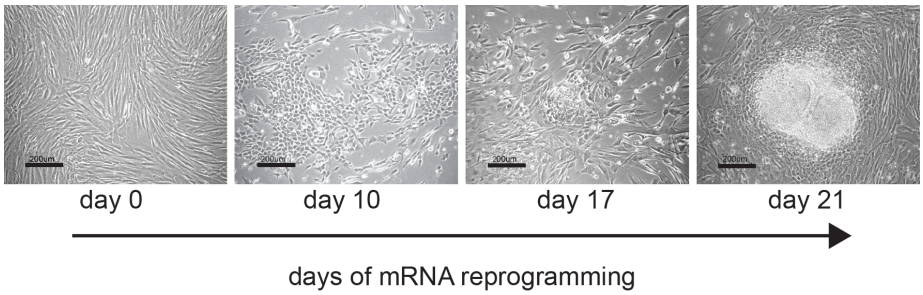

Figure 4. mRNA reprogramming of chondrocytes into iPSCs. On day 0 a monolayer culture of chondrocytes are being transfected with an OSKML cocktail of mRNA molecules. The transfection is performed daily for 21 days and during this period morphological changes in the culture show how the reprogramming is progressing.

The iPSCs are used in several research fields, ranging from tissue engineering applications, to modelling of human diseases and in the search for therapeutic agents[141-143]. The iPSCs also hold advantages over the ESCs in that there are no evident ethical concerns involved, that they can be autologous and that they might retain an epigenetic memory of their origin after reprogramming.

2 AIMS OF THE THESIS

The main objective of this thesis was to gain insight into how articular cartilage healing can be promoted, through improved regenerative methods and inhibition of degenerative processes.

2.1 Specific aims of the included studies

- To study how external factors can induce a regenerative response and increase ECM production in articular chondrocytes, including the use of recombinant growth factors and therapeutic devices, manipulating known regenerative signalling pathways (addressed in study I, II and III).

- To investigate how external factors such as growth factors can prevent progression of OA and through which pathways they function (addressed in study III).

- To implement factorial design of experiments in basic research to study the effect of multifactorial stimuli on anabolic and catabolic factors in articular chondrocytes (addressed in study II and III).

- To find a putative off-the-shelf cell line or personalised cell line for an improved ACI procedure and a model cell line for studying cartilage disease (addressed in study IV).

3 MATERIAL AND METHODS

3.1 The cells

Several different cell types have been used to conduct the studies presented in this thesis. The majority of the work was performed with human articular chondrocytes.

3.1.1 Chondrocytes

Source of chondrocytes

The human chondrocytes used in this thesis were obtained from cartilage biopsies harvested from minor load-bearing areas of the upper femoral condyle of the knee joint of patients undergoing ACI. The donor site showed no macroscopic signs of cartilage defects. The harvested biopsies were transported on ice to the cell culture laboratory in sterile saline solution (0.9% NaCl) supplemented with antibiotics and fungicide[97]. This solution keeps the cells viable at least 48h. At the laboratory, subchondral bone and soft tissues were removed from the biopsies whereafter the cartilage was minced with a scalpel in a sterile petri dish. The minced cartilage was digested over night (20-24 hours) in a collagenase type II solution (0.8 mg/ml) in Ham's F-12 medium at 37°C in 7% CO_2. This resulted in a cell suspension with mostly single cells. These cells were washed twice with Ham's F-12[97]. The ethical committee at the medical faculty at University of Gothenburg approved the donation of tissue. The cells were anonymized and not possible to trace to the individual donors. Age, gender and type of cartilage defect were the only identified parameters.

Expansion of chondrocytes

The isolated chondrocytes were resuspended in chondrocyte culture medium consisting of a 1:1 mix of Dulbecco's Modified Eagle's Medium and Ham's F-12 (DMEM/F12) supplemented with 0.1 mg/ml ascorbic acid, 50 mg/l gentamicin sulphate, 250 µg/ml amphotericin B, 2 mM L-glutamine and 10% v/v human serum, each batch consisting of serum pooled from 10 different donors[144]. After the first passage the gentamicin and amphotericin B was replaced by penicillin (0.1 units/ml) and streptomycin (100 µg/ml) (1% PE/ST). The chondrocytes were seeded at 3×10^3 cells/cm^2 on Primaria™ plastic, a polystyrene surface that improves attachment of cells through incorporated anionic and cationic functional groups. The first medium change was made 6 days after seeding and thereafter two to three times per week.

When cells reached 80%-90% confluence, they were subcultured using a trypsin-etylenediaminetetraacetic acid (EDTA) solution (0.125% trypsin diluted in 0.1M phosphate buffered saline (PBS) with 0.2 g/l EDTA). After the first passage, the cells were cultured in polystyrene culture flasks. To store cells for future use, they were frozen in DMEM/F-12 supplemented with 20% human serum and 10% dimethyl sulfoxide (DMSO).

3.1.2 Induced pluripotent stem cells

iPSC reprogramming

For the reprogramming of human chondrocytes and fibroblasts into iPS cells, the Stemgent mRNA Reprogramming Kit was used, following the manufacturers protocol. 10 000 cells were plated in 6 well plates on top of a confluent layer of NuFF feeder cells. The mRNA for the five reprogramming factors OCT4, SOX2, KLF4, c-MYC and LIN28 were added daily to the culture medium in lipid delivery vehicles[138]. The reprogramming cycle lasted for 21 days for the chondrocytes (17 days for the BJ fibroblasts) after which hESC like colonies could be detected. The reprogramming protocol was conducted in Pluriton reprogramming medium under hypoxic conditions (5% O_2) in a humidified atmosphere at 37 °C, 5% CO_2. Low oxygen tension has proven to yield more efficient reprogramming of iPS cells, compared to regular oxygen tension (21% O_2)[145]. Clonal iPSC lines were established by manually isolating hESC–like colonies.

Expansion of iPSCs

iPSCs, both chondrocyte- and BJ fibroblast-derived, were maintained in human iPS culture medium, which helps to keep the cells in a pluripotent state and supports proliferation of the cells[146,147]. It consists of DMEM/F12 supplemented with 20% knockout serum replacement, 2 mM L-glutamine, 1% v/v non-essential amino acids, 55μM 2-mercaptoethanol, 1% PE/ST and 10 ng/ml human recombinant basic fibroblast growth factor (hrbFGF). Cells were kept at 37°C and 5% CO_2.

Passaging of iPSCs

The iPSCs were mechanically passaged by micro-dissection with a stem cell cutting tool every 4–5 days for the first passages, and enzymatically passaged using Collagenase type IV (200 U/ml) at later passages. When stem cells are dissociated into single cell suspension, they initiate an apoptosis program. Due to this, at the day of passaging, 10 μM Stemolecule Y27632 was added to the medium. It is a Rho-associated kinase (ROCK) inhibitor that prevents this apoptosis[148].

3.1.3 Human embryonic stem cells

hESCs (SA121) were obtained from Cellectis bioresearch, Gothenburg, Sweden

Feeder free expansion

ESCs and iPSCs can be expanded without the use of feeder cells[149]. The culture flasks are in these cases generally coated with gelatin, fibronectin or different laminins as regular stem cell lines do not adhere to ordinary culture plastics[119,150,151], although rare examples of plastic adherence exist[151]. The culture medium used is generally conditioned with feeder cells, allowing for important factors to be released from the feeder cells to the culture medium.

In study IV, the iPS cells were adapted to the DEF feeder free culture system, commercially available at Cellectis Bioresearch.

3.1.4 BJ fibroblasts

The BJ human fibroblasts were purchased from Stemgent and used as control cells during the iPS transfections. They were expanded in BJ medium containing DMEM supplemented with 1% PE/ST and 10% Fetal bovine serum (FBS) and cultured in a humidified atmosphere at 37°C and 5% CO_2. Subculture was performed with Trypsin-EDTA at 80%-90% confluence.

3.1.5 NuFF cells

Mitotically inactivated neonatal Human Foreskin Fibroblasts (NuFF) were used as feeder cells during the iPS reprogramming protocol. Feeder cells supply extrinsic factors that are needed for the propagation of stem cells and the retention of pluripotency of the cells[146,152]. The cells were obtained from Globalstem and plated in NuFF medium consisting of DMEM + GlutaMAX-1 supplemented with 1% PE/ST and 10% FBS and cultured in a humidified atmosphere at 37°C and 5% CO_2. Subculture was performed with Trypsin-EDTA at 80%-90% confluence.

3.1.6 hEL cells

After completed reprogramming, iPS cells were expanded on two different types of irradiated feeders: human diploid embryonic lung fibroblasts (hEL) and a human foreskin fibroblast cell line (HuWIL). The hEL cells[151,153] were expanded in hEL medium consisting of DMEM/F12 supplemented with 0.1 mg/ml ascorbic acid, 1% PE/ST, 2 mM L-glutamine and 10% human serum at 37 °C in 7% CO_2. Subculture was performed with Trypsin-EDTA at 80%-90% confluence.

3.1.7 HuWIL cells

The HuWIL cell line[154] was the second feeder cell line used. It was purchased from Cellectis bioresearch and expanded in HuWIL medium consisting of DMEM/F12, 10% FBS, 0.1 g/l ascorbic acid and 2mM GlutaMAX at 37 °C in 5% CO_2.

3.2 Chondrogenic differentiation

3.2.1 Monolayer differentiation

The iPSCs were differentiated in monolayer culture towards a chondrogenic phenotype using a protocol defined by Oldershaw *et al.*[119]. After adaptation to feeder-free culture the Cellectis Biotech DEF culture system, the iPSCs were stimulated with a series of growth factors known to be important for *in vivo* chondrogenesis, mimicking the differentiation process from the stem cell stage through mesendoderm and mesoderm to a chondrogenic phenotype. The cells were subjected to varying concentrations of Wnt3a, activin A, hrbFGF, BMP4, follistatin, GDF5 and nurotrophin 4, according to the published protocol[119].

3.2.2 Micromass culture

To redifferentiate chondrocytes, and to differentiate iPSCs, the micromass (also known as pellet mass or simply pellet) culture technique was utilized in this thesis. This method provides three features that are important for chondrogenic differentiation: a three-dimensional culture environment, chondrogenic growth factors and a high cellular density[31,155,156]. In this method, the cells are after passaging resuspended in a basic defined medium (BDM) supplemented with a growth factor, usually TGFβ1 or TGFβ3. The BDM consists of DMEM high glucose supplemented with 5.0 µg/ml linoleic acid, 1×ITS-G premix (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid), 1.0 mg/ml human serum albumin, 10^{-7} M dexamethasone and 14 µg/ml L-ascorbic acid. TGFβ1 was supplemented at 10 ng/ml. To form the micromass, 200.000 - 400.000 cells are placed in conical polypropylene tubes and centrifuged at 400 g for five minutes. The tubes are incubated for 48 hours at 37 °C in 5% CO_2, during which the cells attach to each other, pull on each other, effectively forming a sphere. After formation, medium is changed every two to three days.

3.2.3 Scaffold-mediated differentiation

The chondrogenic differentiation can also be matrix assisted, and there is a large range of materials that can be used[157]. In this thesis, an alginate hydrogel was used to retain the cells in place and to provide an appropriate three-dimensional structure that could be used in combination with the arthroscopic plasma-mediated ablation device[158]. Cells were after passage resuspended in a 1.2% w/w sodium alginate solution prepared in PBS at 4×10^6 cells/ml. 575 µl of cell suspension was pipetted into cell culture inserts with a bottom consisting of a porous membrane. The pores in the membrane were 1 µm in diameter, allowing for ions and media to pass over it, but not the cells. The inserts were placed in a culture well containing 800 µl of 102 mmol/L $CaCl_2$ in 0.9% w/w saline solution (NaCl) and placed in the incubator for two hours at 37°C and 5% CO_2. During these two hours, Ca^{2+} ions passed over the membrane into the alginate-cell suspension, crosslinking the negatively charged alginate polymers, forming a firm alginate gel with chondrocytes trapped inside. After two hours, the saline solution was replaced with BDM supplemented with 10 ng/ml TGFβ1. Medium was changed twice per week.

3.3 Verification of pluripotency

To assess pluripotency of the iPS cells in study IV, the iPS cells were cultured in micromass embryoid bodies (EBs). The three dimensional structure and medium used induces spontaneous differentiation of the cells into all three germ layers. The iPSC colonies were detached from culture using a stem cell cutting tool placed into a low-adhesive cell culture dishes in EB medium containing iPS culture medium where the Knockout serum was replaced by 20% FBS. When detached, the colonies form microspheres, or EBs. The EBs were cultured for six days in suspension and are thereafter plated onto gelatin coated cell culture plates and cultured for two weeks. During this period, cells migrate from the EB onto the gelatin-coated plastic and proliferate. These EB derived cells were fixated in Histofix for 24 hours and analysed with immunohistochemistry for endoderm, ectoderm and mesoderm markers (Hepatocyte nuclear factor 3β, βIII tubulin and α-smooth muscle actin, respectively). Further, some EBs were cultured for in vitro teratoma formation in agarose culture in EB medium (as previously described[159]), to further assess pluripotency of the cells. After 8 weeks in culture, cells were fixated with Histofix for 24 hours and analysed with histochemical hematoxylin and eosin stainings for endoderm, ectoderm and mesoderm differentiation.

3.4 Inductive cues

3.4.1 Growth factors

Cell fates during development are governed by several factors, including biochemical and biophysical cues[160]. These aspects can be utilized in tissue engineering of cell constructs, manipulating cells by activating and inhibiting different signalling pathways. The biochemical cues involve several factors, and in this thesis, growth factors have been the main tool for guiding cell fate. The growth factors were added to the BDM at different concentrations depending on protocol: TGFβ1 1-20 ng/ml, GDF5 10-200 ng/ml, Wnt3a 25-50 ng/ml, activin a 10-50 ng/ml, hrbFGF 4-20 ng/ml, BMP4 20-40 ng/ml, follistatin 100 ng/ml and neurotrophin 4 2 ng/ml.

3.4.2 Small molecules

In several areas of the regenerative medicine field, low-molecular weight organic compounds, also called small molecules, are used to regulate biological processes, such as inhibition of cell signalling pathways. In this thesis, the small molecule CHIR99021 was used to inhibit GSK3β, thereby activating the canonical Wnt pathway[62]. The small molecule WAY262611 was used to inhibit DKK-1, also leading to an increased activity of the canonical Wnt pathway. The inhibitors were added to BDM at different concentrations; CHIR99021 0,5-3 μM and WAY262611 0,01-1 μM.

3.4.3 Plasma-mediated ablation

Plasma-mediated bipolar radiofrequency ablation (plasma-mediated ablation or ablation in short in this thesis) is a method that can be used in arthroscopic surgery for the ablation of soft tissues. For chondral lesions and defects in the cartilage, this method can be used to debride, or remove, damaged or diseased tissue and to even out rough cartilage surfaces[161,162] At the tip of the ablation device used, a small electrical plasma field is created when the device is activated in an electrolyte solution, and this field is used to disintegrate tissue in a controlled manner[162].

In study I, human chondrocytes in alginate cultures were exposed to bipolar plasma-mediated radiofrequency ablation by moving an ablation probe over the cells (at 240 V ± 10%), simulating an *in vivo* ablation situation. Samples of exposed cells were harvested for mRNA quantification three and six days after exposure, and stained for viability 1 hour after exposure.

3.5 Viability staining

To determine the viability of cells after being exposed to the ablation device, the cells were stained with propidium iodide (PI) and fluorescein diacetate (FDA) and investigated under the microscope. PI is a dye that stains double stranded DNA by intercalating between the bases. After intercalation it emits fluorescent light with an emission maximum at 617 nm when excited by UV light. It is excluded by viable cells but can cross over damaged cell membranes in dying or dead cells. FDA passively penetrates the cell membrane and once inside the cell it is deacetylated by non-specific esterases into fluorescein. Fluorescein is thereby accumulated in living cells and it emits fluorescent light at 521 nm upon excitation by UV light.

In this thesis, the investigated samples were washed 3 times with PBS and incubated in a 3 μg/ml FDA solution diluted in PBS for 15 minutes at 37°C. The samples were then gently washed once with PBS, and incubated in a 100 μg/ml PI solution diluted in PBS for 2 minutes at 37°C. Samples were finally washed 3 times with PBS and investigated under a microscope for live (green) and dead (red) cells.

3.6 Techniques for Biochemical analyses

3.6.1 Enzymatic digestion

To quantify the extra cellular matrix production by the cells or the amount of DNA in the constructs, the pellet mass cultures or matrix assisted cultures were first digested with 0.3 mg/ml papain enzyme dissolved in a buffer containing 20 mM sodium phosphate buffer, 1mM EDTA and 2 mM dithiothreitol, pH 6.8 at 60°C for 2 hours. Papain is a cysteine protease with broad specificity that cleaves peptide bonds of basic amino acids, leucine and glycine and it hydrolyses esters and amides, and it can thereby degrade cartilage ECM.

3.6.2 Quantification of sulphated proteoglycans

To quantify the sulphated proteoglycans (sGAG) in the cell constructs, a 1,9-dimethylmethylene blue (DMMB) assay was used. The DMMB is a cationic dye that binds to the sulphated GAGs, which results in a change in absorption spectrum[163]. After the papain digestion, the samples were diluted 1:2 in PBS and mixed with an equal volume of DMMB solution, consisting of 16 mg DMMB dissolved in 1 litre H_2O containing 3.04 g lysine, 2.37 g NaCl, and 95 ml 0.1 M HCl. Duplicates of the samples were immediately analysed in a spectrophotometer, where absorbance was determined at 520 nm and sGAG

content was calculated against a standard curve of chondroitin sulphate diluted in PBS. sGAG content was normalised to the amount of DNA in the constructs.

3.6.3 Quantification of DNA

DNA content in the cell is highly regulated and is therefore closely proportional to cell numbers. Quantifying the DNA in a construct can therefore show if cells have proliferated, if compared to adequate controls. The amounts of DNA in the constructs and micromass cultures were determined using Hoechst 33258, a bisbenzimidazole derivative that binds to AT-rich regions of double stranded DNA. After binding, it has a fluorescence emission peak at 460 nm when excited with UV light at 360 nm. The papain-digested samples were diluted 1:2 in PBS and mixed 1:10 v/v of Hoechst 33258 solution (0.2 µg/ml Hoechst 33258, 100 mM Na_2HOP_4, 5 mM EDTA, pH 7.5). Duplicates of each sample were analysed and concentration of DNA was calculated against a standard curve of calf thymus DNA.

3.7 Techniques for gene expression studies

3.7.1 RNA isolation and quantification

To analyse how different genes are expressed in the cell after different stimulations, the mRNA was collected from the cells. For the monolayer cultures, the cells were lysed using RLT lysis buffer (a lysis buffer that inhibits RNAses) and RNA was purified using the RNeasy mini kit, following the manufacturers protocol for animal cells and tissues.

For the three dimensional structures, the tissues were snap frozen in liquid nitrogen. A stainless steel bead, 5 mm in diameter, was added to the tube and the structures were homogenized in a MixerMill. For study I, II and IV, QIAzol lysis reagent was then added to the samples and the cells were further homogenized. The QIAzol reagent is a guanidine and phenol based lysis reagent that allows for RNA extraction through adding chloroform to the lysate. This solution then separates into an organic and one aqueous phase, and the RNA is contained in the aqueous phase. After mixing the aqueous phase in a 1:1 ratio with 70% EtOH, RNA was extracted with the RNeasy mini kit following the manufacturers protocol for animal cells and tissues. For study III, micromass pellets were homogenized in RLT buffer in a MixerMill with a 5 mm stainless steel bead and RNA was extracted from the lysate with the RNeasy mini kit following the manufacturers protocol for animal cells and tissues.

3.7.2 Quantitative real-time PCR/Reverse Transcription PCR

In the four studies included in this thesis, the amounts of mRNA expressed by the cells were quantified using quantitative real-time polymerase chain reaction (qRT-PCR). This method is based on the for molecular biology central PCR technique that allows for amplification of double stranded DNA sequences. This is performed in a cycled reaction where the DNA is amplified in a reaction mix consisting of oligonucleotide primers, deoxyribonucleotide triphosphates (dNTPs) and a thermostable DNA polymerase. The qRT-PCR allows for a sensitive detection and quantification of gene transcripts[164-166]. Before the reaction is initiated, the single stranded RNA is transcribed to a double stranded complementary DNA, cDNA. In this thesis the TaqMan Reverse Transcription reagents, including random hexamer primers, were used according to the manufacturers instructions.

In the quantification of the sequences, probes designed to attach to a specific region of interest are labelled with a fluorescent probe at their 5' end and a quencher at their 3' prevents fluorescent signal as long as the probe is intact. After attaching to the complementary sequence, then a polymerase digests the probe after reaction initiation, resulting in a separation of the fluorescent probe and the quencher and the subsequent fluorescent signal. For each cycle of the PCR reaction, more fluorescent molecules are released and the fluorescent signal intensifies, proportional to the amount of probe target sequences. The fluorescence is quantified after each cycle and compared to a reference. At a certain fluorescence level, a threshold is reached and the cycle number where this happens is denoted as the Ct-value. If a gene has a higher expression, corresponding to more template molecules, that sample will reach the threshold at an earlier cycle, and the relative amounts of starting material can be quantified. To estimate the expression per cell, it is compared to the expression of a reference gene, a gene that is recognised as being relatively stable in the cell and not dependent on outer stimuli.

In this thesis 2.5 ng cDNA was added in each reaction together with TaqMan Universal PCR mater mixture, 1xAssay-on-demand mixes of primers and TaqMan major groove binding probes. After an initial denaturation step at 95°C for 10 minutes, 40 cycles consisting of 95°C denaturation for 15 seconds, and hybridization and extension at 60°C for 1 minute were performed, using the ABI7900 Sequence Detection system. As endogenous control the reference gene Cyclophilin A was used and the relative comparative method was used to analyse the data, according to the Assay-on-demand manufacturers instruction.

3.8 Techniques for protein expression studies

3.8.1 Protein isolation

The proteins in the micromass cultures was isolated through homogenizing the pellet cultures in the presence of ELISA lysis buffer and a 5 mm stainless steel bead in a MixerMill. Protein in culture medium was concentrated using an Amicon Ultra-4 Centrifugal Filter Unit with a cut-off 3 kDa. The filters in these units allow for water and ions to pass over the membrane, leaving proteins larger than 3 kDa on the other side of the membrane, effectively increasing the protein concentrations. For monolayer culture, cells were harvested in ice cold RIPA buffer, containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP40, 1% Na-deoxycholate, 0.1% Na-dodecylsulphate (SDS), pH 7.5.

3.8.2 Enzyme-Linked ImmunoSorbent Assay

To quantify different protein concentrations in medium and cell lysates, Enzyme Linked ImmunoSorbent Assays (ELISAs) were used. The ELISA is an antibody-based assay that is sensitive and cost-effective and therefore commonly used for the quantification of proteins in basic research. In this thesis, dual antibody sandwich ELISAs have been the ELISAs of choice. In this assay, a primary antibody is coated onto a 96 well plate and the antigens in samples that are added to the well are bound to the antibodies. A secondary, enzyme-linked antibody is added that to the plate, binding the antigen creating the antibody sandwich. Unbound antibody is washed away, a substrate for the linked enzyme is added, that upon reaction develops a colour that is read with a spectrophotometer. The amount of colour, usually determined via optical density, is proportional to the antigen concentration[167].

In study III, Quantikine® ELISAs for β-catenin and DKK1 were used, according to the manufacturers instructions. Briefly, 100 µl or 200 µl of sample was added in each well and incubated for 2 hours. After washing 4 times with the supplied wash buffer, 100 µl of horseradish peroxidase (HRP) conjugated secondary antibody were added to each well and incubated for 2 hours. After washing four times, 100 µl of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and the plate was incubated in the dark for 20 minutes. Optical density was determined at 450 nm in a microplate reader after 50 µl acidic stop solution was added to each well. Concentrations of the samples were calculated by comparing them to a standard curve supplied in the assays. Active MMP13 was quantified with the fluorokine® ELISA according to the manufacturers instructions. MMP13 is secreted by cells as an inactive proenzyme, and the enzymes is activated

when the prodomain is removed. This process can be performed by other MMPs, such as MMP-2 and MMP-14. *p*-aminophenylmercuric acetate (APMA) can also activate the enzyme. In the protocol, briefly, 200 µl of sample was added in each well in the pre-coated 96-well plate and incubated for 3 hours. After washing 4 times with the supplied wash buffer, 200 µl of APMA was added for 1 hour to activate the enzyme. The washing step was repeated and 200 µl of enzyme substrate was added and incubated for 18 hours and the relative fluorescence was measured in a fluorescence plate reader.

3.8.3 Alkaline phosphatase activity

Alkaline phosphatase (ALP) is a hydrolase enzyme and its usually highly expressed in stem cells and iPS cells. In this thesis, and the ALP activity of the iPS cells was determined with the Stemgent Alkaline Phosphatase Staining Kit II according to the manufacturers instructions.

3.9 Histological techniques

Histology is the study of microscopic structure and composition of cells and tissues. Tissues to be studied are usually fixed in a 4%-10% formaldehyde solution in PBS. The aldehydes in the solution cross-link proteins in the tissue, which preserves the tissue and cell structure and prevent degradation. After fixation, tissues are embedded in paraffin that allows for sectioning of the tissues with a specific knife, a microtome, into thin sections that are attached to glass slides. These sections are then stained with appropriate tissues stains.

In this thesis, the micromass pellets we fixated in Histofix (6% formaldehyde in PBS) for 24 hours, dehydrated through EtOH and isopropyl alcohol and embedded in paraffin. Sections, 5 µm thick, were cut from each sample at the approximate centre of each pellet, and the sections were attached to glass slides.

3.9.1 Alcian blue van Gieson staining

Alcian blue van Gieson is a histochemical stain that facilitates the observation of cartilaginous tissues under the microscope. Alcian blue is a blue cationic dye that carries up to four cationic groups that interact with the negatively charged proteoglycans in the cartilage tissue. van Gieson is a mix between picric acid and acid fuchsin that stains collagens and connective tissues in a pink nuance and cell nuclei black-brown[168].

After attachment to glass slides, sections were deparaffinised and stained with Alcian blue solution, consisting of 1g Alcian blue in 100 ml H_2O, 100 ml 1% acetic acid and 30 mg phenol. After rinsing in water, acidic EtOH and

95% EtOH, the sections were stained for 2 minutes in a fuchsin solution consisting of 100 mg basic fuchsin, 1 ml 95% EtOH, 10 ml 5% w/w carbol solution, 89 ml H2O containing10% formaldehyde and 10% glacial acetic acid. Finally, sections were mounted with glass cover slips.

3.9.2 Haematoxylin and eosin staining

Haematoxylin-eosin stainings are commonly used in histochemical procedures. The haematoxylin stains nucleic acids in an incompletely understood mechanism, resulting in a blue to violet colour. Eosin is an acidic dye that stains proteins non-specifically, resulting in cell cytoplasm and ECM in a pink colour range.

In study IV, microtome sections of stem cell EBs were attached to glass slides, deparaffinised, stained in Harris' haematoxylin for 4 min, washed and stained with 1% eosin for 7 min. Finally, sections were mounted with glass cover slips.

3.9.3 Immunohisto- and immunocytochemistry

To stain for specific proteins and structures in cells (immunocytochemistry) and tissues (immunohistochemistry), antibodies can also be utilized, which allows for a very specific visualisation. The tissue sections or cells are incubated in a solution with an antibody raised against the specific target antigen. The samples are then incubated with a secondary antibody raised against an epitope on the primary antibody. The secondary antibody has a reporter molecule attached, which usually is a fluorescent compound, but can also be an enzyme or metal[169]. In this thesis, fluorescent molecules were used for the visualization of the antigens. Cell nuclei were visualised using the fluorescent marker 4',6-diamidino-2-phenylindole (DAPI), a DNA-binding molecule that when bound to DNA has an fluorescence emission peak at 461 nm when excited with UV-light.

In study IV, these antibody-based methods were used to verify pluripotency of the iPS cells, and to verify their differentiation into cells from the three different germ layers. Antibodies raised against collagen 2 were used to verify the chondrogenic differentiation of the cells.

After fixation 15 min with Histofix, cells were washed with PBS three times. To reduce unspecific binding of antibodies, the samples were incubated in blocking buffer containing 0.3% Triton X and 5% goat serum in PBS for 1 hour. Samples were incubated in primary antibodies solutions in 1:100 or 1:200 ratio in blocking buffer over night at 4°C. After repeated washing in PBS, the primary antibodies were visualized using fluorescent labelled probes. Cell nuclei were visualised with DAPI and mounted with a ProLong Gold anti-fade solution and a glass cover slip. Samples incubated with isotype specific control antibodies were used as controls.

For the pellet mass cultures, sections were deparaffinised in xylene and rehydrated in a serial dilution of EtOH. For a more efficient detection of the antigens by the collagen II antibody, the sections were digested with hyaluronidase (8000 units/ml) in PBS for 1 hour at 37°C. As the secondary antibody utilized horseradish peroxidase (HRP) for detection, endogenous peroxidase activity was irreversibly inactivated 3% H_2O_2 for 10 minutes. Samples were then rinsed in PBS, blocked with blocking solution containing 3% BSA in PBS for 5 minutes and incubated in primary antibody at a 1:150 dilution in blocking solution over night at 4°C. After addition, the secondary antibody was visualized using a tyramide signalling amplification (TSA) Direct Cy3 kit, according to the manufacturer's protocol. Cell nuclei were visualised with DAPI and mounted with a ProLong Gold anti-fade solution and a glass cover slip. Samples incubated with isotype specific control antibodies were used as controls.

3.10 Factorial design of experiments

Factorial design of experiment (DoE) methods allow for a systematic approach to optimization processes that reduces experimental bias and noise with a limited number of experiments[170] and it is based on defining mathematical relationships between input and output variables in a system. It is used in many parts of the biotechnology field and can be used to optimize e.g. a cell culture medium. As input variables, two or more factors, such as medium additives, are represented at two discrete values: low and high. The factorial designs are often described as a 'factorial 2^n design', where n is the numbers of input variables. Its simplest form with two factors is thus denoted as a factorial 2^2 design and can be visually described as a square setup (Fig. 5). Usually, three or more centre points at mid-concentrations are added to the experimental setup, to validate the stability of the system. The output variables can be everything from cell biomass product yield and productivity to gene expression, as an effect of the input variables. DoE is particularly useful for the optimisation of a system, and to investigate how input variables interact[170,171]. A screening trial is commonly used to define the experimental domain of a research setup. Usually this is done with a fractional factorial design, which is a design with a reduced number of sample points. It allows for the determination of input factors that significantly affect the output variables. This screening process is often followed by optimization steps based on more elaborate designs used to determine true optimal settings for the significant factors. The results are often displayed in response surfaces (RS) that in a visual way show which levels of factors result in the highest or lowest effect on the output variable. For a comprehensive review, read Mandenius *et al*[171].

In study II and III the computer software Modde 8.0 was used to design the factorial experiments, to analyse the results and for the display of results in response surfaces.

In study II, DoE was used to optimize the defined culture medium used to redifferentiate the chondrocytes, to increase yield and purity of the produced biomass by the chondrocytes.

In study III, DoE was used to investigate the interactions between GDF5 and the DKK-1 inhibitor WAY-262611.

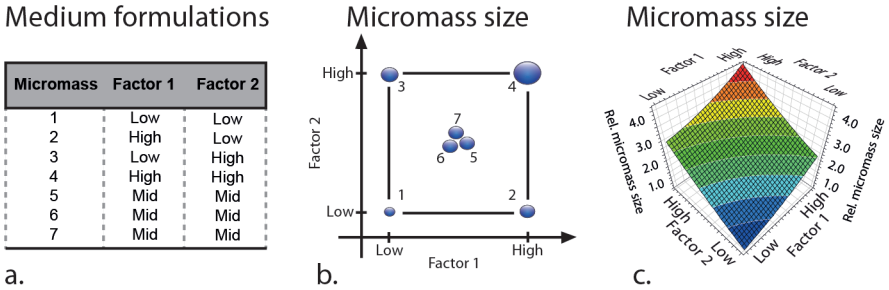

Figure 5. Example of a factorial 2^2 DoE design for optimization of a chondrogenic medium to increase micromass size. a) The medium additives are added at a low, mid or high concentration in seven different medium mixtures. b) Seven micromasses are cultured in the seven separate mixtures, one in each, and the sizes of respective micromass is investigated after 2 weeks in culture, resulting in visual differences. c) Size differences represented in a response surface.

3.11 Statistics

To assess the significance of the results in this thesis, different statistic tests were used. Specific test were selected depending on the type of data and distribution. Samples that had a non-normal distribution were analysed with non-parametric test Mann-Whitney *U* test. Normally distributed data were analysed with Student's t-test, and normally distributed data with multiple groups were analysed with Analysis of variance (ANOVA), and the Tukey post hoc test. Results were expressed with sample mean and standard deviation or 95% confidence interval.

In study I, significance levels were determined with the non-parametric Mann-Whitney *U* test.

For study II-IV, ANOVA was used to assess significance levels where the tests concerned more than two groups, and the Tukey post hoc test. Two-sided Student's t-test was used for normally distributed data and Mann-Whitney *U* tests for non-normal data.

3.12 Ethical approval

All human tissues, cells and serum used in this thesis were donated after informed consent.

Ethical approval to conduct human chondrocyte studies was given by ethical committee at the Medical Faculty at Gothenburg University (Dnr 040-01)

Ethical approval to use human serum for the culture of human chondrocytes was given by the ethical committee at the Medical Faculty at Gothenburg University (Dnr 367-02)

4 SUMMARY OF THE RESULTS

4.1 Study I

In this study, differentiated chondrocytes were exposed to plasma-mediated bipolar radiofrequency ablation, and the effects on viability, proliferation and inflammatory mediator expressions were investigated. The ablation device is clinically used to debride tissue and smoothen rough cartilage surfaces to ease the articulating motion. Viability staining of chondrocytes in alginate gels and in bovine cartilage biopsies revealed a well-defined zone of cell death, ranging 200 μm deep from the ablation-exposed surfaces. Exposing human chondrocytes in alginate gels to the plasma-mediated ablation resulted in an increase in proliferation, determined by quantification of DNA at three and six days after exposure. Further, exposure also increased gene expressions of inflammatory mediators IL6 and IL8. IL6 expression was increased three days after exposure, and elevated levels were maintained also over day six. The IL8 expression showed a burst increase after three days and was reduced again to basal levels after six days. To investigate any degenerative effects of the exposure, the expression of the OA linked inflammatory markers IL1β and TNFα were measured, together with expression of the inflammatory mediator HMGB1. None of these markers were affected by the exposure. Finally, effects on cartilage ECM were investigated through the expression of the degenerative enzyme MMP13 and ECM components COL2A1 and VCAN. None of these markers were affected by the exposure.

4.2 Study II

In study II, factorial DoE and response surface modelling were used to optimize medium content of the standard medium for chondrocyte re-differentiation in micromass cultures, to increased ECM production by the cells. The optimization was performed in a three-step process where each step evaluated how the involved medium factors affected the gene expression of markers of cartilage maturity. The involved markers were SOX9 expression and the expression ratios ACAN/VCAN and COL2A1/COL1A1. The first step in the process was a fractional factorial screen of the factors in the standard medium formulation, resulting in three factors that significantly affected the re-differentiation of the chondrocytes: TGFβ1, dexamethasone and glucose. In the second step, a new design including only the significant factors revealed the optimal level of TGFβ1 to be 13 ng/ml. Dexamethasone

and glucose were optimized in a third step, revealing optimal concentrations for re-differentiation of chondrocytes to be 50 nM for dexamethasone and 5.4 g/L glucose. This resulted in a final medium formulation where TGFβ1 was increased 30%, dexamethasone reduced 50%, and glucose increased 22% compared to the standard formulation. The potency of the optimized medium was validated in a comparative study against the standard medium with surplus cells from five patients undergoing ACI. The optimized medium resulted in micromass cultures with significantly increased expressions of SOX9 and COMP and the ACAN/VCAN ratio. The COL2A1/COL1A1 gene expression ratio was unaffected, as was the COL10A1 gene expression. Further, compared to the standard formulation, the optimized medium resulted in increased production of glycosaminoglycans (GAGs) by the cells as shown by increase in the GAG/DNA ratio. Proliferation of the chondrocytes did not increase after culture in the optimized medium.

4.3 Study III

In the third study, the effect of GDF5 stimulation on the Wnt signalling pathway in human chondrocytes was investigated, together with the expressions of known modulators of cartilage extra cellular matrix.

Stimulating chondrocyte micromass cultures with recombinant human GDF5 resulted in a dose dependent increase in matrix production by the chondrocytes, as shown by GAG/DNA quantifications. The stimulation also resulted in increased proliferation by the cells. An increased expression of anabolic factors was verified with qRT-PCR, revealing upregulation of the SOX9 and ACAN genes. Simultaneously, the stimulation resulted in downregulation of MMP13 and ADAMTS4, indicating decreased matrix degradation. Quantification of amounts of active MMP13 protein in the micromass culture medium revealed reduced protein expression in GDF5 stimulated chondrocyte cultures. Further, after GDF5 stimulation, the chondrocytes showed increased gene expressions of the canonical Wnt signalling pathway inhibitors DKK1 and FRZB, together with increased protein expression of DKK1. Quantifying the amounts of β-catenin in the cells revealed reduced levels of the protein in GDF5 stimulated cells, indicating an inhibition of the canonical Wnt signalling pathway. This inhibition was verified with qRT-PCR, as the canonical Wnt downstream genes FOSL1 and PPARD were downregulated after GDF5 stimulation.

Finally, by blocking the DKK1 protein with a small molecule inhibitor, the suppressed levels of MMP13 were restored in the GDF5 stimulated cultures, verifying that the cause for MMP13 downregulation in these cultures was the inhibition of the canonical Wnt pathway by the GDF5-induced expression of DKK1.

4.4 Study IV

In study IV, chondrocytes from two patients undergoing ACI were reprogrammed to iPSCs using mRNA transfections with synthetic mRNAs encoding OCT4, SOX2, KLF4, C-MYC and LIN28. From 10 000 starting cells, 9 clonal lines were established, resulting in a 0.1% reprogramming efficiency. The established cell lines had high proliferative capacities, and expressed pluripotency markers OCT4, SSEA4, TRA1-60 and NANOG as shown by immunofluorescence staining and qRT-PCR. Karyotyping and genome wide SNP arrays revealed no modifications in the genome after reprogramming. Using EB cultures, pluripotency was verified in all investigated cell lines when outgrowing cells from the EBs on gelatin revealed cells with positive immunohistochemical staining for mesoderm (α-sarcomeric actin), endoderm (HNF3β) and ectoderm (βIII tubulin). Some EBs also developed into spontaneously beating cardiomyocyte clusters (mesoderm) after two weeks in culture, which also showed protein expression of α-sarcomeric actin. Culture of EBs over the course of eight weeks revealed formation of neural epithelium (ectoderm) and columnar epithelium (endoderm). Further, the chondrogenic differentiation capacity of the cells was assessed with two separate protocols. In the first two week protocol, iPSCs in monolayer culture were exposed to varying doses of growth factors in a stage specific manner, thereby guiding the cells through known pathways involved in the development from pluripotent cells to chondrocytes. All investigated cell lines went through the chondrogenic progression, from a stem cell phenotype expressing high levels of markers of pluripotency (*OCT4, NANOG* and *SOX2*) to a chondrogenic phenotype expressing mesodermal and chondrogenic markers (*PDGFRB, SOX6, SOX9, ACAN, COL2A1 types A* and *B*), with an interim stage expressing markers of primitive streak-mesendoderm and mesoderm (*CDH1, GSC, MIXL1*), as assessed by qRT-PCR.

In the second protocol, micromass cultures of iPSCs were used to improve matrix production of the cells. After a pre-differentiation stage, all four investigated iPSC lines showed pre-chondrogenic properties during expansion, however, in a second micromass culture, only one of four lines developed into mature ECM producing chondrogenic cells. This clone showed deposition of proteoglycan ECM at two weeks in culture, which was highly increased at five weeks in culture. The cultures also expressed high amounts of collagen 2 protein and gene expressions of chondrogenic markers *ACAN*, *SOX9* and *COL2A1 types A* and *B* increased throughout the differentiation process, finally reaching the same expression levels as the donor chondrocytes. No expression of pluripotency markers *OCT4* and *NANOG* could be detected after five weeks in culture.

5 DISCUSSION

5.1 Clinical need for assisted healing of cartilage

To date, injuries to the articular cartilage in the knee affect a large number of patients worldwide. Frequencies of injuries increase with age, and OA is one of the most common and symptomatic health problems in middle aged and older people[172]. The numbers of affected patients are expected to increase as the population grows and life expectancy increases[1]. The affected part of the population includes several subgroups, ranging from patients with only minor focal damages to those where entire cartilage surfaces are in need of replacement due to late stages of OA[1,94,173]. As articular cartilage does not heal spontaneously, even the smallest of injuries may progress over time to OA. There are, however, several interventions that hold promise to treat cartilage damages[94,96,97,174]. The goals of these treatments are to reduce symptoms and prevent further degradation, and where possible, restore the cartilage surface with functional repair tissue. There is no general consensus as to which treatment is the best, and methods should be chosen in context to the size and type of cartilage damage, as well as the degree of OA and matrix deterioration. Even though the treatments are in clinical use, there is room for improvement and it is of great value to determine in what manner they function and how they affect cells and tissues on a molecular level. In this thesis, we have further investigated the role of plasma-mediated ablation for cartilage regeneration and how the ACI method can be altered to further promote tissue restoration.

5.2 Plasma-mediated ablation and cell proliferation

For at least 12% of patients with OA in the lower extremities, the development of the disease is a secondary effect of joint trauma, i.e. PTOA[70]. It has been estimated that as much as 44% of patients with articular cartilage fractures develop OA[175], and a generally accepted cause of OA development is the disruption of the smooth articulating surface with lesions and fibrillation that increase wear. It is therefore important to therapeutically smoothen the surface, and for minor defects, both mechanical debridement with a shaving device and plasma-mediated ablation can be used for this purpose[161,162,174]. The mechanical shavers have drawbacks in that it can be

difficult to restore a perfectly smooth surface and that viable tissue is removed along with the unstable or diseased tissue. With the plasma-mediated ablation it is relatively easy to get a smooth surface, but several reports have shown chondrocyte death after the exposure, induced by the heat developed from the devices[176-178]. Similarly, in study I, exposing cartilage biopsies to plasma-mediated ablation caused a defined area of cell death in the tissue close to the exposed area. This effect has been a cause for concern when using these devices, as it damages healthy cartilage[176,178-180], although the molecular events following the ablation have not been thoroughly investigated.

Apart from the cell death, we show in study I that the chondrocytes initiate a short proliferative burst after exposure to the ablation device. This proliferation could stem from either the plasma field itself having a direct effect on a major portion of the cells or from the plasma field affecting a small number of cells locally that excrete soluble factors affecting the main cell population. Looking at the range of cell death, the effect of the ablation appears to be local, only reaching 1/10 of the full cartilage thickness. This implies that the second scenario is the more likely. In several tissues, it has been shown that tissue damage leads to secretion of factors that initiate a compensatory proliferation to maintain tissue homeostasis[181,182]. Compensatory proliferation in chondrocytes is, however, poorly studied, and we could not conclude that the response was of that sort. Despite this, it is intriguing that the detected response could be a part of a compensatory response in the tissue, and that the ablation device could trigger this response, apart from the smoothening of the cartilage surface.

5.3 Plasma-mediated ablation and interleukins

After exposure to the ablation device in study I, the expression of interleukin-6 and -8 (IL6 and IL8) were upregulated by the chondrocytes. Previously, it has been shown that chondrocyte cell death results in cytokine release[183], and that these cytokines regulate the expression of each other[184], which could explain the upregulation in this study. Further, the plasma field formed in the ablation procedure release reactive oxygen species, such as hydroxyl radical and nitric oxide, which in turn have been shown to produce both IL6 and IL8 in chondrocytes[185,186]. The altered expression of cytokines due to the ablation is not exclusive to articular chondrocytes, as in two earlier studies, O'Neill *et al.*[187] and Rhyu *et al.*[158] have shown that plasma-mediated ablation results in altered expression of cytokines also in porcine disc cells.

The role of IL8 in cartilage is poorly studied. In other tissues, it is known to have an anabolic effect, mobilising progenitor cells and inducing wound

healing[188,189]. The roles of IL6 in cartilage are more elaborately studied, although the results diverge. It has been shown to induce proteoglycan degradation in cartilage tissue[190], but also to have a protective role against oxygen radicals and an inhibitory effect to MMP expression via increased TIMP production[191-193]

In the first applications of ACT, the chondrocytes were confined in the defect by a patch of periosteum sutured over the defect. This periosteum has a chondrogenic effect[194] and Brittberg *et al.*[195] have shown that co-culture of chondrocytes with periosteum induces both IL6 and IL8 expression in the chondrocytes, suggesting the anabolic role of the interleukins. Recently, studies in our group have revealed that IL6 induces GDF5 expression in equine chondrocytes (*in manuscript*), and in the light of study III in this thesis, where we show that GDF5 inhibit cartilage matrix degradation, the role of IL-6 as a protective interleukin is strengthened.

In OA, increased proliferation is part of the first events in the development of the disease, along with an altered interleukin expression, including IL-6 and IL-8 expressions[73,196]. The effects of plasma-mediated ablation seen in study I could therefore be an indication of an early onset of OA. However, IL1β, TNFα and MMP13, the most important factors that are upregulated in OA, were not affected after exposure. Taken together, the results imply that using the ablation device to treat minor cartilage defects, apart from the smoothening of the surface, it can also have an anabolic or protective biological effect.

5.4 Chondrocyte dedifferentiation and population doublings

With increased size of focal damages, to only debride the injured cartilage is not enough to prevent tissue degeneration. The defect void also needs to be filled with a new matrix. The ACI procedure is one of the most promising technologies used for this purpose, as it may allow for the formation of hyaline-like cartilage[102]. The low cellularity of the tissue does, however, entail an expansion of the chondrocytes, as the isolated cells are not enough in numbers to produce an implantable construct. As described earlier, the cells go through a process of dedifferentiation during this expansion. The most pronounced profile changes appear during the first 1-4 passages, and it is an ongoing process with continuous reduction in redifferentiation potential[197,198]. Extended expansion results in that the redifferentiation capacity is lost altogether, as is the possibility to use the cultured chondrocytes in a regenerative procedure[129]. A minimal number of cell divisions is thus advantageous, which unfortunately counteracts the purpose of the expansion. Reduced expansion is, however, possible if the ECM

production of each cell is increased. Fewer cells could then be utilised to fill the scaffold with ECM. In study II, a statistical design of experiments approach was used to improve a medium used for the redifferentiation of chondrocytes.

5.5 The Design of Experiments approach

The gold standard medium used for chondrogenic differentiation was determined 15 years ago for the differentiation of mesenchymal stem cells into chondrocytes[45,199]. The medium, relatively simple in composition, contains six additives in a high glucose basic medium. The optimal concentrations of the additives were determined one factor at a time in different species[200-206] and then combined in the chondrogenic medium. Combining different stimulatory into one medium can, however, change their effect as interactions may occur[170,207]. In study II, we optimised the six additives for the differentiation of human chondrocytes simultaneously, increasing the chance of discovering how multiple stimulatory cues affect the cells and how they interact. Interestingly, no synergistic effects were discovered in this process, although new optimal settings for three of the additives were determined, resulting in an increased amount of ECM produced per cell. Arguably, since there were no apparent synergistic effects, these new concentrations would likely have been discovered also in one-factor-at-a-time studies. However, this would have been more time and resource consuming.

As chondrocytes have a multipotent differentiation capacity, with the ability for form not only cartilage but also bone and fat tissues after dedifferentiation[208], it is important that the differentiation protocol induces production of the correct ECM. The ability to in a controlled fashion vary several factors at the same time makes DoE a powerful tool to affect a specific gene or set of genes, and to induce cells to elicit a specific response. This allows for tailoring of, for example, properties such as ECM content, cellularity and mechanical integrity of tissue engineering constructs. Previously, Pritchett *et al.*[209] similarly demonstrated this power of DoE by inducing a specific response in yeast cells, targeting glycosylation of cystatin C, although at the expense of productivity. In study II, we successfully managed to increase the expression of articular cartilage ECM. However, it was not possible to increase both collagenous and non-collagenous parts of the ECM with the factors included. The reason for this is difficult to elucidate, and further studies are required. If at all possible, to further increase matrix deposition would require the addition of other growth factors or other stimuli to the system[210,211]. In proceeding steps, new growth factors known to induce chondrogenic differentiation, such as GDF5[StudyIII,212] or

BMP2[125] could easily be implemented in new factorial designs. In a similar manner, DoE could also be a good instrument for the induction of specific differentiation of other cell types, such as ESCs, MSCs and iPSCs.

5.6 Off-the-shelf cell line

There are still some inherent problems in using chondrocytes as the cell source in the ACI procedure, even after optimized ECM production. As mentioned, the low cellularity of the tissue could have implications for the treatment, due to the restricted proliferative capacity of the chondrocytes, and the dedifferentiation they undertake during this proliferation. There is also a patient dependent variation in quality of the cells, the biopsy in itself is an additional injury to the cartilage surface, and finally, the patient has to undergo two surgical procedures, one for the retrieval of the biopsy and one for the implantation of the cells. Finding a separate cell source, with large inherent proliferative capacity and the ability to form cartilage would be a leap forward in the development of the ACI procedure. The iPSCs could be such a cell source, as we[Study IV] and others[112,213,214] have shown, although efficient complete chondrogenic differentiation is rather difficult to obtain. With the iPSC technology, such personalised stem cells lines are within reach. The future goal for cartilage cell therapy would, however, be an "off-the-shelf" product, a universal donor cell line that, in combination with the proper biomaterial scaffold, could be used instead of the autologous cells. There are several advantages with such a cell line, including reduced variability in cell quality and reduced number of required surgeries. This goal is made possible by the advantage of articular cartilage as an immune-privileged tissue due to the lack of vascularization, which implies that the cell source is not required to be autologous[215,216]. Also in this respect iPSCs hold great promise.

Apart from a high proliferative capacity and the ability to form functional cartilage tissue, an off-the-shelf cell line has to be readily available and clinically safe. The inherent capacity of iPSCs to form tissues from all three germ layers also implies problems, as an incomplete or reversible differentiation of the cells before implantation could result in tumour formation after implantation[123].

5.7 Chondrogenicity of iPSCs

In study IV, we reprogrammed both chondrocytes and fibroblasts into iPSCs through mRNA transfections. Both cell types were then subjected to two differentiation protocols to assess their chondrogenic capacity. The first protocol was based on a previous publication by Oldershaw *et al.*[119] for the

differentiation of ESCs to chondrogenic cells, and was carried out in monolayer culture. After adaption to feeder free culture conditions, the cells were directed through a stepwise differentiation, in an effort to mimic development from the pluripotent stage through primitive streak mesendoderm to mesoderm intermediates to chondrocytes. This was performed through the addition of a series of growth factors that have been shown to induce the different stages of the chondrogenic differentiation *in vitro*. First, we activated the canonical Wnt pathway with a Wnt3a stimulation for the development into primitive streak formation and mesendoderm[27,28], then through BMP stimulation for induction into mesoderm[217], and finally GDF5 stimulation, which is important during mesenchymal condensation and formation of the cartilage anlagen[218]. Through these stimulations, in the end, proper gene expression profile was achieved and chondrogenic cells were obtained. A drawback with this method was however that despite the proper gene expression pattern, there was an apparent lack of ECM production, a trait necessary if the cells are to be used in regenerative applications.

In vivo, there is a vast amount of biochemical cues that are carefully orchestrated to form limbs and joints, and all events of this process are still not known[30,31]. Mechanical and structural cues are just as important as chemical, and for formation of the limb, the mesenchymal condensation is essential[30]. The reason for the incomplete differentiation in the first protocol could be that we were able to mimic the proper differentiation pathway up to the formation of the anlagen, but after that the monolayer culture did not allow for the correct cellular interactions to take place. The second protocol used in study IV therefore included a three-dimensional micromass culture in an effort to mimic the condensation. With this protocol we were able to induce good chondrogenic differentiation, even with simplified growth factor stimulation using only TGFβ1. The protocol resulted in considerable ECM formation by the cells, thereby showing the importance of high cell density in chondrogenic differentiation.

It is, however, important to note that the protocol is in need of further optimization as only 25% of the investigated cell lines differentiated into a chondrogenic phenotype. Unfortunately, the cells also expressed collagen X, indicating a differentiation process towards endochondral ossification[7,14,22]. This could possibly be avoided by adding GDF5 to the culture as this may prevent hypertrophic condensation as shown in study III. Combining the two protocols used in study IV could thus be a solution to the present issues, increasing the chondrogenic differentiation percentage with the first protocol, and mimicking condensation with the second.

5.8 Chondrogenic differentiation downregulates pluripotency markers

The use of ESCs and iPSCs in clinical studies is limited by their ability to form teratomas after *in vivo* implantation[123]. The tumorigenic capability of these stem cells is associated with expression of specific genes of stemness, including genes involved in self-renewal and pluripotency, such as OCT4, SOX2, KLF4, c-MYC, and NANOG[219-222]. These genes include the OKSM factors used is iPSC reprogramming, and after reprogramming the genes are expressed at high levels in the iPSCs, indicating high tumorigenicity[220,223,224]. The expression was, however, rapidly downregulated during the 3D differentiation process. The downregulation also occurred in the Oldershaw monolayer differentiation, albeit in a more gradual fashion. These results are indicative of an abolished tumorigenicity after *in vitro* differentiation, which is an essential trait if the cells are to be used in a clinical setting. Similar effects have been reported previously in retinal and neural differentiation[225,226]. It is, however, important to make certain that no undifferentiated cells remain in the differentiated population, as these cells would display the tumorigenic potential, and even one single cell that remains with these traits could develop into a tumour[227]. It is thus essential to perform *in vivo* studies to examine the tumorigenic nature of the chondrogenic cells derived in study IV, to determine their clinical safety. An indication of their safety was that the proliferative capacity of the cells was highly reduced during the pre-differentiation stage, suggesting that the whole cell population had undergone a diversion from the pluripotent stage.

In this respect, the usefulness of a non-integrative method to reprogram cells should be noted. Using viral vectors, the insertion of the tumorigenic genes into the genome is random. This could lead to unexpected activation of these genes, years after implantation[137]. In study IV, the mRNA transfection allowed for iPSCs without any remnants of the reprogramming factors, securing a stable genome.

5.9 Homogeneity within iPSC populations

In study IV, the iPSC lines that were more deeply investigated showed a similar pluripotent capacity with unlimited proliferation and ability to form teratomas, despite a rather different expressional degree of pluripotency markers. Interestingly, during the chondrogenic differentiation, the different lines showed different capacities for chondrogenic formation. Studies have shown that cells from different tissues or of different ages are more or less easy to reprogram, and once reprogrammed, the cells have different capacities to differentiate into adult tissues and to form teratomas[228-230]. These

abilities are believed to partly be caused by DNA methylation patterns. Methylation controls gene activity through direct methylation of transcription factor genes and through repressor proteins that bind to methylated DNA[231]. These methylations have different patterns in different cells and control the expression of genes and thus the phenotype of a cell. The methylation patterns are also called the epigenetic memory of a cell. During reprogramming, the DNA is demethylated, thereby erasing epigenetic memory. This process is, however, slow and inefficient and varies from cell to cell, allowing for some patterns to remain even after completed reprogramming, and parts of the epigenetic memory are therefore suggested to remain[228]. Due to these, chondrocyte derived iPSCs should have a better chance of being differentiated to a chondrocyte, than fibroblast derived iPSCs. These effects have been shown previously by Marchetto *et al.*[232] and Kim *et al.*[233] in other cell types. In study IV, the A2B chondrocyte iPSC clone showed an outstanding ability to form cartilage, when compared to the other chondrocyte iPSC clones and the fibroblast iPSC clone, and variation in remaining epigenetic memory could be a reason for these differences. Interestingly, this clone also showed the highest expressions of pluripotency markers, indicating that it also retained a greater stemness during the reprogramming.

The variation in differentiation capacity could also stem from differences in the original chondrocytes, as they are harvested from a full thickness biopsy with several different subpopulations[234]. It has been shown that cells from the different subpopulations have different redifferentiation capacities[235]. The different clones could thus stem from the different chondrocyte subpopulations that, already before reprogramming, could possess varying differentiation capacities.

5.10 Using iPSCs for the modelling of OA

The iPSCs have, since the first fibroblast, been reprogrammed from several cell types[133,134,226] and from patients with various diseases, such as amyotrophic lateral sclerosis[142], Parkinson's disease[236] and spinal muscular atrophy[237]. One of the main goals with these disease specific cells is to study the development of the diseases. The cells are reprogrammed to iPSCs, and then differentiated to the adult stage once more. At some point during this differentiation, the diseased cells will, hopefully, diverge from the correct differentiation path and develop the diseased phenotype. Monitoring these differentiation processes could supply important information on disease development[230]. In this fashion, osteoarthritic cells could be used to model development of OA. These methods are, however, likely to be most efficient for diseases that have an early onset and that have a high genetic

penetrance[230]. As OA is a multifactorial disease where age, mechanical stimuli and environmental factors play important roles, the mechanisms involved may be difficult to elucidate.

Despite this complexity, some of the molecular signalling structures involved could still be determined with this technique. The role of genetic susceptibility in OA, including mutations in the GDF5 and FRZB genes[55,86], is an important target, and that osteoarthritic cells expanded *in vitro* show apparent phenotypic and gene expression pattern differences from healthy cells[238,239] open up for interesting possibilities. That the iPSCs from study IV could contain epigenetic memories of their origin is an intriguing thought, although an analysis of the full gene expression pattern of the cells is required, along with the methylation patterns of the DNA to verify this.

Further, the lack of knowledge of the underlying molecular mechanisms leading to OA have made it difficult to determine effective pharmacological treatments in the past[64,74]. Having access to iPSCs could open up for possibilities to find molecular markers that are indicative of disease onset, which would enable earlier detection of the disease *in vivo*, and perhaps the discovery of putative drug targets.

5.11 GDF5 inhibits catabolic processes in articular chondrocytes

Several signalling pathways are interrupted in the OA tissue, leading to the ECM degradation. Both canonical Wnt signalling and GDF5 signalling are important during embryogenesis and have implications on cartilage ECM maintenance[55,59,62].

In study III, we stimulated human chondrocytes in micromass cultures with recombinant human GDF5. The stimulation led to an anabolic effect in the cells, with increases in ECM deposition and proliferation. These results were in agreement with earlier studies by Chubinskaya *et al.*[212] and Bobacz *et al.*[240]. Interestingly, the stimulation also caused a downregulation of MMP13, thereby inhibiting matrix degradation. Previously, other members of the TGFβ superfamily have been shown to prevent cartilage matrix degradation[212,241], and Badlani *et al.*[242] specifically showed that BMP-7 reduced expression of MMP13 in rabbits. The signalling pathways that mediated the reduction were however not further elucidated. In study III, we show an upregulation of the canonical Wnt inhibitors DDK1 and FRZB by GDF5 stimulation, and a simultaneous inhibition of the canonical Wnt pathway. The MMP13 inhibition turned out to be dependent on the DKK1 expression, as small molecule inhibition of DKK1 abolished the GDF5 induced MMP13 inhibition. Future studies are needed to determine if the

other members of the TGFβ family function via the same pathway in their cartilage-protective roles.

GDF5 has previously been implemented to play a role in OA development in several ways. Rountree *et al.*[243] showed that absence of the GDF5 receptor BMPRI leads to a reduced GDF5 response, and due to that the development of OA in a mouse model. Further, in the analyses linking an SNP in the GDF5 gene to OA development, the effect of the SNP is a reduced promoter activity of the GDF5 gene, resulting in reduced GDF5 mRNA levels[56]. How the altered GDF5 expression can cause OA has not been determined, partly because downstream signalling of GDF5 has not been thoroughly investigated. The downregulation of MMP-13 expression by GDF5 shown in study III indicates that GDF5 could counteract the degenerative processes in OA. These results further suggest that the reduced expressions of GDF5 caused by the SNP could lead to impaired regulation of MMP-13 and canonical Wnt signalling, which in turn could help explain why these patients develop OA.

5.12 GDF5 as a pharmacological treatment

Today, there are no approved therapies that can modify OA progression or the structural degradation of the cartilage tissue that it implies. Some symptom modifying treatments exist, however most of them lack any potent long-term effects[64,83,244]. Lack of knowledge of the underlying molecular mechanisms makes it difficult to determine effective pharmacological treatments[64,74].

Previously, several ultimately unsuccessful efforts have been made to combat OA through inhibition of MMPs, with both natural and synthetic inhibitors. The natural inhibitors had difficulties in accessing the tissue or did not have sufficient inhibition capacities[245,246]. The synthetic variants, although effective, suffer from adverse side effects such as being too potent, inhibiting physiological levels of MMPs and thereby causing musculoskeletal toxicity and pain[247,248]. In this respect, GDF5 could be a natural source for MMP13 inhibition, although its *in vivo* potential needs to be verified. The application could also, apart from MMP13 inhibition, induce the chondrocytes to re-enter an anabolic state and rebuild the degraded tissue. In a study by Simank *et al.*[249] a scaffold releasing GDF5 was introduced into rabbit cartilage osteochondral defects and over the course of 24 weeks the healing response was increased, compared to control. The study did not further elucidate the molecular mechanisms involved, and attributed the results to the anabolic effects of GDF5. With our results in mind, it is however intriguing to consider that GDF5 not only supports or induces the anabolic process, but could also play important role in the inhibition of MMP13. This further

implies that GDF could be used as a putative OA disease-modifying drug. Other members of the BMP family are already in clinical use, such as BMP2 and BMP7, and are considered therapeutically safe[250-252], which could simplify the clinical applications of GDF5.

6 CONCLUSIONS

In study I, the plasma-mediated ablation induced a short-term proliferative response in human chondrocytes cultured in alginate gel, and an altered interleukin expression. The ablation device also caused a defined area of cell death reaching 200 μm into the tissue from the exposed surface. The results suggest a potential of plasma-mediated ablation to induce an anabolic response in human articular chondrocytes.

In study II, the standard medium used for chondrocyte redifferentiation was optimized in a three-stage process using factorial design of experiments. The optimized medium resulted in an increased ECM production of the cells. Increasing the productivity of the chondrocytes could possibly increase the defect size that can be treated with the chondrocytes retrieved from a biopsy, and the DoE methodology proved to be an efficient tool for the optimization process.

In study III, the effect of GDF5 on cartilage ECM homeostasis was investigated. The GDF5 stimulation resulted in increased ECM production by the cell and decreased matrix degradation. The reduced expression of matrix degrading enzymes was mediated by an induction of DKK1 expression by the GDF5 stimulation, and the following inhibition of the canonical Wnt signalling pathway. The results shed further light on the role of GDF5 in the progression and development of OA and imply that it could be a putative disease-modifying drug.

In study IV, human chondrocytes were reprogrammed into iPSCs through use of mRNA transfections. After extended passaging, the cells were induced to differentiate into chondrogenic cells, displaying good chondrogenic capacity. The results show that the c-iPSCs represent an interesting platform for the generation of experimental models to study cartilage regeneration and disease. They could also serve as a cell source in tissue engineering applications, as soon as their *in vivo* safety is verified.

Taken together, this thesis reflects upon the intricate problem of a damaged cartilage surface, and that it is difficult to find one method that alone can resolve the questions on how to regenerate the tissue and how to prevent degeneration. Instead tissue damage and the multifactorial OA disease need to be addressed in a multifactorial manner, combining several different regenerative methods. This thesis has, though the discovered results provided further knowledge that can help improve the healing of cartilage.

7 FUTURE PERSPECTIVES

In this thesis, we took one step closer to a personalized cell line to be used in an improved ACI procedure. The reprogramming technology allowed for the formation of iPSCs that are good candidates for such a cell line, although *in vivo* studies are essential in the applicability of these reprogrammed cells, to ascertain their chondrogenic potential. Cell survival and the properties of the synthesized matrix are important aspects that need to be addressed.

The reprogramming of adult cells to the stem cell stage also implies several safety issues. With the high expression of oncogenes and unlimited proliferative capacity, the risk of teratoma formation is an ever-present problem and a threat to their feasibility in clinical applications. In depth clinical studies could also in this case answer many important questions.

The technique makes new research areas possible, including transdifferentiation of adult cells. In the context of this thesis, it would be interesting to induce a chondrogenic phenotype in a cell type that is readily accessible, has a high proliferative capacity, but that is not pluripotent. The skin fibroblast is in this respect an interesting cell type. To obtain an autologous biopsy is a small matter, and the transfection of powerful chondrogenic factors, such as SOX9, into these cells could allow for a transdifferentiation into a chondrogenic cell line. It is likely that other or additional factors are needed for this procedure to work, but a successful transdifferentiation would open up for huge possibilities in the regenerative medicine field.

To be able to use cells in a large scale industrial like production of tissues, factorial design of experiments could prove to be useful, assisting in the finding of the proper scaffold and growth factor cocktail for successful cell differentiation and treatments. The methodology could also be valuable when determining how different drug treatments affect diseased cells and how different drugs can interact.

Finally, OA is one of the major hurdles to tackle when it comes to cartilage regeneration, and will continue to be a difficult issue even in the presence of personalized or off-the-shelf chondrogenic cell lines. The multifactorial nature and the slow development process requires more and deeper studies, targeting cell signalling processes in efforts to find early markers of OA onset and to find treatments. GDF5 has in this thesis showed to be a putative disease-modifying drug, although the safety and in vivo potential needs to addressed. Utilizing scaffolds with a slow release of GDF5 could be a good way to deliver it locally in the joint, targeting mainly the chondrocyte population, and simultaneously providing an improved surface for the articulating motion, reducing further mechanical wear and supporting the healing articular cartilage.

ACKNOWLEDGEMENTS

This work has been carried out at the department of clinical chemistry and transfusion medicine, institute of biomedicine, Sahlgrenska university hospital, Gothenburg, Sweden. I wish to express my sincere gratitude to everyone who has contributed to this thesis.

In particular, I would like to thank:

Anders Lindahl, my supervisor for giving me the opportunity to work on this project, for your open mind and enthusiastic view on science, and for the freedom to pursuit my own ideas. It has truly been a learning experience!

Mats Brittberg, co-supervisor for fruitful scientific discussions, and for all the excellent and quick support throughout these years.

All my co-authors and all former and present colleagues at clinical chemistry and transfusion medicine, in the molecular cell biology research group, the cell and tissue laboratory and the BIOMATCELL project, especially Petra Bergström for being an excellent (former) room mate and for the scientific table tennis; Camilla Brantsing and Marianne Jonsson for your help with qRT-PCR and molecular biology; Hanna Stenhamre for the introduction to the field; Narmin Bigdeli for your help with the stem cells; Pia Andersson, Catherine Concaro, Margret Hagman and Marie Leander for your chondrocyte knowledge and all the cells; Alma Forsman, Eva Skiöldebrand, Stina Simonsson and Ruth Wickelgren for your research input; Vivien Lettry for interesting discussions and for showing me how to take a bone marrow biopsy from a horse; Julia Asp, Joakim Sandstedt, Mikael Sandstedt and Kristina Vukušić for showing me that heart cells can also be interesting…; Cecilia Boreström for proofreading the thesis and for fruitful discussions on past, present and future research.

Anna Thorfve and Emilia Svala, for your encouragement, calm nerves and computer skills; Cecilia Granéli for your encouragement and for making the great Chicago trip even better; Josefine van der Lee for your support and discussions on the important issues.

Johan Stenberg, for proofreading the thesis. Your support, ideas and friendship have been invaluable through all this.

My amazing friends, for all the good times!

Mamma och pappa, vissa saker är omöjliga att översätta. Tack för att ni alltid stöttar mig och för matjorden i mina fickor.

Linus, my brother, for all the things you have taught me, and for what I have yet to learn!

Karin, for all your love. I would be lost without you.

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