On the role of signalling pathways in the pathogenesis of osteoarthritis

Johan Stenberg

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

UNIVERSITY OF GOTHENBURG
2014
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johan.stenberg@gu.se, johanostenberg@gmail.com

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

Correspondence:
Johan Stenberg
Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Bruna Stråket 16
SE-41345 Gothenburg
Sweden


Printed in Gothenburg, Sweden 2014
Ineko AB
…ulcerated cartilage is a troublesome thing and that when destroyed, it is not recovered.

William Hunter, 1743
On the role of signalling pathways in the pathogenesis of osteoarthritis

Johan Stenberg
Department of Clinical Chemistry and Transfusion Medicine
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg
Gothenburg, Sweden

ABSTRACT

The problem with degenerating cartilage tissues is one of the major causes for disability worldwide. The aetiology of the cartilage degenerating disease osteoarthritis is elusive and considered to be multifactorial. The aim of the present thesis was to find new hypotheses regarding the aetiology of osteoarthritis with focus on signalling pathways. In particular, the conducted studies described expressional differences in different grades of cartilage extracellular matrix degradation and in chondrocytes used in successful and failed autologous chondrocyte implantations. These studies were conducted in order to generate new targets for studies of osteoarthritis aetiology and investigate putative biomarkers that could predict clinical outcome of autologous chondrocyte implantation. Further, the role of the osteoarthritis associated growth factor growth differentiation factor 5 in cartilage homeostasis was investigated. Finally, a non-laborious embryoid body culture system for further investigation of the effects of different factors on chondrogenesis was developed.

The different grades of cartilage tissue degradation revealed expressional patterns that may add to the knowledge regarding osteoarthritis aetiology and/or be further investigated for their role as diagnostic markers. There are no apparent differences in gene expressions between chondrocytes used in successful or failed autologous chondrocyte implantations indicating that the cells are seemingly alike before the procedure, which questions the demand for a potency measurement on the cells based on gene expression. Growth differentiation factor 5 showed to balance degenerative processes in
differentiated chondrocytes through inhibiting the collagen II degrading enzyme matrix metallopeptidase 13 via inhibition of the canonical Wnt signalling pathway. This finding further emphasizes the putative role of growth differentiation factor 5 as a future disease-modifying drug against osteoarthritis. The developed three-dimensional culture system improved the formation efficiency and stability of embryoid bodies in a non-laborious way. The culture system may be useful when investigating the role of signalling pathways in early chondrogenesis in the future.

The present thesis adds to descriptions and explanations of the mechanisms behind osteoarthritis and presents a non-laborious embryoid body culture system to investigate questions that can be raised based on results from this thesis.

**Keywords:** Cartilage, osteoarthritis, signalling pathways, Wnt signalling, GDF5 signalling, embryoid body, proteomics, genomics.

**ISBN:** 978-91-628-8967-8
det kliniska resultatet av operationen. GDF5-stimulering resulterade i minskad nedbrytning av broskvävnad via en signalväg som tidigare visat sig betydande för sjukdomsmekanismen bakom osteoartrit. Slutligen visade sig agaros vara ett lyckat stödmaterial för att kunna odla embryonala stamceller från människa på ett effektivt sätt för framtida studier av signalvägar som kan förklara sjukdomsmekanismen bakom osteoartrit. Sammanfattningsvis har denna avhandling utökat kunskapen kring mekanismen bakom osteoartrit och utvecklat ett experimentsystem för att besvara de nya frågor som resultaten gav upphov till.
LIST OF PAPERS

The present thesis is based on the following communications, referred in the text by their Roman numerals.

I. **Quantitative proteomics reveals regulatory differences in the chondrocyte secretome from human medial and lateral femoral condyles in osteoarthritic patients**

II. **Clinical outcome three years after autologous chondrocyte implantation does not correlate with the expression of a predefined gene marker set in chondrocytes prior to implantation but is associated with critical signaling pathways**

III. **GDF5 reduces MMP13 expression in human chondrocytes via DKK1 mediated canonical Wnt signaling inhibition**

IV. **Sustained embryoid body formation and culture in a non-laborious three dimensional culture system for human embryonic stem cells**

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<tbody>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ACAN</td>
<td>Aggrecan</td>
</tr>
<tr>
<td>ACI</td>
<td>Autologous chondrocyte implantation</td>
</tr>
<tr>
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<td>Activin A receptor type II-like 1</td>
</tr>
<tr>
<td>ACVR2A</td>
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<td>ALK-7</td>
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<td>AMHR</td>
<td>Anti-Mullerian hormone receptor, type II</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
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<td>Description</td>
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<td>--------------------------------------------------</td>
</tr>
<tr>
<td>APMA</td>
<td>p-aminophenylmercuric acetate</td>
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<td>B2M</td>
<td>Beta-2-microglobulin</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Carnitine deficiency-associated gene expressed in ventricle 3 homolog (mouse)</td>
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<td>COL10A1</td>
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<td>COMP</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<td>CYTL1</td>
<td>Cytokine like protein 1</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenyldole</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf Wnt signalling pathway inhibitor 1</td>
</tr>
<tr>
<td>DMD</td>
<td>Dystrophin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DoE</td>
<td>Design of Experiments</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DVL-1</td>
<td>Dishevelled segment polarity protein 1</td>
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<tr>
<td>EB</td>
<td>Embryoid body</td>
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<td>EFEMP1</td>
<td>EGF containing fibulin-like extracellular matrix protein-1</td>
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</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosrobent assay</td>
</tr>
<tr>
<td>ERG</td>
<td>v-ets avian erythroblastosis virus E26 oncogene -homolog</td>
</tr>
</tbody>
</table>
ES  Embryonic stem
EtOH  Ethanol
FABP3  fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
FAIM2  Fas apoptotic inhibitory molecule 2
FGFR3  fibroblast growth factor receptor 3
FOSL1  FOS-like antigen 1
FRZB  Frizzled related protein
FSTL1  Follistatin-like 1
FZD  Frizzled
GAG  Glycosaminoglycan
GCLC  Glutamate-cysteine ligase, catalytic subunit
GDF5  Growth differentiation factor 5
GRIK2  Glutamate receptor, ionotopic, kainate 2
GSEA  Gene set enrichment analysis
GSK3β  Glycogen synthase kinase 3β
HA  Hyaluronic acid
HepG2  Hepatocellular carcinoma cell line
hESC  Human embryonic stem cell
HOX  Homeobox
bFGF  Basic fibroblast growth factor
IGFBP7  Insulin-like growth factor binding protein 7
<table>
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<td>insulin-like growth factor binding protein 4</td>
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<td>Immunohistochemistry</td>
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<td>IL-1β</td>
<td>Interleukin-1β</td>
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<td>Interleukin 3</td>
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<td>IFNB1</td>
<td>Interferon, beta 1</td>
</tr>
<tr>
<td>IFNK</td>
<td>Interferon, kappa</td>
</tr>
<tr>
<td>IKDC</td>
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<tr>
<td>IL8</td>
<td>Interleukin 8</td>
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<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenium</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KOOS</td>
<td>Knee Injury and Osteoarthritis Outcome Scores</td>
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<tr>
<td>KS</td>
<td>Keratan sulphate</td>
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<tr>
<td>LEF</td>
<td>Lymphoid enhancer factor</td>
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<tr>
<td>LRP 5/6</td>
<td>Low-density lipoprotein receptor related protein 5 / 6</td>
</tr>
<tr>
<td>acronym</td>
<td>full name</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>LYST</td>
<td>Lysosomal trafficking regulator</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMP13</td>
<td>matrix metalloproteinase 13</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>N-cadherin</td>
<td>Neural cadherin</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin-Streptomycin</td>
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<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<td>PHLDA1</td>
<td>Pleckstrin homology-like domain, family A, member 1</td>
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<td>PPARD</td>
<td>Peroxisome proliferator-activated receptor delta</td>
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<tr>
<td>PRLR</td>
<td>Prolactin receptor</td>
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<tr>
<td>PTPRF</td>
<td>Protein tyrosine phosphatase, receptor type, F</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Secernin 1</td>
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<tr>
<td>SECI</td>
<td>Subjective evaluation of clinical improvement</td>
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<td>SMAD</td>
<td>Mothers against decapentaplegic homolog</td>
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<td>Full Name</td>
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<td>-----------</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SOX5</td>
<td>Sex determining region Y-box 5</td>
</tr>
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<td>SOX6</td>
<td>Sex determining region Y-box 6</td>
</tr>
<tr>
<td>SOX9</td>
<td>Sex determining region Y-box 9</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein, acidic, cysteine-rich (osteonectin)</td>
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<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor β</td>
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<td>Transforming growth factor, beta receptor II</td>
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<td>TIMP3</td>
<td>Tissue inhibitor of metallopeptidase 3</td>
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<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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# INTRODUCTION

Freedom of movement is made possible through the musculoskeletal system where the bones articulate through joints. Joints consist of bone, articular cartilage covering the articulating bone surface, synovium, synovial fluid and ligaments and may be considered as an integrated organ\(^1\). Joint pain is a common trait of various joint diseases and causes disability to many individuals around the world with high economical costs to the society due to healthcare expenditure and loss of work ability\(^2,3\).

## 1.1 Synovial joint development

As all organogenesis, joint formation is a continuous process but can for descriptive purposes be divided into separate stages that are dependent on different stimuli and signalling pathways. Joint formation originates by formation of the lateral plate mesoderm during embryonic development and the subsequent migration of mesoderm-derived mesenchymal stem cells (MSC) to sites of bone formation\(^4\). The joint formation process can roughly be divided into three stages; pre-cartilaginous condensation also called mesenchymal condensation, formation of the interzone and the essential morphological event of cavitation. During mesenchymal condensation the cartilaginous skeletal anlagen are formed, determining the characteristics of the skeletal elements\(^5\). Before the condensation process MSCs express collagen type I (COL1) and hyaluronic acid (HA), which effectively keeps the cells separated, preventing cell-cell interactions. During the condensation phase the cells change their gene expression pattern together with an increased hyaluronidase activity resulting in a lowered amounts of HA which brings the cells closer to each other enabling cell-cell interactions through gap junctions\(^6-8\). Neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) are essential for cell-cell adhesion during mesenchymal condensation. These proteins are mainly found in the perichondrium at later stages and are vital for chondrogenesis as disturbing the function of these proteins negatively affects chondrogenesis\(^9-12\).

The SRY (sex determining region Y)-box 9 gene (SOX9) has an important role as a signalling agent in the condensation phase. Without SOX9 the condensation phase does not proceed and there is increased apoptosis in the mesenchyme. As a stimulant of condensation, SOX9 has been proposed to increase expression of N-cadherin\(^13\). However, unpublished data suggest that SOX9 is dispensable for the expression of N-cam and N-cadherin as a double
SOX9 knock out mouse showed comparable expression levels of the adhesion molecules compared to control\(^{14}\). Nevertheless SOX9 is one of the key signalling agents in joint formation and cartilage development as it regulates expression of important cartilage extracellular matrix (ECM) components as Col2a1, Col1a2 and aggrecan (ACAN)\(^{15,16}\). Sox9 also seems to be important after the mesenchymal condensation since mouse embryos experience a generalized chondrodysplasia if Sox9 is deleted after the mesenchymal condensations\(^{14}\). Finally, SOX9 has been proposed to act in consort with SOX5 and SOX6 to promote chondrogenesis \textit{in vitro}\(^{17}\).

The chondrogenic growth factor TGF-\(\beta\) positively regulates expression of SOX9, data thus connect these two important chondrogenic growth factors\(^{18}\). Furthermore, TGF-\(\beta\) mediate important cell-matrix interactions during mesenchymal condensation through induction of fibronectin, a glycoprotein highly expressed during the condensation phase and is believed to regulate N-CAM expression and control migration of MSCs\(^{19,20}\).

Joint sites are unrecognizable during mesenchymal condensation and little is known about how joint site cells initiate formation of the joint. Holder showed that if the tissue at the site of the future interzone is removed, the chick elbow joint will not form suggesting that cells at joint sites are pre-specified and develop in an autonomous fashion\(^{21}\). The BMP antagonist Noggin has been shown to be important for joint formation as ablation of Noggin in mouse embryos disrupts joint formation\(^{22}\). Also, the homeobox (Hox) genes have been proposed to determine joint sites and especially intersection points of different Hox gene expressions have been of interest\(^{23}\).

Joint forming sites are subsequently characterized by a local dedifferentiation, where closely packed cells regain a mesenchymal phenotype with morphological changes into an elongated shape. Loss of collagen type II expression and induction of collagen type I expression mark initiation of the interzone phase\(^{24}\). Cells within the interzone have been described as structurally divided into an intermediate zone flanked by two outer layers forming a sandwich structure. The outer layer cells have been shown to be incorporated in the epiphyses and the intermediate zonal cells develop into articular cartilage\(^{23,25}\). Many signalling pathways have been studied in association with the interzone formation including TGF superfamily members BMP2, BMP4, GDF-5, and GDF-6; Wnt-4, Wnt-14, and Wnt-16; BMP antagonists Chordin and Noggin; fibroblast growth factor family member FGF-2, FGF-4, and FGF-13; transcription factors Cux-1 and ERG; and other molecules (Autotaxin and Stanniocalcin) which have been reviewed previously\(^{8,26}\).
Growth differentiation factor 5 (GDF5) belongs to the TGF-β family and was originally described as mutated in brachypod mouse mutants where some joints are absent\textsuperscript{27}. GDF5 is interesting during joint formation not for specifying the joint but for promoting initiation of chondrogenesis and chondrocyte proliferation and for maintaining the early joint as reviewed by Archer \textit{et al.} \textsuperscript{8}.

The actual cavitation process where the joint cavity is formed is not fully understood, however mechanical forces, apoptosis, HA and lubricin production have been proposed as important factors\textsuperscript{28-31}. The most believed hypothesis is that differential synthesis of HA and its cell surface receptor CD44 inhibits aggregation of cells and mediates formation of a fluid-filled cavity. Lubricin has been proposed by Pacifici \textit{et al.}\textsuperscript{23} to be expressed in the interzone of mouse embryo limbs (unpublished data) and it is believed that the lubricating properties of lubricin can facilitate the cavitation process. However the actual role of lubricin during cavitation needs further investigation as Rhee \textit{et al.} found the expression of lubricin to be initiated after cavitation in mouse joint development\textsuperscript{32}.

Morphogenesis of joint structures is the last step of joint formation and was previously believed to be initiated after cavitation however recent studies by Nowlan \textit{et al.} suggest that joint morphogenesis precedes cavitation\textsuperscript{33}. One important process during joint morphogenesis is the so-called appositional growth, which is believed to be responsible for the reorganization of immature isotropic cartilage into the well-defined structure of mature cartilage. The appositional growth basis is proposed to be resorption of immature cartilage in all cartilage tissue zones and vascular invasion from the subchondral bone from which only the superficial zones are spared. Extracellular matrix is continuously added by progenitor cells in the superficial zone, increasing the amount of cartilage tissue, meanwhile the deep zones become infiltrated by vessels, hence the appositional growth\textsuperscript{34}. Bone is formed through endochondral ossification, at two sites proximal to the joint; first the primary ossification centre in the diaphysis and secondly the secondary ossification centre in the epiphysis. Chondrocytes at the ossification centres stop proliferating, enter hypertrophy and change their gene expression program to synthesise collagen type X. Hypertrophic chondrocytes starts the transformation of the tissue by initiating mineralization, vascularization, direct osteoblast differentiation of perichondrial cells and attract osteoclasts from the macrophage lineage. Hypertrophic chondrocytes then undergo a controlled cell death and it is under debate whether it is apoptosis or another process morphologically distinct from apoptosis\textsuperscript{35}. The left ECM from hypertrophic cells functions as a scaffold for osteoblasts when they produce bone matrix.
Chondrocytes continue to proliferate in the tissue between the two ossification centres in long bones. This cartilage forms the growth plate that is generally arranged in separate zones. Starting furthest away from the ossification border, the zones are characterized by: resting chondrocytes, proliferating chondrocytes, pre-hypertrophic chondrocytes and hypertrophic chondrocytes closest to the infiltrating bone tissue. The proliferating chondrocytes and the matrix producing pre-hypertrophic and hypertrophic chondrocytes cause the elongation of the bones. The growth plates continue to elongate the bones until adolescence when growth plates disappear and are replaced by bone\textsuperscript{16,35}.

Increased knowledge of the embryonic basis for joint formation could hypothetically be used to regenerate damaged tissue through tissue regeneration and tissue engineering. Furthermore, the search for genes associated with OA has been rather unsuccessful, resulting in weak and irreproducible results. However, the genes that have been found are in many cases related to joint development and apparently none of the OA associated genes encode proteins involved in cartilage ECM degradation\textsuperscript{36}.

Therefore it seems like the well-studied degrading traits of OA are not the biggest problem, it may very well be the lack of appropriate regenerating mechanisms that with time destroys joint function.

1.2 Articular cartilage

Prerequisites for smooth locomotion are low friction and chock absorption between the moving bones of the skeleton. Articular cartilage is a connective tissue that covers the ends of moving bones and has unique properties that enables low friction and chock absorption. This is made possible due to a seemingly simple tissue structure that lacks neurons and vascularisation. Cartilage tissue consist of relatively few cells compared to the tissue volume they produce and has been measured to as few as 1–2\% of the volume\textsuperscript{37,38}. The ECM thus constitutes the bulk volume and its properties enable the tissue to harbour large quantities of water giving the tissue biomechanical properties that can bear compression. The organized structure of the ECM together with synovial fluid also give cartilage ability to withstand shear forces.

1.2.1 The chondrocyte

Cartilage is uniquely built and maintained by one sparingly distributed cell type, the chondrocyte. The chondrocyte phenotype varies in shape and gene expression depending on where in the cartilage chondrocytes reside. At the
cartilage surface facing the synovial fluid the cells are elongated laterally to the
surface and deeper down in the tissue the cells become rounded or polygonal
ending with large round cells at the bone boundary. The cells are distributed
throughout the tissue as single cells or as small groups located inside of
lacunae. The lacunae are believed to protect the cells by serving as fluid filled
chock absorbers that also buffer osmotic and physiochemical changes during
dynamic loading\(^\text{39}\). The chondrocyte experience no cell-cell contact from other
cell types, as cartilage is an avascular and immune privileged site, however it
has been shown that they can communicate with each others through gap
junctions\(^\text{40}\). The chondrocyte rarely divide during normal conditions and has
an anaerobic metabolism due to the low oxygen levels in cartilage ranging
from ~10% at the surface to ~1% near the subchondral bone\(^\text{39,41-43}\). The lack
of blood in the tissue creates low oxygen levels and also makes the
chondrocytes dependent on diffusion for exchange of nutrients and
metabolites. Despite their low numbers and limited activity, chondrocytes
create the ECM that is a prerequisite for proper joint function.

1.2.2 Articular cartilage extracellular matrix

The impressive load bearing properties of cartilage comes mainly from two
fundamental constituents of the ECM namely large proteoglycan (PG)
molecules and collagen triple helix fibres (Figure 1). The PGs are highly
hydrophilic and swell by binding water, thus creating a force in the opposite
direction of the compressing load. This swelling force would not be effective
if the tensile strengths from the cross-linked collagen network would not
restrain the swelling nature of PGs. The combination of these swelling and
enclosing forces creates a pressure within the tissue that withstands the
compressive forces of locomotion\(^\text{44,45}\). This mechanism is made possible
through the structures of these molecules. Proteoglycans consist of a protein
core that functions as a binding structure to one or several glycosaminoglycan
(GAG) polysaccharides or oligosaccharides. Aggrecan is the most abundant
PG in cartilage and is constructed by a protein backbone of three globular
domains with the longest inter globular structure between globular domain
two and three. This outstretched region has covalent binding sites for the
negatively charged hydrophilic GAGs keratin sulphate (KS) and chondroitin
sulphate (CS)\(^\text{46}\). The GAGs stretch out from the protein core of ACAN like
the branches from a Christmas tree, catching and holding water molecules
through their negative charge. The first globular domain of ACAN represents
the base of the Christmas tree and has an attachment region for hyaluronic
acid and the small glycoprotein link protein (LP). Widespread PG complexes
are formed through the attachment of many ACAN molecules to HA like
Christmas trees on a string. The attachment between HA and ACAN is
stabilized by LP through the HA and ACAN binding sites. LP consequently has an important stabilizing function in cartilage. The HA string is also decorated with chondrocytes attached through the cell surface receptor CD44. Other important PGs in cartilage include the small leucine-rich repeat PGs; decorin, fibromodulin, biglucan and lumican. These smaller PGs govern important regulatory processes in cartilage e.g. collagen fibril diameter, regulation of proteolytic degradation of collagens and interactions between fibrils. Importantly, it has been suggested that different growth factors are trapped and kept within cartilage in order to mediate their effect through the small leucine-rich repeat PGs.

The collagen content in adult cartilage is mainly constituted by collagen type II but a number of different types are important for the ECM, e.g. collagen type I, II, VI, IX, X and XI. The collagen type II gene has 54 exons and is expressed in two splicing variants during development where type IIA (including exon 2) is the main splicing variant in pre-chondrocytes and as the chondrocyte matures into an adult phenotype the splicing variant changes to type IIB (excluding exon 2). The collagen fibril is formed from many collagen molecules that in turn are right handed super helixes from three polypeptide α-chains. The rope like super helix is stabilised with peptide bonds and hydrogen bonds, which make the structure durable and turnover of collagen is close to zero. The collagen structures are supported by cartilage oligomeric matrix protein (COMP), which is a molecule shaped like a bouquet constituted by five arms. COMP has binding regions to collagen II, I and IX, which facilitates its possible involvement in collagen fibril formation and maintenance of the ECM. Collagen type I is predominantly expressed in the superficial zone while collagen X is the dominant collagen in hypertrophic cartilage in the deep calcified zone. Collagen IX and XI are associated with the collagen II fibril and are believed to have supportive and growth limited functions. Collagen VI is mainly located in the pericellular matrix compartment and is most probably involved in anchoring the chondrocyte to the ECM.
Figure 1. Schematic view of the major constituents of articular cartilage with a chondrocyte and the surrounding ECM.
1.2.3 Histology of articular cartilage

Normal adult cartilage is traditionally described as divided into four characterized layers namely from the bone towards the joint cavity; calcified zone, radial zone, transitional zone and the superficial zone (Figure 2). At the base by the subchondral bone lies the calcified zone harbouring round hypertrophic chondrocytes, i.e. the cells have increased in size. The cells mainly express collagen X arranged perpendicular to the cartilage surface and the mechanical properties of the tissue are gradually shifting from bone-like to cartilage-like further away from the subchondral bone. The calcified zone is separated from the radial zone by an undulating line called the tidemark, which marks the mineralized cartilage from the unmineralized cartilage. Above the tidemark is the radial zone where the few chondrocytes are mainly situated in perpendicular columns to the cartilage surface. The collagen expression is shifted from type X to type II and the fibrils are mainly oriented perpendicular to the cartilage surface. The PG amount peaks in the radial zone making the compressive buffering the highest. Approximately 30% of the total cartilage volume constitutes of the radial zone. Approaching the joint cavity, the radial zone is shifted into the transitional zone where the collagen II fibrils have a more isotropic distribution. The cell abundance is still low and morphology is round, but the columnar organisation is shifted into a more isotropic distribution. The biomechanical properties are still anti compressive but start to shift into increased resistance to shear stress. The transitional zone represents approximately 40-60% of the cartilage volume. The last zone before the low friction surface of the cartilage is called the superficial zone. The chondrocytes in the superficial zone are more abundant and the morphology is more elongated than in the other zones. Collagen expression is mainly concentrated to collagen I that form a network of thin fibrils running side by side in parallel to the surface. This network of collagen I has great tensile properties and work together with the lubricant lubricin to manage the shear forces from locomotion. The most superficial zone, that faces the joint cavity is very thin and was originally described as the lamina splendens. However the superficial zone may be constituted of three layers where the outermost layer comprises an amorphous substance suggested to contain lipids and proteins. The deeper second layer may be questioned but is described as a low-electron density layer. The deepest layer of the most superficial zone is stabilized by collagen I, II and III.
Figure 2. Illustration showing a cross section of articular cartilage from the subchondral bone up to the superficial layer facing the joint cavity.

1.3 Cartilage reparative abilities and osteoarthritis

The joint does not have to endure a triple jump in track and field to be exposed to great forces. Normal walking gives rise to a pressure of 40-50 atmospheres to the cartilage surface\textsuperscript{39,63}. The joint is designed to withstand these forces from locomotion but cartilage is a low regenerating tissue that
mainly forms a fibrous scar tissue with poor biomechanical properties when damaged. A contributing factor to the poor reparative ability of cartilage upon damage is believed to be the lack of bleeding. Had the tissue been vascularized, repair cells and/or growth factors would have been distributed through bleeding and could theoretically participate in a healing process. This is the reasoning behind the surgical techniques that punctuates the subchondral bone in order to create a bleeding in to the defected cartilage.

Osteoarthritis (OA) is the major joint degenerative disease and has been estimated to affect approximately 18% of the female and 10% of the male population aged over 60 worldwide. The pain and loss of function in the affected joints are considered to cause serious trouble for the society as an ageing population is expected to make OA among the leading causes of disability by 2020. Traditionally OA is divided into primary OA where there are no obvious underlying factors or abnormalities and secondary OA where focal damages due to mechanical stress caused by anatomical deformities, known structural abnormalities in the ECM or trauma is the initiating cause. Damaged cartilage is likely to degenerate if left untreated and development of arthritis is likely although initiation of symptoms ranges from a few years if the injury is large to decades if the injuries are minor. The breakdown of the matrix in OA is believed to start from the superficial zone and work its way down the cartilage layers.

The most prominent risk factor for developing OA is age. Several other factors have been suggested as predisposing to OA including altered loading of the joint, joint injury, obesity related metabolic implications together with obvious mechanical overload and mutations causing malfunctioning ECM molecules. The traditional explanation to OA is the wear and tear hypothesis, which is supported by the overload risk factor and in vitro studies that show the increased breakdown of collagen type II after repetitive loading of bovine cartilage explants. Recently, genome-wide association studies (GWAS) have been made to investigate the genetic background of OA. The results suggest that there are several individual risk alleles that in concert contribute to the general susceptibility to OA development. Further, studies on inheritance demonstrate that OA may be attributed to genetic factors and OA has thus been considered a multifactorial polygenic disease.

There are several traits to OA regardless of whether it is a trauma or the mineralisation of cartilage and decreased PG synthesis related to age that are the initiating events (Figure 3). The progression of OA is due to lost homeostasis in the tissue where catabolic events overrun the anabolic
attempts. OA is characteristic of an unregulated expression of matrix degrading enzymes where collagen degrading matrix metalloproteinase (MMPs) and PG degrading aggrecanases (A disintegrin-like and metalloproteinase with thrombospondin type I motif, ADAMTS) are the most common. Collagen II is an important target and MMP13 is considered the most dangerous collagen II degrading enzyme. ADAMTS4 and 5 are the most active ACAN degrading enzymes and are thus considered large contributors to OA progression. ADAMTS4 also cleaves other ECM molecules including COMP, fibromodulin and decorin. Aggrecans are the first molecules to be degraded in OA as the collagens are more rigid and lose their integrity at the later stages of OA. The degrading events in OA trigger the expression of inflammatory mediators like interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α), which in turn induce expression of MMPs and even suppress expression of ECM components. The chondrocytes attempt to repair the damaged ECM by abandoning their adult arrested phenotype and increase proliferation, which explains the chondrocyte cloning seen in OA. The increased cell numbers do not help in mending the damage as the cells do not initiate a massive ECM production. The cells even change their matrix expression pattern from the important collagen IIB to collagen I, IIA, III, V, VI and the marker for hypertrophic chondrocytes collagen X. Tissue inhibitors of metalloproteinases (TIMPs) are the counterbalance to the catabolic mediators in cartilage. There are four known TIMPs where TIMP1 is the main inhibitor that balances MMP13 activities and TIMP3 counteracts ADAMTS4 and ADAMTS5. The imbalance between the matrix degrading enzymes and their inhibitors has been suggested to be an important field of pharmacological treatments. The net effect of the above mentioned events together with a plausible effect from apoptotic events result in a slow degeneration of cartilage and ultimately joint failure.

Pharmacological treatment of OA is mainly restricted to palliative interventions and the joint is finally replaced when the disease reaches an end stage. However, there are surgical interventions that aim to regenerate cartilage before joint replacement is inevitable e.g. autologous chondrocyte implantations (ACI) originally developed by Brittberg et al., micro fracture techniques that stimulate bleeding from the bone marrow and various tissue engineering attempts. Furthermore, some patient groups may benefit from preventive surgical treatments e.g. to lower the impact of hip impingements and thus reduce the rate of OA in these individuals, which emphasize the importance of correct joint alignment.
1.4 Signalling pathways in osteoarthritis

Genome-wide association studies are performed to link certain genetic variants to diseases thereby elucidating the mechanisms behind the aetiology. When OA patients were investigated for enriched genetic variants the associations were weak and differed between populations. However, the interesting conclusion was that the hit genes were often not associated with ECM degrading enzymes as expected but with processes involved in synovial joint development. Furthermore, many of the genes were associated with TGFβ and Wnt signalling e.g. the TGFβ1 inhibitory protein asporin (ASPN), the TGFβ super family included bone morphogenetic protein 2 and 5 and BMP14 also known as growth differentiation factor 5 (GDF5), frizzled-related protein (FRPB) and WNT1 inducible signalling pathway protein 1 (WISP1). The traditional characterization of OA with primary and secondary classification is being questioned as the genetic association with OA suggests that the genes associated with OA are involved in joint formation and thus may cause small joint abnormalities that with time leads to OA.
1.4.1 WNT

Wnt proteins are 19 known glycoproteins that activate one of basically two signalling pathways, the canonical β-catenin mediated pathway and the collectively classified non-canonical pathways e.g. the planar cell polarity pathway and the Ca$^{2+}$/CamMKII pathway$^{109-111}$. The canonical pathway is the most studied and when inactive the intracellular transducer β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) together with a range of co-proteins; adenomatous polyposis coli (APC), caseine kinase 1-α (CK1α) and Axin, in a destruction complex$^{110}$. The phosphorylation leads to ubiquitin-mediated degradation of β-catenin by the proteasome and thus no transduction of the signal. The destruction complex is neutralized by binding to activated Wnt receptors at the cell membrane when Wnt signalling is initiated. The Wnt ligand binds to the receptors serpentine seven transmembrane frizzled (FZD) family receptors and co-receptors including low-density lipoprotein receptor-related protein (LRP) 5 and 6, which mediates the interaction between the receptors and the destruction complex through the protein disheveled. Newly synthesized β-catenin is consequently sheltered from the destruction complex and free to pass through to the nucleus. In the nucleus β-catenin interacts and binds T cell factor/lymphoid enhancer factor (TCF/LEF) proteins and also displaces groucho, which is a transcriptional co-repressor to TCF transcription factors. The β-catenin also interacts with several other transcription factors, co-activators and co-repressors to form a multiprotein complex that can regulate target gene expression e.g. peroxisome proliferator-activated receptor delta (PPARD) and FOS-like antigen 1 (FOSL1) $^{111,112}$. Wnt signalling inhibitors mainly act in the extracellular region by binding either Wnt e.g. frizzled related protein (FRZB) or the LRP co-receptors e.g. dickkopf 1 (DKK1)$^{111}$. Wnt signalling has proven important through out the chain of embryogenesis, organogenesis and the homeostatic maintenance of tissues. During skeletogenesis, Wnt expression keeps progenitor cells in a proliferative state and prevents chondrogenic differentiation. Wnt14 (same as Wnt9a and Wnt4)$^{113}$ is expressed in the joint interzone and is believed to inhibit the chondrogenic differentiation of the interzone cells and guide them into synovial connective tissue$^{114,115}$. Furthermore, Wnt signalling is believed to stimulate chondrocyte hyperthrophy and reduce expression of SOX9, the important factor during chondrocyte differentiation mentioned above$^{110,116}$. However, Wnt signalling is not only negative for cartilage but is probably a signalling pathway that needs proper fine-tuning, as hampered β-catenin signalling results in chondrocyte apoptosis and tissue damage in a mouse model$^{117}$ and active signalling leads to an OA like phenotype in mice$^{118}$. 
1.4.2 TGFβ

Two subfamilies are known within the transforming growth factor beta (TGFβ) family i.e. TGFβ/Activin/Nodal subfamily and the BMP/GDF/Muellerian Inhibiting Substance (MIS) subfamily\(^{119,120}\). There are seven different type I receptors (ALK-1 - ALK-7) and five different type II receptors (BMPRII, ACVR2A also known as ActRII, ACVR2B also know as ActRIIB, TGFBR2 and AMHR. These receptor types exist as homodimers when no TGFβ1 is present. Upon binding of TGFβ1 a receptor complex is formed with any type I receptor dimer and any type II receptor dimer, which subsequently results in the phosphorylation and activation of the type I receptor by the type II receptor\(^{119}\). The possibility to mix the configuration of members of different receptor types makes a great variation potential. The signal is transduced downstream via SMAD proteins subdivided into three classes; the receptor-regulated SMADs (SMADs 1, 2, 3, 5, and 8), the co-SMAD (SMAD4) and the inhibitory SMADs (SMAD6 and -7). The receptor-regulated SMADs are upon signal initiation phosphorylated by the type I receptor on two serines at the COOH-terminus. The phosphorylation initiates one or several mechanisms including SMAD release from the receptor complex and the SMAD anchor SARA, and formation of heteromeric complexes with SMAD4 and subsequent accumulation in the nucleus where the complex associates with co-activators or co-repressors to positively or negatively regulate gene expression. Inhibitory SMADs antagonize TGFβ-signalling through counteracting effects of receptor-regulated SMADs\(^{119}\). TGFβ-signalling is possibly important for expression of SOX9 and thereby chondrogenesis\(^{121}\). Regulation of TGFβ-signalling pathway has been suggested to be important for osteoarthritis progression\(^{122}\).

1.4.3 GDF5

The discovery of a 290 amino acid protein from cartilage explants that could induce cartilage and bone formation in subcutaneous implants is important to the field of cartilage research\(^{123}\). The protein was originally designated cartilage-derived morphogenetic protein-1 (CDMP-1) and is also known as BMP14 and Growth differentiation factor 5 (GDF5) and belongs to the BMP sub-family of the TGFβ superfamily. GDF5 signalling is transduced through binding to the BMP receptor IB and further mediated via SMAD1, 5 and 8 and also the p38 MAPK pathway\(^{124,125}\). GDF5 has been implemented in joint formation as a mutation in the gene is suggested to be responsible for brachypodism\(^{27}\), which is a trait that affects length and number of the appendicular skeleton and as the name implies especially shortens the feet of mice\(^{126}\). Furthermore, GDF5 is considered a key regulator of mesenchyme condensation, chondrocyte differentiation and joint maintenance\(^8\). The role of
GDF5 and Wnt signalling may be summarised as having opposite roles in joint formation where GDF5 signalling is part of the chondrogenic stimulation and Wnt signalling participates with an anti-chondrogenic stimuli of GDF5 expressing progenitor cells during joint formation\textsuperscript{127}.

GDF5 is also an important candidate to be associated with OA by a single nucleotide polymorphism (SNP) (rs143383 T/C) in the 5'-UTR that reduces the transcriptional activity of the GDF5 promoter resulting in lowered protein levels\textsuperscript{103,104}. This again shows that proper regulation of signalling pathways and not just the presence or absence of signalling molecules is of utmost importance to gain the proper phenotype. The whole mechanism behind GDF5 association to OA is not known and also the role of GDF5 in the homeostasis of mature cartilage and healing of cartilage is unknown\textsuperscript{36}. A putative role of GDF5 in cartilage homeostasis as a MMP13 inhibitor is elucidated in the discussion section of the present thesis.

### 1.5 Regeneration of articular cartilage

Several approaches have been developed to slow down tissue matrix degradation and initiate a repair response that can heal the tissue in focal defects to articular cartilage. Examples are bone marrow stimulation techniques (e.g. Pridie drilling, abrasion chondroplasty and microfracture), ACI\textsuperscript{93} and tissue engineering approaches. One of the most promising ideas is to use various stem cell types in combination with a suitable scaffold and the right culture methods to mediate a cartilage construct that can be surgically implanted in the defect. Although promising, guidance of the stem cell differentiation is a challenging task that is far from resolved in stem cell based regenerative techniques. Further, it is not just cartilage matrix of any type that needs to be rebuilt or engineered but a whole tissue construction from subchondral bone integration to the surface zone of the superficial layer\textsuperscript{128}. The different tissue structures that need to be rebuilt by the stem cells require most probably different signalling pathways or at least different fine-tuning regulations of the important pathways. Also, different stem cell types need different signalling pathways in order to initiate chondrogenic differentiation demonstrated by TGFβ3 stimulation for adult MSCs and BMP2 stimulation for fetal MSCs\textsuperscript{129}. Interestingly the developing field of small molecules that can direct signalling pathways has been implemented on stem cell based cartilage repair\textsuperscript{130}. Furthermore, induced pluripotent stem (iPS) cells have recently been developed from different cell types and may be promising candidates for a variety of tissue engineering and regenerative medicine applications\textsuperscript{131}. Human iPS cells from a fibroblast origin have been
implemented in an animal model of osteochondral defect repair with promising results\textsuperscript{132}. Moreover, human articular chondrocytes have recently been reprogrammed into iPS cells by Boreström et al. and successfully redifferentiated into cartilage ECM forming cells\textsuperscript{133}. Also, iPS cells have potential as disease model systems when studying disease mechanisms\textsuperscript{134}. However, human embryonic stem (ES) cells may be the natural stem cell choice if the whole chain of events during cartilage development is to be studied \textit{in vitro} as small differences between iPS cells and ES cells may hypothetically affect their differentiation path.

\section*{1.6 Embryonic stem cells as model system for chondrogenesis}

Modelling the embryonic development \textit{in vitro} seems like a study set-up that would struggle with its validity, as it is not technically possible to control and monitor all the developmental events. However, studying the chondrogenic event of embryonic development via ES cells \textit{in vitro} is an interesting approach that should not be left out. The ES cells are considered a self-renewing cell type that is pluripotent i.e. can give rise to the three germ layers and essentially all the cell types that constitute the body \textsuperscript{135}. This characteristic should be distinguished from totipotent that is the ability to give rise to an entire new organism including extra embryonic tissue and the placenta\textsuperscript{136}. Many researchers have aimed at differentiating ES cells into chondrocytes with various results\textsuperscript{137-139}. The different approaches have all achieved results towards articular cartilage but much remains to be elucidated. Involvement of multiple signalling pathways in chondrogenic differentiation of human ES cells is evident and fine-tuning of the signalling during different time points most probable. Regardless of which approach that is used for the purpose of generating new matrix producing and matrix maintaining chondrocytes, it is inevitable to understand the mechanisms behind chondrogenic differentiation.
2 AIM

The general objective was to improve knowledge regarding the aetiology of osteoarthritis with focus on signalling pathways.

2.1 Purposes of the studies include

- To describe how secretomes from chondrocytes differ between different levels of cartilage degeneration in order to find early markers of osteoarthritis and altered expression of secreted signalling pathways (study I).
- To investigate if gene expression in chondrocytes prior to ACI could be associated with clinical outcome and thus explain the regenerative mechanisms behind ACI (study II).
- To study new biological markers that predict clinical outcome of ACI (study II).
- To investigate the effect of the OA associated growth factor GDF5 on differentiated chondrocytes in order to study the role of GDF5 in extra cellular matrix homeostasis (study III).
- To characterize potential crosstalk between GDF5 and the OA-associated Wnt signalling pathway (study III).
- To improve the culture methods used for embryoid bodies in order to develop a non-laborious and cost effective model system for future studies on signalling pathways implemented during chondrogenesis (study IV).
3 MATERIAL AND METHODS

Detailed information regarding methodology can be found in the papers as listed below and the following sections discuss the general aspects.

3.1 Isolation and expansion of human chondrocytes and human embryonic stem cells

3.1.1 Chondrocyte source, isolation and expansion

Obtaining healthy human articular chondrocytes is troublesome as a control biopsy is not possible to harvest from healthy volunteers as such procedures will permanently damage the healthy joint. Therefore healthy chondrocytes used in paper III were obtained from patients undergoing ACI where healthy cartilage is harvest from a minor load-bearing area of the knee joint, for example the superolateral or superomedial area of the upper femoral condyle. These healthy ACI biopsies had no macroscopic signs of cartilage defects such as tissue damage, mitosis or staining artefacts of the extracellular matrix. OA cartilage used in paper I was obtained from the medial and lateral compartment of the femoral condyle from OA patients undergoing total knee replacement. The biopsies were taken from the load-bearing area of each compartment, anonymized and Mankin scored in order to conclude a difference in OA progression between the medial and lateral side. The one healthy biopsy used in paper I was obtained from an amputated leg where the knee had no disease history. The Mankin score of the healthy knee cartilage showed that the medial compartment was slightly affected by OA while the lateral showed no signs of OA. This healthy sample gave us the unique opportunity to study an early OA process in the medial femoral compartment as compared to the unaffected lateral compartment within the same patient.

Chondrocytes were isolated according to a previously described protocol. The cartilage biopsies were immediately submerged in a sterile saline solution (0.9% NaCl) kept at 4°C and transported to the cell culture laboratory. The saline solution preserves the viability of the chondrocytes up to 48 hours. The saline solution is preferred to culture medium because the phenol red used in many media will stain the biopsy making it difficult to distinguish the cartilage from the subchondral bone. The cartilage biopsies were freed from subchondral bone and soft tissue and subsequently minced with scalpel. The
minced cartilage was enzymatically treated over night with collagenase type II (0.8 mg/ml) in Ham’s F-12 medium at 37°C in 7% CO₂ and 95% humidity in order to isolate the chondrocytes. The collagenase treatment results in mainly single cells that were washed twice with Ham’s F-12 medium before expansion. The washed cells were resuspended in a 1:1 mix of Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 (DMEM/F-12) supplemented with L-ascorbic acid (0.1 mg/ml), gentamicin sulphate (50mg/l), amphotericin B (250µg/ml), L-glutamine (2mM) and 10% human serum where each batch is serum pooled from 10 different donors[140]. Gentamicin sulphate and amphotericin B were replaced by penicillin (0.1 units/ml) and streptomycin (100 µg/ml) i.e. PE/ST (1%) (PAA Laboratories, Linz, Austria, http://www.paa.at) after the first passage. Primaria™ plastic was used as supportive surface for the chondrocytes as this polystyrene surface has incorporated anionic and cationic functional groups that improve attachment of the cells. The chondrocytes were first seeded at 3×10³ cells/cm² and the first medium change was made six days after the initial seeding and subsequently two to three times per week. The cells were subcultured when they reached 80-90% confluence by using a trypsin and ethylenediaminetetraacetic acid (EDTA) solution i.e. trypsin (0.125%) diluted in 0.1M phosphate buffered saline (PBS) with EDTA (0.2g/l). EDTA is a chelating agent that binds calcium ions and thereby prevents the binding of chaderins between cells and between cells and the surface. Trypsin hydrolyses adherent proteins at the carboxyl side of arginine and lysine. After the first passage the cells were transferred to regular polystyrene culture flasks. Cells were stored in liquid nitrogen after freezing in DMEM/F-12 supplemented with 20% human serum and 10% dimethyl sulfoxide (DMSO) as cryoprotectant. All chondrocyte cultures were incubated in a humidified atmosphere at 37°C and 7% CO₂.

3.1.2 Embryonic stem cell source, isolation and expansion

The five human ES cell lines SA002, AS034, AS038, SA121, SA167 used in this thesis were derived and characterized at Cellartis (Cellartis AB, Gothenburg, Sweden)[141]. Human ES cells are isolated from the blastocyst stage of the embryo development. In short the inner cell mass of the blastocyst is dissected from the surrounding trophoblasts by microsurgery or immunosurgery. The freed inner cell mass constitutes of approximately 30-34 cells and are subsequently plated on mitotically inactivated mouse embryonic fibroblast (MEF) or human embryonic fibroblasts (HEF) in 80% KnockOut D-MEM (Dulbecco Modified Eagle Medium) supplemented with 20% KnockOut serum replacement, L-Glutamine (2mM), β-mercaptopoethanol
(0.1mM), nonessential amino acids (1%), human recombinant basic fibroblast growth factor (bFGF) (4ng/ml) and penicillin (0.1 units/ml) and streptomycin (100 µg/ml) (PE/ST). Subculture is made mechanically by carefully dissecting the undifferentiated human ES cells from the differentiated and plating them on a fresh layer of MEF or HEF in fresh medium every 4-5 days\textsuperscript{141}. All human ES cell cultures were incubated in a humidified atmosphere at 37°C in 5% CO\textsubscript{2}.

\section*{3.2 Cell culture techniques}

\subsection*{3.2.1 Chondrocyte secretome isolation}

Serum is a vital component of cell culture media when cell proliferation is wanted as serum contains several important factors for cell survival e.g. hormones, growth factors, lipids, vitamins, amino acids often bound to carrier proteins like albumin and transferrin. Serum was included in the chondrocyte cultures in paper I during the proliferation phase but excluded during the medium conditioning as the serum protein content, mainly albumin, may interfere with the proteomic analysis. In the case of MS techniques highly abundant serum peptides may interfere with the detection of other low concentration peptides as they may fall out of the dynamic range of the mass spectrometer. Chondrocytes were expanded in SILAC-labelled ($^{13}$C\textsubscript{6}-Lysine and $^{13}$C\textsubscript{6}-Arginine) or SILAC-unlabelled ($^{12}$C\textsubscript{6}-Lysine and $^{12}$C\textsubscript{6}-Arginine) media for 5-6 cell doublings and subsequently the cells were conditioned in serum-free medium for 24 hours. Extra care was taken to wash the cells with 37°C sterile PBS before the conditioning with serum-free media. Also the serum used for the expansion is a dialyzed serum in order to avoid incorporation of unlabelled amino acids during the labelling procedure. The serum free conditioned media were collected and stored in -80°C prior to MS-analysis.

\subsection*{3.2.2 Chondrogenic differentiation}

Chondrocytes are known to dedifferentiate when cultured in monolayer\textsuperscript{142-144}. Redifferentiation of the monolayer-cultured chondrocytes was performed with a micromass culture technique (also known as pellet mass or pellet culture technique). The pellets are formed by centrifugation at 400g for five minutes of 2-4×10\textsuperscript{5} cells resuspended in a basic defined medium consisting of DMEM high glucose supplemented with linoleic acid (5.0µg/ml), 1×ITS-G premix (6.25 µg/ml insulin, 6.25µg/ml transferrin, 6.25µg/ml selenious acid), human serum albumin (1.0mg/ml), dexamethasone (10\textsuperscript{-7} M), L-ascorbic acid (14µg/ml). This basic defined medium is supplemented with a growth factor usually TGFβ1 or TGFβ3, the micro mass cultures in this thesis were
supplemented with TGF\(\beta\)1 (10ng/ml). All micro mass cultures were conducted in a conical polypropylene tube, or a 96-well plate when many pellets were needed. The pellets in the tubes or the 96-well plate are incubated in a humidified atmosphere for 48 hours at 37°C in 5% CO\(\text{2}\). During this period the cells orientate each other into a sphere. Medium is changed every two to three days starting after the sphere formation.

3.2.3 Signalling pathway stimulation

Several factors both chemical and physical influence the developmental fate of cells\(^{145,146}\). Many of these factors influence developmental signalling pathways and may thus be used in tissue regeneration and engineering. In study III the growth factors GDF5 (10-200ng/ml) and Wnt3a (50ng/ml) were added to the basic defined medium. Study III also utilized low-molecular weight organic compounds, also designated small molecules that are used to regulate biological processes through affecting cell-signalling pathways. The small molecule CHIR99021 (0.5µM) inhibits GSK3\(\beta\) in the Wnt signalling pathway, thereby activating the pathway\(^{112}\). The small molecule WAY262611 (0.01-1µM) was also used in study III to inhibit DKK-1, which results in an activation of the Wnt signalling pathway as well.

3.3 Factorial design of experiments

It can be difficult to optimize an experiment where several input variable, e.g. growth factors, can affect the readout in different ways. Experimental bias can interfere with the result analysis if one factor is evaluated at a time. It is more interesting to study the input variables at the same time, which can be done by factorial design of experiment (DoE). This method gives a systematic approach to optimization processes and is based on defining mathematical relationships between input and output variable in a system\(^{147,148}\). The input variables should be two or more factors, e.g. factors affecting signalling pathways, added in two values: high and low. The simplest form of DoE is symbolised as a factorial 2\(^2\) design where the superscript figure is the number of input variable but can be extended to several variables and DoE is therefore often described as “factorial 2\(^n\) design”. The factorial 2\(^2\) design can be visualised as a square set-up (Figure 4). The system is often validated through three or more centre points with a medium concentration between high and low. The output variables can be anything that is affected by the input variable and is measurable, e.g. bio mass, gene expression, proliferation rate or cell death. The start-up experiment is usually a screening trial to narrow the range of the experiment. The screening trial is usually performed with a fractional factorial design, basically a design with reduced number of sample
points that determines which input variables that significantly affect the output variables. The trial is followed by extended designs with many sample points where the true optimal settings for the significant input variables are determined. For an understandable illustration of the results a response surface is drawn, which show how the different levels of the input variable affect the output variables. Mandenius et al. give a comprehensive review on DoE\textsuperscript{148}. In study III the computer software Modde 8.0 was used to investigate the interactions between GDF5 and the DKK1 inhibitor WAY-262611 and their effect on the expression of MMP13 and ACAN. Multiple linear regression (MLR) using least square methodology was used for the statistical analysis in Modde 8.0 and models were considered significant at $p<0.05$ and without a significant lack of fit at $p>0.05$.

![Medium formulations](image1.png)

![Micromass size](image2.png)

**Figure 4.** Schematic illustration of a set-up for factorial design of experiment where the aim is to maximize the micromass size. The mediums are supplemented with the additives in a low, mid and high concentration in seven different mixtures (a). The seven different mediums are used for micromass culture during a standard period of two weeks and the micromass sizes are measured (b). A response surface is calculated from the results and illustrates the optimal concentration for each additive (c).

### 3.4 Multivariate analysis

Statistical techniques that analyses data that arises from multiple variables are denoted multivariate analysis. In study II the multivariate analysis using principal component analysis created models of the array result where dominant patterns in the data are identified without discriminating variables. Analysing the success and failure groups in study II were performed with Discriminant analysis using Orthogonal Partial Least Square analysis (OPLS-
DA). This analysis uses the discriminating variable “groups” to analyse which transcripts that may differ between the two groups. All multivariate analysis in study II was performed with the SIMCA-P 12+ software.

3.5 Embryoid body formation

Embryoid bodies (EB) are a common culture method to induce differentiation of ES cells into a desired adult cell type and thus a platform to study the effect of different signalling pathways during development. To induce EB formation, whole human ES cell colonies were removed using a stem cell cutting tool. The colonies were cut with a margin to the MEFs in order to avoid mouse embryo fibroblast contamination and to achieve an even size of the starting colony and subsequently cultured in agarose, hanging droplet and suspension. The EB formation efficiency was calculated by dividing the number of formed EBs from the individual experiments with the number of colonies originally placed in each culture condition. The EBs were analysed with an inverted light microscope. Pictures were taken with a digital camera using Nikon ACT-1 version 2.12 software.

3.5.1 Agarose culture of embryoid bodies

Agarose was used as a supportive three dimensional culture system for EB formation and culture in study IV. Agarose was chosen as it is commonly used in laboratories and because chondrocytes, the target cell type for the stem cell studies, are known to grow and redifferentiate in agarose. In short, a 2% agarose solution was cooled to 60°C and mixed 1:1 with room tempered VitroHES™. This agarose solution was mixed 1:1 with detached human ES cell colonies suspended in 37°C VitroHES™ medium without human recombinant basic fibroblast growth factor. The agarose/human ES cell-colony suspension was subsequently poured into a culture dish precoated with 1% agarose. The agarose culture was left to settle at 4°C for 30 min and the semisolid agarose layer was subsequently overlaid with VitroHES™ medium without bFGF (Figure 5). The culture medium was changed once a week and the EBs were cultured for 7 days and 8 weeks, at 37°C, in 5% CO₂ and a humidified atmosphere.
3.5.2 Hanging Droplet Culture of embryoid bodies

Hanging droplet cultures were performed in study IV as controls to the agarose technique. The inside of a Petri dish lid was used and 25 IL droplets of VitroHES™ medium without bFGF were gently attached with a pipet. One detached human ES cell colony was placed into each droplet. Subsequently, the lid was gently inverted and placed on the Petri dish, which contained 10 ml VitroHES™ medium without bFGF to prevent evaporation from the droplets. The EBs were cultured for 7 days in a humidified atmosphere at 37°C and in 5% CO₂.

3.5.3 Suspension Culture of embryoid bodies

Suspension culture was also used as control to agarose culture in study IV. The detached human ES cell colonies were placed in VitroHES™ medium, without bFGF supplement, in bacteriological Petri dishes and cultured for 7 days in a humidified atmosphere at 37°C and in 5% CO₂.
3.5.4 Teratoma Formation

_in vivo_ teratoma formation is a technique to assess the pluripotency of stem cells and was used in study IV as a comparative technique to agarose cultures. The teratoma of SA002, AS034, SA121, SA167 human ES cell lines were performed as previously described^{141}. Briefly, undifferentiated human ES cell colonies were cut and injected under the kidney capsule or in the testicular lumen in SCID mice. The resulting tumours were allowed to develop for 8 weeks before the experiment was terminated and the resulting teratomas were dissected and fixated.

3.6 Histological methods

Histology is studied to understand the microscopic structure of cells and ECM within a tissue or any other construct made up by cells and scaffolds. The architecture of the tissue to be studies needs to be preserved in some way and this is usually done by fixation in a 4-10% formaldehyde solution in PBS. The proteins become cross-linked by the aldehydes, which thereby preserve cellular structures in their _in vivo_ relationship. Soluble proteins are also made insoluble through fixation to structural proteins. Paraffin resin is usually used as embedding material when soft tissues are sectioned in a microtome and mounted on glass slides. For harder specimens different plastic resins are needed but the plastic may interfere with subsequent immunohistochemical stainings. Throughout this thesis the fixation was done in Histofix™ (6% formaldehyde in PBS), dehydrated through 70% EtOH and isopropyl and embedded in paraffin. The specimens (chondrocyte pellets and embryoid bodies) were sectioned (5µm thick) at the approximate centre and the sections were attached to glass slides. Sections were subsequently stained.

3.6.1 Safranin-O staining

Safranin-O (3,7-Diamino-2,8-dimethyl-5-phenylphenazinium chloride) is used to stain PGs in cartilage tissues. It is a monovalent cationic dye that interacts with negatively charged PGs. Safranin-O binds stoichiometrically with one dye molecule on either chondroitin-6-sulphate or keratan sulphate^{150}. The result is that sulphated proteoclycans are stained orange to red, cytoplasm blue-greenish and nuclei black. The procedure is in short; deparaffinisation, rehydration and staining with Weigerts hematoxilin (1:1 of solution A and B) for 7 minutes, rinsed in water, washed with acidic EtOH (10ml HCL, 990 ml 99.5% EtOH) and rinsed again in water. Sections were fast green stained for 3 minutes (0.1 g fast green in 100 ml water), rinsed with acetic acid and stained with Safranin-O (0.1 g Safranin-O in 100 ml water) for 5 minutes. Sections were subsequently dehydrated and mounted.
3.6.2 Alcian Blue van Gieson Staining

The combination of Alcian blue and van Gieson staining makes it easy to detect cartilaginous tissues in a light microscope and is commonly used in histological examinations of cartilage specimens. Alcian blue is like Safranin-O a cationic dye but makes PGs blue and it contains four cationic groups, which probably enhance the affinity to the PGs. The van Gieson dye is a mix between the acid fuchsin and picric acid, which stains connective tissue and collagens pinkish and the cell nucleus black-brown. The sliced sections were after deparaffinisation stained with Alcian blue solution (1g Alcian blue in 100 ml H$_2$O, 100 ml 1% acetic acid and 30 mg phenol). The first wash was performed in water, acidic EtOH and 95% EtOH. The sections were stained in 2 minutes with a solution containing 1g basic fuchsin, 10 ml 95% EtOH, 100 ml 5% carbol solution (5 g phenol in 100 ml water) diluted 1:10 in water containing 10% formaldehyde and glacial acetic acid. A second wash in water, acidic EtOH and 95% EtOH was performed before counterstaining with Weigert’s hematoxylin (Weigert A and B in a 1:1 mixture). Sections were rinsed gently in water before van Gieson staining (10 ml acidic fuchsin in 100 ml water mixed with 90 ml saturated picric acid). The sections were finally rinsed in water and dehydrated with a 95% EtOH bath.

3.6.3 Hematoxylin-Eosin

Hematoxylin-Eosin is commonly used to stain the nuclei and protein content of tissue sections. Haematoxyline stains the nucleus in a blue to violet tone and Eosin stains proteins nonspecifically in pink tones. The sections made in study IV were deparaffinised, stained in Harris’ haematoxylin for 4 minutes, washed and stained with 1% eosin for 7 minutes and mounted with glass cover slips.

3.6.4 Size assessment of pellets

In study III the pellets in the 96 well plates were investigated under the DIAPHOT300 microscope and photographed with a DXM1200 camera. The size of the pellet was determined with the ImageJ software (National Institutes of Health, USA).

3.6.5 Mankin Scoring

Mankin et al. developed a scoring method for microscopic OA histopathological assessment in 1971. The method uses a 14 point score for evaluation of cellular changes (proliferation, cloning), histochemical presence of Safranin-O staining, and structural changes (i.e. erosion, vessel penetration through the tidemark and fissures). This method is well accepted and was used
in a modified version without tidemark inspection in paper I as one inclusion/exclusion criteria for the used biopsies. The tidemark inspection was excluded, as the biopsies did not include the subchondrial bone. The modified Mankin score only reaches the maximum score of 13.

3.7 BIOCHEMICAL ANALYSES

Quantification of proliferation by measurements of DNA content and quantification of extra cellular matrix production in study III was done by digestion of the pellet mass culture with papain enzyme, which is a cysteine protease that cleaves the peptide bonds of basic amino acids leucine and glycine and also hydrolyses esters and amides. This digestion dissolves the tissue into a solution, which can be analysed for its biochemical content. The papain digestion solution was prepared with papain enzyme (0.3 mg/ml) dissolved in sodium phosphate buffer (20mM), EDTA (1mM) and dithiothreitol (2mM), pH 6.8 at 60°C for 2 hours.

3.7.1 Sulphated proteoglycan quantification

In order to assess the influence on extra cellular matrix production of different stimulus in study III the sulphated proteoglycans (sGAG) were quantified with a 1.9-dimethylmethylene blue (DMMB) assay. An absorption spectrum is changed up on the binding of the cationic dye DMMB to the sulphated GAGs. The papain dissolved samples were diluted 1:2 in PBS and mixed 1:1 with DMMB solution prepared from 16 mg DMMB dissolved in 1 litre of water containing lysine (3.04 g), NaCl (2.37 g) and 95 ml HCl (0.1 M). The absorbance was immediately determined from a sample duplicate at 520 nm. The standard curve from which the sGAG content was calculated against was prepared from chondroitin sulphate diluted in PBS. The amount of DNA in the pellets was used for normalization of the sGAG content.

3.7.2 DNA quantification

Proliferation can be measured by many techniques e.g. detection of cells synthesising DNA in the S phase by incorporation of nucleoside analogs that are visualized by antibodies or chemical reactions, counting viable cells through flow cytometry or measurement of total DNA compared to control as DNA content with in one cell can be calculated. The DNA content was measured in study III by total DNA quantification through incorporation of Hoechst 33258, a bisbenzimidazole derivate that binds to double stranded DNA at AT-rich regions. The Hoechst 33258 molecule has after binding to DNA a fluorescence emission peak at 460 nm when excited with 360 nm. The samples dissolved with the papain solution were diluted 1:2 in PBS and mixed
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1:10 with Hoechst 33258 solution containing Hoechst 33258 (0.2 µg/ml), Na$_2$HOP$_4$ (100mM), EDTA (5 mM), pH 7.5. Duplicate measurements were made of each sample and DNA concentration was calculated against a calf thymus DNA standard curve.

### 3.8 Proteomic techniques

#### 3.8.1 Immunohistochemistry

Visualization of specific proteins in cells and ECM of tissues using antibodies attached to reporter molecules, e.g. enzymes, metals or fluorescent compounds, is called immunohistochemistry. Immunocytochemistry is the same technique applied on cell. The tissue sections or cells are incubated with a target antibody that is specific for the antigen, most often an antibody without a reporter molecule called primary antibody. The primary antibody is then targeted with a secondary antibody that is conjugated with the reporter molecule. Secondary antibodies ensure for a strong staining as usually many more secondary antibodies can bind the primary antibody than the primary antibody can bind the target antigen. The specificity of the antibodies is important in order to succeed with a specific staining of the target and to avoid unspecific staining of the surrounding tissue. Monoclonal antibodies are therefore often used as primary antibodies while the more unspecific polyclonal antibody is used as secondary antibody as the unspecific binding makes many secondary antibodies with the reporter molecule attached bind all-over the primary antibody$^{153}$. Immunohistochemistry was used in study IV in order to assess the presence of the three germ layers in the embryoid bodies i.e. alpha fetoprotein (AFP) for endoderm, vimentin for mesoderm and b-tubulin for ectoderm, 46-diamidino-2-phenylindole (DAPI) was used as nuclear stain.

All slides were deparaffinised in xylene and hydrated through a series of 99, 95 and 70% ethanol baths and subsequently incubated in PBS for 10 min. The sections were boiled in 0.1 M TRIS- buffer pH 9 for first 8 min in 750 W and subsequently for 15 min in 350 W in a microwave oven for antigen retrieval, left to cool down to room temperature and rinsed in PBS. Antigen retrieval procedures may some times be necessary and are performed in order to restore the antigen after the fixation and must be designed specifically for each antigen. Endogenous peroxidase was quenched with 3% H$_2$O$_2$ for 10 min. The slides were washed in PBS before individual treatment with one of the three germ layers marker antibodies as described below. Isotype control IgG was used as negative control on each analysed slide and applied in the same concentration as the primary antibody. Negative control antibody was:
monoclonal mouse isotype IgG₁.

3.8.2 Alpha fetoprotein staining
Permeabilisation of the cell membrane for AFP staining was done with 99.5% ethanol for 2 min. Blocking was done with blocking reagent supplied in the TSA™ Cyanine 3 System kit for 30 min at room temperature. The monoclonal IgG₁ mouse anti-human-AFP antibody was mounted and incubated overnight in humidity chamber at 4°C. At day 2, the sections were rinsed three times in PBS for 5 min each before they were incubated with the horse radish peroxidase coupled rabbit anti-mouse IgG₁ antibody for 45 min in humidity chamber at room temperature. The slides were rinsed in PBS and incubated with fluorophore tyramide working solution supplied in the TSA™ Cyanine 3 System kit according to manufactures instructions. The sections were counterstained with DAPI for 3 min, rinsed in PBS and mounted in p-phenylenediamine antifade solution (PPD11).

3.8.3 Vimentin and β-Tubulin staining
The procedure for the vimentin and β-tubulin analysis was performed in the same way as for AFP with the following modifications. Cell membrane permeabilisation was conducted with 0.5% Triton-X in PBS for 5 min and the primary antibodies were: monoclonal IgG₁ mouse anti-human-vimentin antibody for vimentin and monoclonal IgG₁ mouse anti-β-tubulin antibody for β-tubulin. The sections were counterstained with DAPI for 3 min, rinsed in PBS and mounted in PPD11. All sections were finally analysed and photo-documented using a Nikon Eclipse 90i fluorescence microscope with the NIS elements D 3.0 imaging software.

3.8.4 Protein Isolation
In study III the proteins from the pellet mass cultures were isolated through homogenization in a MixerMill of the pellet with a 5 mm stainless steel bead in ELISA lysis buffer. The protein isolation from medium in study III was performed through centrifugation of 600 µl medium to 250 µl with Amicon Ultra-4 Centrifugal Filter Unit, cut-off 3kDa according to the manufacturers protocol. These filters allow water and ions to pass through the membrane and leave proteins larger than 3kDa on the sample side of the membrane, thus concentrating the sample.
3.8.5 Enzyme-linked ImmunoSorbent Assay

Quantification of specific protein amounts in medium and cell lysates with Enzyme-linked ImmunoSorbent Assay (ELISA) is non-laborious, sensitive and cost effective. In study I and III double antibody sandwich ELISAs were used. The name sandwich comes from the structure of the ELISA. Primary antibodies that capture the antigen is coated to the bottom of preferably a 96-well plate; the sample is then poured into the well where it is bound to the primary antibody and a second enzyme linked detector antibody is subsequently bound to the antigen. Unbound antibody is washed away to avoid saturation of the enzyme driven reaction where an added substrate is transformed into a colour by the enzyme attached to the second antibody. The amount of the generated colour is proportional to the antigen concentration and is usually determined via optical density by a spectrophotometer. In study I the samples were diluted 1:49 for the TIMP1 ELISA and used undiluted for the MMP2 ELISA. Optical density of each well was determined with an Infinite® F50 Absorbance microplate reader set to measure 450 nm with the wavelength correction set to 540 nm. Measurement data and protein amounts were calculated using the Magellan™ V6.6 software.

Quantikine® ELISAs for β-catenin and DKK1 were used according to manufactures instruction in study III. In short up to 200 µl sample was added to the capture antibody in the well and incubated for 2 hours. The wells were thoroughly washed with the supplied ELISA wash buffer and 100 µl of horseradish peroxidase (HRP) conjugated antibody was added to the well and incubated for 2 hours. After another round of thorough washing with ELSA wash buffer 100 µl of the HRP substrate 3,3´, 5,5´-tertramethylbenzidine (TMB) was added to each well and the plate was incubated for 20 minutes in the dark. Acidic stop solution (50 µl) was finally added to each well and the optical density was measured at 450 nm on a microplate reader. A supplied standard curve was used for sample concentration calculations.

Active MMP13 in the medium collected in study III was quantified with the fluorokine® ELISA according to manufactures instruction. In order to measure active MMP13 the secreted inactive proenzyme needs to be activated by removal of the prodomain. This is naturally done by other MMPs, e.g. MMP-2 and MMP-4 but can be done by p-aminophenylmercuric acetate (APMA). Briefly 200 µl sample was incubated for 3 hours in the pre-coated well. The wells were washed thoroughly and 200 µl of APMA was added for activation of MMP13 during one hour. The wells were washed again and 200 µl of enzyme substrate was added and incubated for 18 hours. The resulting relative fluorescence was measured in a fluorescence plate reader.
3.8.6 Stable isotope labelling by amino acids in cell culture

Stable isotope labelling by amino acids in cell culture (SILAC) is a metabolic labelling technique that incorporates stable isotope labelled amino acids in all newly synthesized proteins\textsuperscript{154}. The isotope labelled proteome from one cell culture can be qualitatively and quantitatively compared to another culture condition or cell line cultured with normal amino acids using mass spectrometry. Hence, SILAC combined with MS makes a powerful tool capable of analysing global proteomic variances.

In study I, SILAC was used to label the chondrocytes harvest from tissues with different Mankin score. In short the extracted chondrocytes from differently Mankin scored tissues of the femoral condyle of the knee were seeded, at 16 × 10^3 cells/cm\textsuperscript{2}, in SILAC DMEM/F12 medium. For the chondrocyte cultures from the medial femur condyle the SILAC DMEM/F12 medium contained 0.1 mg/ml of stable isotope labelled arginine and lysine (U-13C6-Arginine and U-13C6- Lysine) and for the chondrocyte cultures from the lateral femur condyle the SILAC DMEM/F12 medium contained 0.1 mg/ml of normal arginine and lysine. In addition, the low Mankin scored cells were also cultured with the inverted labels. Medium was changed every third day. All cultures were conducted in a humidified atmosphere at 37°C, and in 5% CO\textsubscript{2}. 

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Before the media samples are analysed with MS the proteins need to be isolated, roughly separated and digested with trypsin. Briefly the samples from labelled and unlabelled media were mixed in equal volumes (2 ml) and the volume was reduced from 4 ml to approximately 50 μl on the Amicon Ultra-2 centrifugal concentrators with a molecular cut-off of 3,000 Da. The concentrated protein secretome in the retentate was separated by denaturing one-dimensional polyacrylamide gel electrophoresis. For in-gel trypsin digestion each gel lane was divided into 15 equally sized gel slices and subjected to automated trypsin digestion on the BioMek 2000 workstation equipped with a vacuum manifold. Finally, peptides were eluted in two times 40 μl of 60% acetonitrile in 0.1% trifluoroacetic Acid (TFA) and the eluted fractions were evaporated to dryness in a speedvac centrifuge. Prior to LC-MS/MS analysis samples were redissolved in 0.1% formic acid. For experimental line-up see (Figure 6).

Figure 6. Schematic view over the experimental procedures in study I.

3.8.7 Mass spectrometry analysis

**Protein enrichment and digestion**

Before the media samples are analysed with MS the proteins need to be isolated, roughly separated and digested with trypsin. Briefly the samples from labelled and unlabelled media were mixed in equal volumes (2 ml) and the volume was reduced from 4 ml to approximately 50 μl on the Amicon Ultra-2 centrifugal concentrators with a molecular cut-off of 3,000 Da. The concentrated protein secretome in the retentate was separated by denaturing one-dimensional polyacrylamide gel electrophoresis. For in-gel trypsin digestion each gel lane was divided into 15 equally sized gel slices and subjected to automated trypsin digestion on the BioMek 2000 workstation equipped with a vacuum manifold. Finally, peptides were eluted in two times 40 μl of 60% acetonitrile in 0.1% trifluoroacetic Acid (TFA) and the eluted fractions were evaporated to dryness in a speedvac centrifuge. Prior to LC-MS/MS analysis samples were redissolved in 0.1% formic acid. For experimental line-up see (Figure 6).
**LC-MS/MS analysis**

The fractions of trypsin digested and formic acid dissolved peptides were chromatographed and analysed in-line with an electrospray ionization linear ion trap quadrupole fourier transform ion cyclotron resonance (ESI-LTQ-FTICR) instrument. The ions generated in the ESI (precursor ions) were measured for mass in the FTICR analyser, the 7 most intense peaks in each full mass scan, with charge state \( \geq 2 \) and intensity above a threshold of 100, were put through fragmentation in the linear ion trap, LTQ, by collision induced dissociation (CID). The collision takes place between the precursor ion and neutral helium molecules, which generates peptide backbone fragmentations. The new fragments (product ions) are mass measured by LTQ. The measured ions from the peptides were identified with the Andromeda search engine integrated into the MaxQuant package version 1.2.0.18. Searches were performed against the IPI human database (version 3.68, 8,7061 sequences). Median of all peptide ratios assigned to the protein are used to determine SILAC protein ratios. For quantification a minimum peptide ratio count of two was set for each protein.

**Data analysis of MS results**

The mass spectrometer generates bulk of data that needs to be broken down and put into context. This was done in study I by the use of the ProteinCenter software version 3.8.2014. The data sets from the explant cultures were compared to the data sets generated from the monolayer cultures to investigate the differences between explant and monolayer cultured secretomes. Additionally, the biological context of the proteins that were differentially abundant when comparing the medial and lateral femoral condyles were investigated by gene ontology (GO) analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7\textsuperscript{155,156}. DAVID is an Internet based, user-friendly application that analyses the possible enrichment of gene ontology terms in a selected gene or protein list compared to a background list, in study I the total human genome. This allows for a fast analysis of the biological processes that are active or mute among the genes or proteins in the result list of an experiment. Briefly, the lists of differently abundant proteins were uploaded to DAVID with official gene symbol as identifier and the gene ontology term Biological Process FAT (GOT ERM_BP_FAT) was chosen for gene ontology analysis. The functional annotation chart selection within the application was used for analysing the functional annotation results. Genetic association database for disease was used in DAVID to analyse if the proteins that were present in both monolayer chondrocytes and explants were associated with any known disease.
3.9 Transcription analysis techniques

3.9.1 RNA isolation and quantification

Gene expression studies were performed on transcription level in study II and III. The total RNA was extracted from the monolayer cultured cells by first cell lysis through incubation with RLT lysis buffer, which lyse the cells and simultaneously inhibits RNAses. The RNeasy mini kit was then used to purify the total RNA according to the manufactures instructions for animal cells.

The pellet mass cultures were first homogenized in RLT buffer using a MixerMill with a 5 mm stainless steel bead and the total RNA was subsequently isolated with the RNeasy mini kit following the manufactures protocol for animal cells.

The total RNA amount and quality were measured with a Nano Drop®. The wavelength ratio between $\lambda_{260}/\lambda_{280}$ is considered a purity measurement and a value between 1.9 and 2.1 was considered to be an acceptable quality of the total RNA fraction.

All extracted RNA samples included in the present thesis were treated with Deoxyribonuclease I (DNaseI) using an on-column procedure according to the manufactures recommendations. RNA integrity was investigated before microarray analysis with an Agilent Bioanalyzer and a RNA integrity number (RIN) was assigned to each RNA sample. The Agilent Bioanalyzer works as an electrophoresis gel but on a microfluidic capillary chip with a fluorescent dye bound to the RNA. An electropherogram (fluorescence over time) and a gel-like image are generated. Several characteristics of the electropherogram are used to generate the RIN value e.g. height of the 28s, 18s and marker peak, areas and ratios of specific regions. This method is considered to be more adequate than judging the 28s (5kb) : 18s (2kb) rRNA ratio, where a ratio of 2 is considered intact good quality total RNA.

3.9.2 Real time PCR

Transcription levels were measured through a two-step real time polymerase chain reaction, often denoted quantitative PCR (qPCR) in order to distinguish the abbreviation from reverse transcription PCR (RT-PCR). The first step is a reverse transcription of total RNA into one copy of its complementary DNA (cDNA). The synthesis of cDNA was performed with the High Capacity cDNA Reverse Transcription Kit with random hexamer primers according to the manufactures instructions on a 2720 Thermal Cycler set on initial temperature 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5
minutes. The second step is the actual real-time PCR, which was initially described by Holland et al. where the 5′→3′ exonuclease activity of Thermous aquaticous (Taq) polymerase is the main feature of the technique\textsuperscript{157}. A probe labelled at the 5´end with a fluorescent reporter dye and at the 3´end with a fluorescence quencher is designed to anneal between a forward and reverse primer on a specific region on the cDNA of interest. The used reporter dye was 6-carboxyfluorescein (FAM) in this thesis. When the probe is intact the quencher is close enough to absorb the fluorescence from the reporter. During the amplification the probe is cleaved by the Taq polymerase, releasing the reporter dye that can freely fluorescent while not tethered to its quencher. With each cycle of amplification more and more reporter dye molecules are cleaved from their probes and the increasing amount of fluorescence is proportional to the amount of produced amplicon. The higher gene expression, the higher specific mRNA content and thus cDNA content in the starting material, the more amplicon is produced and the sooner the fluorescence is detected. The reaction proceeds until reagents run out but the amount of amplified products are calculated while reagents are in excess and the reaction is presumably 100% i.e. the exponential phase. The fluorescence is measured continuously through out the amplification cycles and when the fluorescence for each sample reaches a certain threshold in the exponential phase an amplification cycle number, or so called cycle threshold (Ct) is determined. The more starting material the sooner the fluorescence threshold is reached, which means that a gene that has a high expression receives a lower Ct value than a low expressed gene. Samples were analysed in duplicates and a random sample was run on a regular agarose gel to check that the correct amplicon was produced. Each plate was run with a positive control (a RNA mixture from different donor chondrocytes and chondrocyte progenitor like cells) in order to compensate for inter-assay variations. An endogenous reference gene was analyzed with each sample. The reference gene needs to be stably expressed through out the experiment and the gene Cyclophilin A was chosen for the experiments in this thesis based on in house tests. In study II and III 2.5 ng cDNA was mixed with TaqMan Universal PCR master mixture, 1×Assay-on-demand mixes of primers and TaqMan major groove binding probes in a total reaction volume of 10 µl. The reaction was run on a Lifetechnologies 7900HT Real-Time PCR System with an initial denaturation step at 95°C for 10 minutes followed by 40 cycles of 95°C denaturation for 15 seconds and hybridization and extension at 60°C for 1 minute. The relative gene expression of each gene was calculated using the Comparative CT method according to the assay-on-demand manufactures instructions.
3.9.3 The microarray technology

The microarray technology has since the 1990s made it possible to simultaneously analyse multiple gene expressions in a sample. The possibility to even analyse the expression of the total genome in one run has revolutionized the analysis of several biological processes including differentiation, tissue generation and disease aetiology. The technology is based on the complementary hybridization between a mobile, labelled transcript and an immobilized probe on the array surface. The microarray is basically build by many probes organized in to features (i.e. locations) and each feature harbours an excess of probes that are complementary to a certain transcript. The procedure may vary between different arrays but is mainly performed through initial reverse transcription of the RNA into cDNA, which is labelled with a fluorophore. The labelled cDNA solution is applied onto the array surface and the cDNA hybridise to its corresponding probes. The scanning of the microarray is performed after non-hybridized transcripts have been washed away. The fluorescence intensity for every feature, which is directly proportional to the amount of gene transcripts that have hybridised to that feature, is measured during the scanning. There is a considerable amount of data generated from a microarray experiment and it needs to be processed before it can be interpreted. The microarray data processing typically includes subtraction of background; normalization, log₂-transformation (to achieve normal distribution of the data) and outliers are evaluated for exclusion. In study II the Affymetrix whole transcript Gene ST 1.0 array was used to search for transcriptional differences between chondrocytes that were either used in successful or failed ACIs. Briefly the fragmented cDNA (reversely transcribed from the total RNA) was hybridised at 45°C for 16 hours to whole transcript Gene ST 1.0 arrays. The microarrays were scanned on a GeneChip Scanner 3000 7G and expression signals were extracted and normalized using the Expression Console™ applying the Robust Multichip Average (RMA) normalization method.

3.9.4 Microarray data analysis

In the present thesis the bioinformatics analyses that follows the initial preprocessing of the data included; clustering, identification of differentially expressed genes and pathway analysis and functional annotation. Clustering is used to identify co-expressed and functionally related transcripts in a large dataset. Hierarchical clustering was performed using Pearson correlation and average linkage in study II. Different filtering thresholds of background expression were evaluated. There are other clustering techniques that can be used e.g. K-means and self-organizing maps. Identification of differentially expressed genes between the success and the failure groups in study II was
performed using a combination of Students t-test and mean fold change (FC) values between the two groups. Genes with p-values < 0.05 and with a FC of more than 20% were defined as differentially expressed. Another commonly used method for identification of differentially expressed genes is the Significance Analysis of Microarray data (SAM). The SAM algorithm measures differences between conditions and assigns a score to each gene represented on the microarray. The score is relative to the standard deviation and repeated measurements. A pathway analysis was performed to identify differentially expressed sets of genes between the two groups in study II by applying the Gene set enrichment analysis (GSEA) using KEGG pathways as gene sets. GSEA scores a defined set of genes, e.g. a pathway, based on the different expression levels and is especially useful when searching for subtle but coordinated expression changes.

Although the microarray technology is powerful for global transcriptional analysis it has limitations that scientists should be aware of. These include;

- Cross-hybridisation of transcripts to probes that are designed to detect other transcripts. On the Affymetrix chip there are multiple probe pairs for each target transcript in order increase the specificity.
- Poor sensitivity for transcripts with low expression has been reported for various microarrays.
- Fold change compression can be noted with the array technology compared with qPCR due to signal saturation and limited dynamic range.

### 3.10 Statistics

The statistical tests in this thesis were conducted depending of the type of data and the data distribution.

The PCR validation of the microarray in study II was statistically analysed with Unpaired Student t-tests to compare both groups.

The data in study III were analysed by students t-test or one-way ANOVA with the Tukey post hoc test where the test ranged over more than two groups and p-values were considered significant at p<0.05.

In study IV statistical analysis was performed using the Chi Squared test for trend and the two-sample student’s t test assuming equal variances. Statistical significance was set to p < 0.05.
3.11 Ethical approval

All studied material (tissues, cells and serum) throughout this thesis was donated after informed consent. The ethical approval to conduct studies on human chondrocytes and to use human serum when culturing chondrocytes were granted by the institutional review board at The Sahlgrenska Academy at The University of Gothenburg (Dnr 040-01 for studying human chondrocytes; Dnr 367-02 for using human serum when culturing chondrocytes).
4 RESULTS

4.1 Chondrocytes from different stages of osteoarthritic degeneration express proteins differently (study I)

Study I was designed to investigate the total secretome of chondrocytes isolated from cartilage in different stages of osteoarthritic degeneration. The study was a hypothesis generating approach to find biomarkers and pathways correlated to cartilage degeneration and thus osteoarthritis disease stage. Monolayer cultured chondrocytes from the medial and lateral femoral condyle were analysed for their secretome composition as cartilage explants were not compatible with the SILAC labelling procedure. Although chondrocytes dedifferentiate during monolayer culture, demonstrated by presence of collagen type I alpha 1 and 2 subunits and absence of collagen type II subunits in the monolayer secretome, the result from study I show that 172 proteins were identified in both monolayer cultures and cartilage explants. These 172 proteins represented 54% of the proteins identified in the explant secretome. Among the proteins identified in both explant and monolayer cultures were several cartilage ECM remodelling proteins, e.g. cartilage oligomeric matrix protein (COMP), MMP-1, -2, -3 and −10, metallopeptidase inhibitor (TIMP) 1 and 2 and several isoforms of collagen. The genetic association for disease term osteoarthritis was also highly connected to the 172 proteins found in the secretome of both monolayer cultures and cartilage explants. The most enriched terms from gene ontology analysis for biological process of the 172 common proteins were involved in ECM organization and inflammation processes. The monolayer cultures were considered eligible as model to study osteoarthritic processes. The subsequent analysis resulted in two comprehensive secretomes. One high Mankin scored secretome with 825 proteins and one low Mankin scored secretome with 528 proteins. The high Mankin scored secretome showed significant differences in the protein amounts of 69 protein groups, among these, 28 protein groups were medially abundant and 41 were laterally abundant. The significantly regulated high Mankin scored secretome showed the highest statistically significant enrichment of the gene ontology terms GO:0006954 inflammatory response and GO:0009611 response to wounding.
The medial femoral chondrocytes of the low Mankin scored individual showed a higher abundance of 34 protein groups. Gene ontology analysis of biological process showed statistically significant enrichment of the terms GO:0001501 skeletal system development, GO:0042127 regulation of cell proliferation, and GO:0030198 extracellular matrix organization. The femoral condyle lateral chondrocytes from the low Mankin scored individual secreted 166 protein groups at a higher level than the compared medial chondrocytes. The laterally abundant protein groups showed significant term enrichment results in gene ontology analysis for GO:0030036 actin cytoskeleton organization e.g. Destrin, GO:0006006 glucose metabolic process e.g. Enolase 1 and GO:0006916 anti-apoptosis e.g. Annexin a1. Interestingly, the femoral medial and lateral protein SILAC ratios seen in the low Mankin scored individual were lost in the high Mankin patients, i.e. the SILAC ratio between the medial and lateral femur condyle compartments were close to one. Thus, quantitative differences in protein secretion between the medial and lateral compartments found in the low Mankin scored individual are normalized in the medial and lateral compartments in the high Mankin scored individual.

The chondrocyte secretomes from high and low Mankin scored cartilage were analysed for unique proteins only found in the low Mankin scored individual, which resulted in 25 unique proteins where 5 were found in both labelled experiments (EPB41L2, AKAP12, FABP3, SCRN-1, CDV3).

4.2 Gene expression profiles in expanded chondrocytes do not correlate with clinical outcome of autologous chondrocyte implantation (study II)

The aim of study II was to evaluate if the expression of a predefined set of genes in chondrocytes prior to ACI could be associated with clinical outcome. Furthermore, the study aimed at investigating if there were any transcriptional differences in chondrocytes prior to implantation that could explain the biological mechanisms leading to the clinical outcome. Five clinically success and five clinically failure ACI patients were included in the study. The expression pattern in chondrocytes before implantation was analysed with microarray in order to investigate expressional differences that may influence the clinical outcome.
Neither the comparative microarray analysis nor the principal component analysis of the array data showed expressional disparities that could separate success and failure groups. Additionally, the predefined set of gene markers suggested by Dell’Accio et al.\textsuperscript{160} to predict chondrogenicity did not contribute to any separation of the two groups. The same result was found for the genes identified to be important for chondrogenic capacity by Dehne et al.\textsuperscript{161}. A student T-test identified 541 transcripts to be differentially expressed between the groups. A total of 346 transcripts were induced in the success group and 195 transcripts in the failure group. The list of 541 transcripts was filtered to remove genes with small fold changes (< 1.2). This approach resulted in 39 genes that were induced and 38 genes that were repressed in the success group. The gene ontology analysis in DAVID showed the GO biological process term GO:0007185 “transmembrane receptor protein tyrosine phosphatase signaling pathway” to be enriched among the induced genes in the success group. The repressed genes in the success group showed enrichment of several terms related to a reparative potential e.g. the GO biological process terms: GO:0042981 “regulation of apoptotic process” and the term GO:0043062 “extracellular structure organization”. Finally, the array data was analysed for gene set enrichment, which enables investigation of small expressional changes of genes involved in for instance signalling pathways. The analysis showed 15 enriched gene sets in the failure group and zero enriched gene sets in the success group. Among the 15 enriched gene sets in the failure group, the cytokine cytokine-receptor interaction pathway showed enrichment of several cytokines e.g. IL3, IL8, IL19, IL20, IL22 IFNA16, IFNA21, IFNA8, IFNB1 and IFNK.

4.3 GDF5 stimulation represses expression of MMP13 through inhibition of the Wnt signalling pathway in human chondrocytes (study III)

The aim of study III was to investigate whether GDF5 stimulation affected the Wnt signalling pathway in human chondrocytes, as these pathways are known to be involved in OA pathogenesis. The study also aimed to investigate effects of GDF5 stimulation on ECM production of human chondrocytes. Recombinant GDF5 stimulation of chondrocyte micromass cultures resulted in an anabolic state in the cells as shown by increased GAG/DNA ratio, more intense Alcian Blue van Gieson staining, increased DNA production and pellet size, upregulation of the anabolic genes SOX9
and ACAN and decreased expression of the catabolic genes MMP13 and ADAMTS4. Reduction of active MMP13 levels was also seen in the culture medium of GDF5 stimulated pellets. GDF5 stimulation increased the expression of Wnt signalling inhibitors DKK1 and FRZB. Increased DKK1 levels were also seen in the medium of pellet mass cultures stimulated with GDF5. Experiments to verify that the Wnt signalling pathway was inhibited by GDF5 stimulation showed reduced levels of total β-catenin in GDF5 stimulated pellets. Downstream target genes of Wnt signalling, FOSL1 and PPARD showed decreased expression levels after GDF5 stimulation. Also, the reduced MMP13 levels were restored in GDF5 stimulated pellet cultures when the GDF5 induced DKK1 protein was blocked by a small molecule. Taken together the results show that GDF5 stimulation reduces MMP13 expression in pellet mass cultures via inhibition of Wnt signalling by induced DKK1 expression (Figure 7).
Figure 7. Illustration over the crosstalk between GDF5 and Wnt signalling where GDF5 increases expression of the Wnt inhibitor DKK1, which reduces Wnt signalling and consequently MMP13 expression.
4.4 Agarose culture of embryoid bodies from human embryonic stem cells enables stable embryoid body formation and efficient differentiation for long-period cultures (study IV)

In study IV, the agarose culture of embryoid bodies aimed at improving the EB culture procedure for future model systems of chondrogenic signalling pathways. The three dimensional environment of agarose culture improved the EB formation efficiency and increased growth by up to three times of the EB grown in agarose as compared to both suspension and hanging droplet cultures. Finally, agarose culture enabled for long term cultures of up to eight weeks and supported pluripotent differentiation as shown by development of the three germ layers verified by expression of AFP for endoderm, vimentin for mesoderm and β-tubulin for ectoderm.
5 DISCUSSION

5.1 Osteoarthritis aetiology is elusive.

Musculoskeletal defects, osteoarthritis being one of the most common, are major reasons for impaired movement and pain in the locomotion system. Impaired movement may also affect the overall health status of affected individuals, as these patients are unable to perform health beneficial exercise, a field that needs to be studied further. The pain and loss of movement, combined with the growing life expectancy of the population worldwide are a financial burden to the society. Damaged cartilage has limited healing abilities, and even the smallest damage can be a starting point for OA development. There are several treatment approaches for osteoarthritis but they are predominantly performed at a late stage of the disease, as the onset of symptom is late, and they mainly aim at reducing the symptoms and possibly slowing down the progression. Therefore there is a need to extend the knowledge about OA aetiology in order to find new interventions that can stop the degeneration process before the tissue is beyond recovery and maybe even initiate healing of the tissue. Several large studies have investigated the inheritance of OA, resulting in a few candidate genes that increase the risk of OA, e.g. GDF5, asporin (ASPN) and deiodinase, iodothyronine, type II (DIO2). The present thesis aimed at investigating the chondrocyte secretome and transcriptome for new biological markers of disease and possible disease and regenerative mechanisms. Further the interplay between two important signalling pathways in joint development and osteoarthritis was studied together with a non-laborious model system for additional signalling pathways studies using human ES cells.

5.2 Different osteoarthritis degeneration stages may reveal new early disease markers (I)

Cartilage is avascular, immune privileged and lack neurons; therefore there are no systems in the body that can detect the degenerative process of early osteoarthritis. The lack of alarming symptoms lets the disease progress until the cartilage tissue is damaged to such extent that the disease involves subchondral bone and synovium with severely diminished joint function as a result. The slow and masked degeneration process is important to detect and turn at an early stage, as the healing capacity of cartilage is limited. Study I was
therefore designed to quantitatively compare, using SILAC technology, the secretome from chondrocytes isolated from cartilage in different degeneration stages in order to investigate proteins that could serve as early biological markers. Further the different osteoarthritis stages of the biopsies could reveal signalling pathways that drive the progression of OA.

The Mankin score system was used to assess the degree of osteoarthritis in the biopsies. The Mankin score of the biopsies from the asymptomatic amputation patient with no clinical or macroscopical signs of OA indicated that although the low Mankin scored patient was asymptomatic from any joint disease the medial compartment was weakly affected and could hypothetically be regarded as a mild early stage of OA. The biopsies harvested from OA patients also showed differences in Mankin scores between the medial and lateral sides and could be regarded as a late stage of OA, with the most propagated disease in the medial side. The differences in protein secretion between the medial and lateral sides could thus be interesting as potential markers of early OA. IGFBP7, EFEMP1, FSTL1, CHI3L1, IGFBP4, SPARC and B2M were selected as putative markers of early OA as the SILAC ratio showed an elevated abundance in the medial compartment in the patient with no joint disease history. These medial and lateral differences were lowered and eventually equal when the disease propagated and included the lateral compartment. This was shown in the OA patients where SILAC ratios from the OA patient with the lowest Mankin score resembled SILAC ratios from the amputation patient the most.

5.2.1 Different OA disease stages may reveal disease mechanisms (I)

The different stages of OA disease as shown by Mankin score could reveal differences in the abundance of proteins that may affect the disease progression. IGFBP4 was elevated in the secretome from the medial chondrocytes of the low Mankin scored individual. IGFBP4 is suggested to inhibit the canonical Wnt signalling pathway, which is an interesting finding as stimulated Wnt signalling is known to be involved in OA pathogenesis. The elevated IGFBP4 level may indicate a counteracting process to an OA initiation in the medial femur condyle of the low Mankin scored individual, which may have balanced cartilage homeostasis in this aged and sex matched individual who never developed clinical signs of OA. Taken together with the results from study III it appears promising to regulate the Wnt signalling pathway in order to promote anabolic processes in chondrocytes. The results from study III are discussed later in this section. Another interesting finding was the regulation of dystrophin (DMD) that was present at lower levels in
the medial than the lateral femoral condyle of OA patients. Lack of DMD may indicate an escalating tissue degeneration as DMD deficient female mice show significantly reduced cartilage thickness and cartilage tissue area on the proximal femur head compared to wild type control upon voluntary exercise\textsuperscript{171}. Lack of DMD expression could thus indicate a more progressed OA stage or the presence of DMD could indicate a better prognosis of OA. The use of DMD as prognostic marker of OA would be interesting to investigate further. Cytokine like protein 1 (CYTL1) was only found in two HM patients with an elevated level on the femur lateral side but was also elevated in the medial compartment of the patient with no joint disease. CYTL1 is a novel autocrine regulatory factor that regulates chondrogenesis of mouse mesenchymal cells\textsuperscript{172}. In a recent knockout study, deletion of the CYTL1 gene did not affect chondrogenesis or cartilage development. However, CYTL1 knockout mice were more sensitive to osteoarthritic cartilage destruction and expression levels of CYTL1 were markedly decreased in OA cartilage of humans and experimental mice\textsuperscript{173}. It can be argued that CYTL1 is not an adequate biological marker for OA as it is elevated in early OA as compared to healthy cartilage in the patient with no joint disease and the expression is again reduced when the disease progresses as shown by the low levels in the medial compartment of OA patients. However CYTL1 is interesting from a disease mechanism perspective as the protein is elevated during the initial OA and when the disease progress into a late stage of OA the protein amount is decreased as compared with a less degenerated tissue. Taken together with the results from the above-mentioned animal studies CYTL1 appears to have a role in cartilage tissue maintenance, which grants for further investigation of the mechanisms behind the function of CYTL1.

5.2.2 Limitations of study I

The results from the individual with no joint disease history are well experimentally documented by repeated independent cultures from the medial and lateral compartments in both labelled and unlabelled medium, respectively. In this way we could analyse the secretome twice with inverted labelling conditions, which gave strong technical data that supported the validity of the results. However no statistical conclusions could be drawn regarding the state of the secretome within normal knee joints, as only one specimen was analysed. Also, the experimental set-up would benefit from explant cultures instead of monolayer cultures as the latter change the morphology of the chondrocytes into a dedifferentiated state\textsuperscript{142}. The effort to fully label the secretome of explants failed, possibly due to the slow cell cycling of chondrocytes, retention of secreted proteins in the ECM and/or leakage from the ECM of unlabelled proteins that were translated before the
labelling attempt. This could be addressed with thinner explant slides that would easier release the newly synthesised proteins or with animal models of osteoarthritis that could be fed labelled amino acids for a fully labelled proteome. An advantage of using explants is the serum free culture. Due to the limited dynamic range in the mass spectrometric analysis, high abundant proteins may hinder identification of low abundant proteins relevant for the disease mechanism. The serum supplement in monolayer cultures contain high amounts of albumin, which can interfere with the analysis. The presence of serum is thus a limitation to study I. If the explants can be fully labelled the serum aspect would be avoided as explants can be cultured without serum.

Lastly, a well-known problem in the mass-spectrometric analysis of any complex protein mixtures is the “under-sampling effect” of the mass spectrometer, which results in missed identifications of low abundant proteins.

5.3 Gene expression in chondrocytes prior to implantation does not predict clinical outcome (II)

The mechanisms behind a successful ACI is not known with regard to what factors are important for the integration, differentiation and finally maintenance of the implanted chondrocytes. The expression pattern of a set of genes (FGFR3, BMP2, COL2A1, ACAN, CD44 and ACVRL1) has been proposed to be predictive of the cells’ chondrogenic capacity. A method to predict the chondrogenic potency of the implanted cells would be beneficial for the patient as it would increase the success rate and is necessary due to European legislations. Study II aimed at investigating if there are any expression patterns in chondrocytes before implantation that could be associated with the clinical outcome of ACI. Chondrocytes from five successful and five failed ACIs were compared for expressional differences at the time of implantation. The analysis did not address the expressional differences that take place after implantation but investigated if there were any gene expression patterns that could be predictive of clinical outcome. The microarray results showed no clustering of patients indicating that there are no large transcriptional differences between the two groups. Additionally, analysis of the genes found to be important for chondrogenicity (FGFR3, BMP2, COL2A1, ACAN, CD44 and ACVRL1) by Dell’Accio et al. showed no intergroup expressional differences further emphasizing that the chondrocytes are seemingly alike before surgery; with no possibility to asses their chondrogenic potency based on gene expression. These results are contradictory with the results from other groups showing that the
chondrogenic capacity can be evaluated with the expression of the genes included in study II\textsuperscript{175-178}. The study from Saris et al.\textsuperscript{178} found the patients that had been evaluated with a high gene profile score to achieve greater improvement from baseline at 36 months compared to lower gene scores. However, this correlation was not corroborated in the recent five-year follow-up report\textsuperscript{179}. Other groups have made studies with results that are in accordance with the results from study II. Pestka et al. studied 252 consecutive ACI patients and correlated chondrocyte phenotype to patient characteristics in order to find parameters that could predict the cell quality from different individuals. Neither the expression of CD44, ACAN or COL2A1 nor cell density or viability after monolayer expansion seemed to correlate with the grade of joint degeneration, defect etiology or patient gender\textsuperscript{180}. In a similar study, Niemeyer et al. investigated the influence of cell quality on clinical outcome after ACI based on expression of CD44, ACAN and COL2A1\textsuperscript{181}. Postoperative International Knee Documentation Committee (IKDC) subjective Knee Form scores were reported to be significantly influenced by COL2A1 expression ($P < 0.05$) at 6, 12, and 24 months and CD44 expression at 24 months. Interestingly, ACAN expression did not correlate with clinical outcome at any follow up time point and cell viability as assessed by 7AAD incorporation did only negatively correlate with clinical outcome at the 12 months follow up. It seems that predicting the clinical outcome of ACI with a golden bullet such as the expression pattern of a set of genes is unlikely to be successful. It appears more interesting to take several additional factors into account e.g. defect size, patient age, preoperative joint function, preoperative activity grade, and patient BMI when predicting success of ACI.

### 5.3.1 Signalling pathways and clinical outcome of ACI (II)

Study II also aimed at investigating if any gene expression patterns could be associated with clinical success. The hopes were set at finding differential expression in a signalling pathway that would have set the chondrocytes off towards the right regenerative path. Therefore study II searched for a more subtle expressional difference between the two groups and found 39 genes to be induced and 37 genes to be repressed in the success group. Protein tyrosine phosphatase, receptor type, F (PTPRF) was induced in the success group and belongs to a family of protein tyrosine phosphatases involved in the regulation of a variety of cellular processes such as focal adhesion, migration, cell growth, differentiation, mitosis and apoptosis\textsuperscript{182-184}. PTPRF is interestingly suggested to be a negative regulator of β-catenin tyrosine phosphorylation, which preserves focal adhesion via prevention of β-catenin release from E-cadherin\textsuperscript{185}. This would mean that PTPRF have a negative effect on β-catenin
mediated signalling i.e. Wnt signalling. Some of the repressed genes in the success group are apoptosis related genes \textit{GCLC, PRLR, GRIK2, LYST, AGT, FAIM2} and \textit{PHLDA1}. It is interesting to hypothesise that the chondrocytes used in unsuccessful ACI had an increased apoptotic profile before implantation, however Zheng \textit{et al.} showed that chondrocytes used for ACI contain a low level of apoptotic cells\textsuperscript{186}. Nevertheless there may exist small differences in the regulation of apoptosis in the transplanted cells that may influence the chondrogenicity of the cells and thus clinical outcome. The role of apoptotic mechanisms in ACI needs further investigation. Finally genes involved in the cytokine system, which is known to be involved in the pathogenesis of osteoarthritis and has serious adverse effects on cartilage extracellular matrix\textsuperscript{82,187}, showed to be induced in the chondrocytes from the failure group according to GSEA. This strengthens a recent result from the study by Albrecht \textit{et al.}, which showed that \textit{IL-1\beta} expression in the transplanted chondrocytes negatively correlate with clinical outcome\textsuperscript{188}. In summary, study II is as study I a hypothesis generating approach and an appealing conclusion would be to further investigate the role of Wnt signalling modulation together with control of apoptotic processes and cytokine regulation in cells used for ACI.

\section*{5.3.2 Limitations of study II}

The right inclusion criteria are a prerequisite for good validity in any study. In study II the inclusion criteria for the success group were a statistically significant improvement in Knee Injury and Osteoarthritis Outcome Scores (KOOS), the Lysholm knee rating scale and a subjective evaluation of clinical improvement (SECI). Although the selection of failed ACI were based on patients requiring a re-intervention due to graft failure within three years after surgery without a traumatic or other apparent cause for failure, a heterogeneity within the failed group could exist due to unknown factors such as lack of graft integration, a disturbed joint homeostasis, lifestyle and/or a different compliance to rehabilitation. The comparative analysis with the success group could thus be hampered. However the clustering analysis did not show strong homogeneity within the success group either, indicating that the chondrocytes from both groups show large transcriptional homogeneity.

In addition, statistical power is dependent on the sample size and the number of included patients may not give sufficient statistical power to separate the two groups on the basis of gene expression differences.

Regarding the clinical impact of very small gene expressional differences one can argue that the relatively low expressional differences between the two
groups might have little or no effect on the clinical outcome. However, as no clear recipe of gene expression has been shown for chondrogenic differentiation of chondrocytes it would be interesting to emphasize the possibility that fine-tuning of gene expression may lead to new insights in the cellular behaviour after ACI. This is exemplified by the above mentioned gene PTPRF that is subjected to such expressional fine tuning where high ectopic expression leads to activation of caspase driven apoptosis\textsuperscript{184}. As discussed above, Müller et al. reported that even such low differences as twice the endogenous levels of PTPRF were enough to reduce the free pool of β-catenin\textsuperscript{185}. Fine-tuning of Wnt signalling during cartilage regeneration is an interesting field for future research.

5.4 Crosstalk between GDF5 and wnt signalling (III)

Crosstalk between the Wnt signalling pathway and GDF5 has been studied previously resulting in contradictory results where Guo et al. proposed that GDF5 expression and thus joint formation in mice is dependent on canonical Wnt signalling via overlapping and complementary expression of Wnt4, Wnt14 and Wnt16\textsuperscript{189}. This was later questioned by Später et al. who showed that Wnt9a and Wnt4 is not required for formation of the joint interzone in a mouse double knock-out model\textsuperscript{114}. Später et al. further showed that GDF5 expression was not altered in the joint interzone in a mouse model where the entire limb mesenchyme was depleted of β-catenin suggesting that GDF5 expression in joint formation is not dependent on canonical Wnt signalling. Furthermore, crosstalk between TGF-β/BMP and Wnt signalling pathway is known through generally three mechanisms. First, the TGF-β/BMP and Wnt signalling pathway reciprocally regulate their ligand production exemplified by BMP2 dependent downregulation of Wnt7a and β-catenin in chicken embryonic mesenchymal cells, which enhances chondrogenesis\textsuperscript{190}, and oncogenic β-catenin induced BMP4 expression in human colon cancer cells\textsuperscript{191}. Second, the signalling transduction proteins, generally Smad4 for TGF-β/BMP signalling and β-catenin for canonical Wnt signalling, crosstalk in the nucleus where they form a protein complex with lymphocyte enhancer factor (LEF) and synergistically activate transcription of the Xenopus homeobox gene twin (Xtwn) in HepG2 cells\textsuperscript{192}. A third novel crosstalk mechanism is cytoplasmic interaction between BMP and Wnt signalling elements, which is exemplified by reduction of Wnt signalling upon BMP-2 stimulation through a mechanism where Smad1 forms a complex with dishevelled segment polarity protein 1 (Dvl-1) and thereby blocks the GSK-3β inhibiting effect of the Dvl-1 protein. The antagonized inhibition of GSK-3β leads to continued
ubiquitination and degradation of β-catenin and thus inhibited Wnt signalling\textsuperscript{193}. Study III aimed at further investigating the crosstalk between GDF5 and Wnt signalling in human chondrocytes in an adult cartilage model as both pathways are implemented in OA as described in the introduction of this thesis.

The most interesting parts in study III were the decreased MMP13 expression and the simultaneous increased expression of the Wnt signalling inhibitors DKK1 and FRZB with a confirmed Wnt signalling inhibition. DKK1 turned out to be the key mediator of the MMP13 inhibitory effect of GDF5 stimulation as shown by the rescue of MMP13 expression when a small molecule that inhibits DKK1 was added simultaneously with GDF5 to the pellet mass medium. The MMP13 inhibitory effect of GDF5 further strengthens the role of BMPs as modulators of ECM degradation as BMP7 have previously been shown to decrease the expression of MMP13 in rabbit knee joints\textsuperscript{194}.

### 5.4.1 GDF5 shifts chondrocyte homeoastasis (III)

The first visible result of human recombinant GDF5 stimulation on human chondrocyte micromass cultures was an anabolic effect in the pellets with increased proliferation and ECM production. The anabolic result confirms the results from other groups that also indicate an anabolic effect of GDF5 stimulation\textsuperscript{195}-\textsuperscript{197}. However the anabolic effect on chondrocytes seen by GDF5 stimulation in study III may not be the most important as the expressional level of \textit{COL2A1} and \textit{ACAN} in the stimulated pellets is considerably lower than in biopsies from normal and OA cartilage. The more interesting effect of GDF5 stimulation is the reduced expression of MMP13 in the pellets which adds to the anabolic effect as MMP13 is one of the major cartilage ECM degrading enzymes\textsuperscript{87}. Taken together the anabolic and MMP13 inhibitory effect of GDF5 stimulation in chondrocytes indicates that the growth factor shifts the chondrocyte homeostasis into an anabolic state. These features of GDF5 may be coupled to OA and GDF5 has previously been associated with OA. Absence of the GDF5 receptor \textit{Bmpr1a} in mice leads to a hampered GDF5 signal and eventually OA\textsuperscript{198}. The SNP in the GDF5 gene discussed in the introduction of this thesis reduces the promoter activity of the GDF5 gene and thus the transcribed GDF5 mRNA levels\textsuperscript{199}. It is not known if this reduction in promoter activity is the reason why GDF5 has been linked to OA. Nonetheless, it is an interesting hypothesis that the reason why the patients with the GDF5 SNP develop OA is partly because the reduced GDF5 expression leads to an impaired regulation of MMP13 and anabolic
processes shifting the cartilage homeostasis towards a catabolic state and eventually OA development in theses patients. The results from study III adds to the knowledge regarding the elusive effect of GDF5 on adult cartilage homeostasis, which is generally considered unknown.

5.4.2 GDF5 as an osteoarthritis disease-modifying drug (III)

Given the anabolic properties of GDF5 and the connection between low GDF5 expression due to a SNP in the promoter and OA it would be interesting to study whether GDF5 administration to the joint could slow down or even turn an OA progression. Simank et al. have addressed the regenerative properties of GDF5 in a study where a GDF5 releasing scaffold was implanted into rabbit cartilage osteochondral defects. The GDF5 releasing scaffold increased the healing response at the early time points of four and eight weeks, however the Pineda score was not different between the GDF5 treated group and control after 24 weeks. The results from Simank et al. indicate that GDF5 has a positive effect on cartilage regeneration although it may not be the exclusive solution to the mechanism. Nevertheless, as other members of the BMP family e.g. BMP2 and BMP7 are considered clinically safe GDF5 administration could make it to the clinic as one intervention to modify disease progression of OA.

5.4.3 Limitations to study III

The pellet cultures used in study III do not express ACAN or COL2A1 to a high degree compared to explants, which implies that these cells have gone far in the dedifferentiation process during the monolayer expansion phase. This can be considered a major limitation to the study and the role for GDF5 in fully differentiated chondrocytes in vivo remains to be determined. However, it should be noted that all pellets in study III were cultured without TGFβ1, which is the main anabolic supplement in the pellet mass culture system originally developed for cartilage differentiation of MSCs and validated for human articular chondrocytes by Tallhed et al. The absence of TGFβ1 may have hampered the anabolic processes by the chondrocytes but was excluded in order to not mask the effects of the examined GDF5 signalling.

Hypothetically, the reduced amounts of active MMP13 protein in the medium could have been due to a decreased MMP13 release or increased breakdown of the protein. This could be considered unlikely as we show a simultaneous effect on both mRNA and protein level.
Study III does not describe if GDF5 treatment would have any physiological relevance but as the effect of GDF5 treatment was prominent in vitro although the cells had endogenous expression levels of GDF5 the effect may have physiological relevance in vivo since the expression was comparably low in cartilage biopsies.

5.5 Human ES cells as model system for pathway studies (IV)

The present thesis demonstrates that signalling pathways and their regulation may have implication for osteoarthritis and cartilage regeneration. Therefore it is interesting to further study fine-tuning of different signalling pathways in chondrocytes to elucidate OA disease mechanisms and test different cues that stimulate chondrogenesis. The pellet mass system is a well-established chondrogenesis model but the model has limitations e.g. poor availability of normal cartilage donors and genetic variations between donors. Therefore a robust and potent model system would facilitate studies on signalling pathways during chondrogenesis. Human ES cells have potential to serve as cell source to many different cell types and could serve as a model system over chondrogenesis. It is common that differentiation protocols of human ES cells and other sources of stem cells use an EB stage to initiate differentiation. Study IV aimed at rendering the EB formations process more effective in a cost effective and non-laborious method as formation and long-term culture of EBs are troublesome in suspension. Study IV demonstrated that EB formation and growth are enhanced in an agarose based 3-D environment compared to the hanging droplet system and the regular suspension culture in bacteriological Petri dishes. Properties of agarose culture that putatively enhances formation efficiency are the prevention of cell clusters from attaching to the culture dish and agglomerate and supply of mechanical stability and support, which enables the EBs to form without the risk of dissolving into smaller unproliferative cell clusters or single cells. The agarose culture of EBs made it possible to perform long-term cultures as compared with suspension cultures and supported pluripotent differentiation. Agarose culture allowed for differentiation into the three germ layers suggesting that the agarose system does not direct differentiation into a specific lineage. In summary, the results indicate that the system is valid for differentiation studies over an extended time period. The results confirmed the reported variation in differentiation propensity among human ES cell lines. This trait of ES cells lines should be taken into account when studying differentiation and may be assessed more cost efficiently with EB agarose cultures than with teratoma formation in mice. Agarose culture has been used
in several studies of chondrogenesis where the three-dimensional support enabled long-term cultivation, application of mechanical stimuli and growth factors \(2^{13-215}\). It may be interesting to bring this knowledge together and use a screening set-up with the agarose culture model from study IV in order to investigate the different effect of various chondrogenic cues on the differentiation capacity of human ES cells.

### 5.5.1 Limitations to study IV

Study IV only investigates the prerequisites of an efficient EB formation and does not strengthen the knowledge about chondrogenic differentiation potential of human ES cells. However, the aim was to develop an easy and cost effective culture method of human ES cells that could address these questions in future studies. It is interesting to note that Messana et al. showed that small EB size could guide the differentiation of mouse cells into a more chondrogenic lineage than larger embryoid bodies\(2^{16}\). This would be a shortcoming for the agarose system if used as a chondrogenic catalyst as the EBs cultured in agarose grew larger than in suspension and hanging droplet. However the EB size is easier to monitor in the agarose system than in suspension as the agarose fixates the EB in one spot and the EB size is probably controllable with the number of seeded cells.
6 CONCLUDING REMARKS

Many studies have been conducted in the field of OA research and yet no resolution of the problem is in sight. A realistic goal would be to find ways of diagnosing the disease in an early stage and slow down the disease progression to a pace that gives the patient acceptable joint function throughout their life, without the need of surgical interventions. For this, the clinicians reasonably need a reliable biomarker for the disease onset (i.e. see the smoke) and a disease-modifying drug (i.e. water to cool down the fire with). The present thesis aimed at increasing the knowledge regarding diagnostic biomarkers and disease mechanism and presents interesting data that serves as a base for future research. Of special importance are the expressional shift seen in cartilage affected by different degrees of degradation (study I). These findings indicate the altered gene expression throughout the disease process and may work as disease markers and/or improve the knowledge about the disease mechanisms. The finding that expression patterns of chondrocytes isolated for ACI procedures are very similar regardless of the clinical outcome has great importance for the ACI inclusion criteria and should be taken into account when making regulating legislations for the procedure (study II). The role of GDF5 in adult cartilage has been puzzling and is not negligible as an SNP in the UTR of the gene is associated with OA. It is plausible that the present thesis sheds some light on this matter as GDF5 stimulation of differentiated human chondrocytes lowered the pressure from Wnt signalling by upregulating the Wnt inhibitor DKK1, which resulted in decreased expression of the ECM degrading enzyme MMP13. It is interesting to argue that the reduced GDF5 promoter activity seen in the rs143383 T/C SNP results in a reduced ECM preservative function and thus OA in the long run (study III). None of the findings in the present thesis are definite and must be studied further. Various techniques can be utilized for this matter and a cost-effective screening of the effects of various signalling pathways on chondrogenic differentiation may be performed with human ES cells cultured in a supportive three-dimensional culture system using agarose (study IV).

If there were a single factor or gene that caused OA, the problem would most probably have been resolved already. The results from the present thesis shed further light on OA as a multifactorial disease by putting some pieces together and possibly revealing new pieces in this puzzle.
7 FUTURE PERSPECTIVES

Global perspectives on proteomics and genomics are hypothesis-generating approaches that need to be validated. It would be interesting to measure the, in the present thesis, proposed early markers of OA in synovial fluid and serum in order to assess if the marker could predict an OA diagnosis. Possibly by following a population in a cohort setting. An alternative approach would be to measure these early disease markers in OA animal models and investigate if their presence could predict the disease onset.

The potency aspect of the ACI intervention lays not likely in the transplanted cells as cells from clinical success and failures are seemingly alike. Other factors are likely to govern clinical result from ACI as even chondrocytes from characterized OA cartilage have been shown to be potent cells for ACI procedures. Therefore it would be interesting to perform an analysis on the signaling pathways that are active at the graft site in the joint in order to elucidate in what milieu the chondrocytes are inserted. This is an important aspect as today all hope is put in the inserted material and little is known about the factors the graft faces in the transplanted joint. It may well be that it is the potency of the recipient and not the potency of the transplanted cells that needs to be regulated in legislations.

Further investigating the effects of GDF5 is one of the most interesting future perspectives of the present thesis. GDF5 may become a disease-modifying drug after its effects in vitro, potency in vivo and clinical safety have been thoroughly addressed. Local injections of GDF5 in the joints of OA animal models would be a first attempt to assess the in vivo potential of GDF5 as a reducer of the disease progress. Furthermore, utilizing a scaffold that slowly released GDF5 would be an interesting approach to assess the regenerative properties of GDF5. The scaffold would assure that the GDF5 stimulation is localized to the chondrocytes, however it would not only work as a vehicle for GDF5 release but also provide structural support for tissue regeneration. The signaling pathways found interesting throughout the present thesis need to be further investigated in how they affect chondrogenesis. This may be addressed with the proposed agarose system from study IV.

Finally, macrophages have recently been suggested to self-renew by a local process of proliferation of adult cells (recently reviewed by Sieweke et al.). An interesting scenario would be to directly transform existing mature chondrocytes into self-renewing cells that can give rise to progeny of self-renewing cells and adult ECM producing cells in a manner similar to the
macrophages. Such a scenario would hypothetically be able to mend articular cartilage damages and it would be interesting to apply the signal pattern that control the macrophages onto chondrocytes to investigate this possibility.
ACKNOWLEDGEMENT

Many people have contributed to the present thesis that was carried out at the department of clinical chemistry and transfusion medicine, institute of biomedicine, the Sahlgrenska academy at the University of Gothenburg. I express my deep gratitude towards all and in particular:

**Anders Lindahl**, who supervised and made my education possible. Anders has made the time as PhD student fun and educational through his research ideas, analytical mind, enthusiasm and common sense. I hope to meet his kind in the future.

Johan Kärrholm and Sven Inerot, who took this thesis under their wings as co-supervisors.

Göran Larson, who is the head of the department of clinical chemistry and transfusion medicine and has generated great facilities and an atmosphere of collaboration and respect between the research groups and clinical personnel at the Sahlgrenska University Hospital department of clinical chemistry.

All co-authors: Ulla Rüetschi, who made study I possible through her outstanding skills with the MS instrument, knowledge and diligence during data analysis and sharp mind during manuscript writing. I am grateful for this time with Ulla and for the help with proof reading this thesis. Jane Synnergren, who has excellent knowledge in micro array technology, which she generously shares. Her work with study II made me more experienced in array data analysis and writing. I am also grateful for the help with proof reading this thesis. Lars Enochson, his analytical and technical skills are an asset to any research constellation and his support and friendship important to me. Maria Elovsson, who introduced me to ES cell culture. Lars Hynsjö, who showed me the power of multivariate analysis. Mats Brittberg, who generated good scientific discussions and quick and helpful support. Tommy de Windt, for fruitful and fun collaboration. Josefine van der Lee for important support in scientific and laboratory matters and friendship. Daniel Saris, Lars Peterson, Eva Sköldbrand, Raimund Strehl, Eva Kilmare and Johan Hyllner for great collaboration.

All former and present colleagues at clinical chemistry and transfusion medicine, Cellartis and especially the awesome friends at molecular cell biology research.
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group and the cell and tissue laboratory; Marianne Jonsson and Camilla Brantsing for your consideration and all help with every matter from Q-PCR to molecular biology, Hanna Stenhamre for analytical discussions regarding data and technical help, Kristina Vukšić and Narmin Bigdeli for knowledge on cell culture and laboratory techniques and humour, Catherine Concaro, Pia Andersson and Margret Hagman for all help regarding all thinkable cell culture matters, Alma Forsman, Ruth Wickelgren, Stina Simonson, Julia Asp for research input, Joakim and Mikael Sandstedt for discussions regarding research and all technical support, Vivien Lettry, Emilia Svala and Anna Thorfve for fun discussions regarding science and stuff, Katarina Junevik for generous help with instruments. Petra Bergström for being a discussion partner as well as a partner in scientific table tennis. Ola Hammarsten for scientific input.

Cecilia Granéli for friendship and your important help and discussions regarding study design, manuscript writing, analysis of proteomic data and proof reading this thesis.

Cecilia Boreström for friendship and all your help on study design, proteomic techniques and manuscript writing also for proof reading this thesis and manuscripts.

Marie Leander for sharing your knowhow and proof reading this thesis.

The former and present members of the Glyco group, Gustaf Rydell, Jonas Nilsson, Fredrik Noborn, Adnan Halim, Alejandro Gomez Toledo, Johanna Nilsson.

The former and present members of the Hammarsten group. Pegah Johanson, Susanne Nyström, Karin Starnberg and Sherin Mathew.

Anders Ståhlberg for generously sharing his skills in PCR.

Carl-Henrik Andersson for fun discussions and stimuli.

Henrik Svensson and Birgitta Odén for generously sharing writing space and discussions

Anna Staffas, Alexandra Abramsson and Maria Olsson for generous help in the lab
Christian Hüls and the rest of the skilled toxicology gang

Eva Flenner, Ulla Strandberg, Kristina Härdner, Annika Larsen and Linnea Wallgren

Marianne Wallgren and Bertil Hjalmers

Anne Marie Svennerholm and Åsa Sjöling, role models!

Anders Janzon for sharing your analytical mind

Lillemor and Thomas Holmquist for support and excellent writing facilities

Kristina Stenberg and Sten Stenberg for inspiration to and support during my studies. This thesis is to you.

Cecilia and Thomas Håkansson, you know it!

Friends, you know what to do and Nicklas you are next!

Isabel Holmquist, Doris and Harald I love you!
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