Bone and Cartilage Regeneration

Wnt Signaling Pathway in Healing

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UNIVERSITY OF GOTHENBURG

Gothenburg 2014

Cover illustration: *β-catenin protein structure.*

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ISBN 978-91-628-8882-4 (Print)
ISBN 978-91-637-4689-5 (Electronic)

Printed in Gothenburg, Sweden 2014
Ineko AB, Gothenburg

Printed in 200 copies

To my beloved parents

- for your endless love and support

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ABSTRACT

The Wnt signaling pathway plays a central role in bone and cartilage embryonic development, processes that are recapitulated during regeneration. Imbalance in such well conserved and complex system often contributes to numerous diseases, whereas controlled modulation of the Wnt signaling activity is an attractive target e.g. for improved fracture healing therapies. The first aim of the present thesis was to increase the knowledge of the underlying mechanisms that lead to cellular alterations in osteoarthritis (OA), resulting in cartilage degeneration. In particular, we investigated the genome-wide expression profile of Wnt related markers in human OA cartilage and the effect of the pro-inflammatory cytokines IL-1β and IL-6 in the context of Wnt signaling pathway, thereby revealing mechanisms for OA modulation therapies. As a second aim, we studied if a local release of the canonical Wnt activator Li^+ from hydroxyapatite (HA) or poly(lactic-co-glycolic acid) (PLGA) modulated the Wnt pathway and subsequently enhanced the bone regeneration around the implants. The results indicated that the Wnt signaling pathways were dysregulated in OA cartilage, with a partly inhibited canonical Wnt signaling and an active non-canonical Wnt cascade. We were able to demonstrate that WNT5A was excessively expressed in degenerative cartilage, and that the pro-inflammatory cytokine IL-6 possessed cartilage protective properties by reducing β-catenin and canonical Wnt signaling. The canonical Wnt pathway was activated by HA but the osteoinductivity of HA itself overridden the Wnt modulating capacity of Li^+. Finally, a global gene expression profiling demonstrated that the controlled release of Li^+ from PLGA activated the canonical Wnt signaling. In conclusion, the present findings may be used to develop gene targeted OA treatments and serve as a basis for further improvement of Li^+ based therapies associated to fracture repair. This thesis sheds further light on the ambiguous influence of Wnt signaling in osteochondral homeostasis and repair mechanisms.

Keywords: Wnt signaling pathway, osteoarthritis, bone regeneration, lithium

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Celler kommunicerar med varandra genom olika signalvägar som reglerar processer såsom migration, tillväxt, struktur och överlevnad. Wnt signaleringsvägen är väl konserverad genom evolutionens gång och styr viktiga aspekter under den embryonala utvecklingen av olika organismer. Den består av lösliga Wnt ligander som binder till receptorer i cellmembranet och kan delas in i tre olika vägar; den kanoniska Wnt vägen, samt de två icke-kanoniska Wnt vägarna (Wnt5a/Ca^{+2} och PCP). Ligand/receptor interaktionen initierar impulser från cellens utsida till cellkärnan där de aktiverar genuttryck. Wnt signalvägen är mycket central bl.a. under skelettutvecklingen och dessa viktiga processer som sker under fosterstadiet efterliknas till stor del när brosk och ben regenereras - en obalans i systemet är således ofta en bidragande faktor till olika sjukdomsförlopp, som t.ex. cancer och osteoartros (OA). Följaktligen är Wnt signalvägen attraktiv att modifiera för att återställa balansen, och det är av stor vikt för framtida behandlingar att öka kunskapen om denna signalväg i olika sjukdomar. Generellt sätt är en låg dos av Wnt signalvägen positivt för broskbildning, medan en stark signal inducerar benbildning. Detta kan liknas vid att Wnt signaleringen utövar ett slags yin-yang förhållande rörande brosk och ben. Med tanke på detta skulle det t.ex. vid läkning av benfrakturer vara intressant att modulera Wnt signalvägens aktivitet för att på detta sätt förbättra individens läkningsförmåga. Ett välkänt och enkelt sätt att aktivera Wnt signaler är litium (Li^{+}) behandling. Syftet med denna avhandling avspeglar Wnt signalvägens yin-yang liknelse, och vi har studerat Wnt markörer i OA brosk, dess förhållande till de inflammations associerade cytokinerna IL-1β och IL-6, och hur en lokal frisättning av Li^{+} från ortopediska implantat påverkar Wnt signalvägen och följaktligen implantatinläkning i ben. Vi såg att den kanoniska Wnt signalvägen var delvis inhiberad, medan Wnt5a/Ca^{2+} vägen var aktiv med ett högt uttryck av liganden WNT5A i OA brosk. Vidare såg vi att ett flertal Wnt markörer inducerades genom IL-1β påverkan, medan IL-6 inhiberade dessa och uppvisade positiva egenskaper för brosk. Frisättningen av Li^{+} från implantat aktiverade Wnt vägen, men inducerade ingen förbättrad benläkningsförmåga. Sammanfattningsvis har denna avhandling bidragit med mer kunskap om de komplexa regleringsmekanismer som Wnt signalvägen utövar i skelettvävnaderna, och visat WNT5A som en möjlig målkandidat att inhibera för att bromsa OA processerna i brosk. Våra resultat kan också ligga till grund för ytterligare förbättringar av Li^{+} baserade terapier associerade till benfrakturläkning.

LIST OF PAPERS

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

I. Thorfve. A, Dehne, T, Lindahl, A, Brittberg, M, Pruss. A, Ringe. J, Sittinger. M, and Karlsson. C, *Characteristic Markers of the Wnt Sgnaling Pathways Are Differentially Expressed in Osteoarthtitic Cartilage*. Cartilage 2012; 3: 43-57.

II. Svala. E, Thorfve. A, Ley. C, Barreto Henriksson. H, Synnergren. J, Lindahl. A, Ekman. S, and Skiöldebrand. E, *Effects of Interleukin-6 and Interleukin-1β on Expression of Growth Differentiation Factor-5 and Wnt Signaling Pathway Genes in Equine Chondrocytes.* Am J Vet Res 2014; 75: 132-140.

III. Thorfve. A, Lindahl. C, Xia. W, Igawa. K, Lindahl. A, Thomsen. P, Palmquist. A, and Tengvall. P, *Hydroxyapatite Coating Affects the Wnt Signaling Pathway during Peri-implant Healing in vivo.* Acta Biomater 2014; 10: 1451-1462.

IV. Thorfve. A, Bergstrand. A, Ekström. K, Lindahl. A, Thomsen. P, Larsson. A, and Tengvall. P, *Gene Expression Profiling of Peri-implant Healing of PLGA-Li^+ Implants Reveals an Activated Wnt Signaling Pathway in vivo.* In manuscript, submitted to PLOS ONE.

MY CONTRIBUTIONS TO PAPERS I-IV

I. Main author. Active part in the formulation of the hypotheses and design of the study. Performed all analyses involved, apart from the experimental microarray analysis and normalization. Performed the presentation of the data, statistical analysis and first draft of the manuscript.

II. Second author. Active part in the planning and design of the study. Conducted several experiments, and I was in particular responsible for the Wnt related analyses (interpretation and summarization of microarray data, IHC). Active part in the presentation of the data, contributed to the writing process (drafting and editing the manuscript).

III. Main author. Active part in the formulation of the hypotheses and design of the study. Performed the majority of the analyses involved (histomorphometry, qPCR, IHC and ELISA) apart from implant preparation and characterization. Active part in the *in vivo* surgical procedure and biomechanical analysis. Performed the presentation of the data, statistical analysis and first draft of the manuscript.

IV. Main author. Active part in the formulation of the hypotheses and design of the study. Performed the majority of the analyses involved (interpretation and summarization of microarray data with related bioinformatics analyses, qPCR and IHC) apart from implant preparation and characterization. Active part in the *in vivo* surgical procedure. Performed the presentation of the data, statistical analysis and first draft of the manuscript.

Papers not included in the thesis

Granéli. C*, Thorfve. A*, Ruetschi. U, Brisby. H, Thomsen. P, Lindahl. A, and Karlsson. C*, Novel markers of osteogenic and adipogenic differentiation of human bone marrow stromal cells identified using a quantitative proteomics approach.* Stem cell research 2013; 12(1):153-165.

* indicates that the authors contributed equally to the study and should be considered as first authors.

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ABBREVIATIONS AND GENE SYMBOLS

3D	Three-dimensional
ACAN	Aggrecan
ADAMTS	A disintegrin-like and metalloproteinase with thrombospondin type 1 motif
ADAMTS5	A disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 5
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ASPN	Asporin
BA	Bone area
BCA	Bicinchoninic acid
BIC	Bone-implant contact
BMP	Bone morphogenetic protein
BMP14	Bone morphogenetic protein 14
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CAMKII	Calcium/calmodulin-dependent protein kinase II
CCDC88C	Coiled-coil domain containing 88C
cDNA	Complementary DNA
COL1A1	Collagen, type I, alpha 1
COL1A2	Collagen, type I, alpha 2
COL2A1	Collagen, type 2, alpha 1
DAAM2	Dishevelled associated activator of morphogenesis 2
DAB	3,30-diaminobenzidine
DAPI	4’,6-diamidino-2-phenylindol
DKK1	Dickkopf Wnt signaling pathway inhibitor 1
DKK2	Dickkopf Wnt signaling pathway inhibitor 2
DKK3	Dickkopf Wnt signaling pathway inhibitor 3
DNA	Deoxyribonucleic acid
DVL	Dishevelled segment polarity protein
DVL2	Dishevelled segment polarity protein 2
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FAM	6-carboxyfluorescein

FC	Fold change
FGF	Fibroblast growth factor
FOSL1	FOS-like antigen 1
FRA1	FOS-related antigen 1
FRZB	Frizzled related protein
FZD	Frizzled
GAG	Glycosaminoglycan
GDF-5	Growth differentiation factor 5
GO	Gene ontology
GP	Glycoprotein
GSK-3β	Glycogen synthase kinase 3 beta
HA	Hydroxyapatite
HE	Hematoxylin eosin
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IHC	Immunohistochemistry
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
LEF	Lymphoid enhancer-binding factor
LEF1	Lymphoid enhancer-binding factor 1
LiCl	Lithium chloride
Li_2CO_3	Lithium carbonate
LRP5	Low density lipoprotein receptor-related protein 5
LRP6	Low density lipoprotein receptor-related protein 6
MBG	Minor binding groove
MBGS	Mesoporous bioglass
MMP	Matrix metallopeptidase
MMP13	Matrix metallopeptidase 13
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
Na_2CO_3	Sodium carbonate
ND	Normal donor
NFAT5	Nuclear factor of activated T-cells 5, tonicity-responsive
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2

OA	Osteoarthritis
OCF	Osteochondral fragment
OCN	Osteocalcin
ON	Osteonectin
OPG	Osteoclastogenesis inhibitory factor
OPN	Osteopontin
PBS	Phosphate buffered saline
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PG	Proteoglycan
PLGA	Poly(lactic-co-glycolic acid)
PPARD	Peroxisome proliferator-activated receptor delta
PPI	Protein-protein interaction
qPCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor κ B
RANKL	Receptor activator of nuclear factor κ B ligand
RMA	Robust multichip average
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Revers transcription
RTQ	Removal torque
RUNX2	Runt-related transcription factor 2
SEM	Scanning electron microscope
SFRP	Secreted frizzled-related protein
SFRP1	Secreted frizzled-related protein 1
SFRP2	Secreted frizzled-related protein 2
SFRP3	Secreted frizzled-related protein 3
SFPR4	Secreted frizzled-related protein 4
SLRP	Small leucine-rich proteoglycan
SNP	Single nucleotide polymorphism
SOX17	SRY (sex determining region Y)-box 17
SOX9	SRY (sex determining region Y)-box 9
TAXIBP3	Tax1-binding protein 3
TCF7	Transcription factor 7 (T-Cell Specific, HMG-Box)
TCF7L2	Transcription factor 7-like 2 (T-Cell Specific, HMG-Box)
TEM	Transmission electron microscopy

TF-XRD	Thin-film X-ray diffractometer
TGF-β	Transforming growth factor beta
TLE4	Transducin-like enhancer of split 4
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
TNF-α	Tumor necrosis factor alpha
tRNA	Transfer RNA
TSA	Tyramide signal amplification
UV	Ultraviolet
UVO	Ultraviolet ozone
VEGF	Vascular endothelial growth factor
WIF1	WNT inhibitory factor 1
WISP1	WNT1 inducible signaling pathway protein 1
WISP2	WNT1 inducible signaling pathway protein 2
WNT	Wingless-type MMTV integration site family
WNT1	Wingless-type MMTV integration site family, member 1
WNT11	Wingless-type MMTV integration site family, member 11
WNT3A	Wingless-type MMTV integration site family, member 3a
WNT4	Wingless-type MMTV integration site family, member 4
WNT5A	Wingless-type MMTV integration site family, member 5a
WNT5B	Wingless-type MMTV integration site family, member 5b
WNT7A	Wingless-type MMTV integration site family, member 7a

1 INTRODUCTION

Most people will during their lifetime experience a degenerative joint disease such as osteoarthritis (OA) or a bone fracture. Due to the present demographic shift towards an elderly population these issues have become increasingly important. Bone and cartilage are often gathered within the term "the skeleton", and although related to one another they exhibit different structure and function. Hallmarks of OA are joint pain and reduced mobility due to the restricted ability of the chondrocytes to repair the erosion of the cartilage extracellular matrix (ECM). In contrast, bone tissue holds the unique ability to repair fractures without an apparent scar formation and damaged bone is removed and replaced with new, leaving no or a few traces of impaired functionality behind. Intriguingly, these different tissues are mainly regulated by the same mechanism; the Wnt signaling pathway, which is a well conserved signaling pathway that exerts ambiguous roles in growth and maintenance of bone and cartilage. Below follows an introduction that has the aim to guide the reader to some insights and issues related to our present knowledge of molecular mechanisms in cartilage and bone regeneration.

1.1 Cartilage

Cartilagenous tissues exist throughout the human body and are present in three different types depending on their ECM composition; elastic cartilage, fibrous cartilage and hyaline cartilage. Elastic cartilage is associated with the ear and the larynx, whereas fibrous cartilage is related to the knee menisci and the intervertebral disc. Hyaline cartilage is the most abundant cartilagenous tissue and is mostly associated with the skeletal system. When found at the interface between the gliding bony surfaces in the articular synovial joints, such as the knee, it is referred to as articular cartilage[1,2].

Articular cartilage

Articular cartilage covers the ends of long bones and acts as a shock absorber, thereby minimizing peak pressure on the subchondral bone while providing frictionless movement of the joint. At a first glance, articular cartilage appears as a rather simple tissue that unlike most tissues is avascular with no blood supply. This tissue has a low cell-to-matrix ratio and consists of only one single cell type – the chondrocyte. However, the apparent simplicity is a contradiction since the articular cartilage, which at a first glance appears as a very homogenous tissue, exhibits in fact a broad spectrum

of mechanical properties, organization and composition of the ECM, as well as a cellular morphology that varies with depth from the articular surface. The heterogeneity contributes to unique biomechanical properties that resist compression, tension and shear during normal locomotion. The articular cartilage consists of chondrocytes, ECM and water. The chondrocytes that originate from the mesenchymal stem cells (MSCs) are highly specialized and metabolically active cells that produce the ECM, and are located within small cavities in the ECM called lacunae. Lacunae are fluid filled and believed to reduce the mechanical changes during dynamic loading, thus protecting the cells. The main components of the ECM are different collagens and glycoproteins (heavily glycosylated proteins), whereas the chondrocytes represent only 2% of the adult human cartilage mass[3-5].

Articular cartilage extracellular matrix

The most abundant matrix protein in articular cartilage is type II collagen that represents 90-95% of the collagens. The type II collagen network is extremely stable with a turnover close to zero, and is considered to provide tensile strength to the articular cartilage. In addition to type II collagen also collagen type I, VI, IX, X and XI are found, these are less well studied but participate in the stabilization of the collagen fibril network[3].

Articular cartilage contains a variety of proteoglycans (PGs) that are essential for the normal function of the tissue. Aggrecan is the most abundant PG (95% of total proteoglycan mass) and the largest in size. Aggrecan interacts with hyaluronic acid to form large aggregates via link proteins. Hyaluronic acid then interacts with the collagen network and chondrocytes. Aggrecan is highly negatively charged and attracts positively charged ions in tissue fluids, and forms together with water a hydrogel with space filling and load distribution properties. Hence, providing the cartilage with its critical ability to resist compressive loads. Water is the most abundant component of articular cartilage and since water is trapped within the tissue, it will support most of the loading. The flow of water through the cartilage and across the articular surface transports nutrients to the cells and provides lubrication. Smaller non-aggregating PGs also exist in articular cartilage. These interact with collagen and are involved in tissue integrity and metabolism[2,4].

Articular cartilage structure

The organization of articular cartilage reflects its functional role. Structurally, the articular cartilage can be divided into distinct zones with respect to the depth of the tissue, i.e. the superficial, the middle, the deep and the calcified zones (see Fig. 1)[3]. In the superficial zone, which is the thinnest zone the chondrocytes are flattened, aligned parallel to the surface and more numerous

than elsewhere in the cartilage. The cells within this zone produce lubricin, a protein with essential roles for the almost frictionless joint movements, and the cells are surrounded by a network of collagen fibrils (mostly type I collagen). The fibrils are 20 nm in diameter[4] and are aligned parallel to the articular surface and to each other, providing a web with high tensile properties. The superficial zone has a low concentration of aggrecan, but the highest water concentration and the greatest ability to resist shear stresses found in the tissue, thereby protecting the deeper layers. Hence, disruption of this superficial layer alters the mechanical properties which contributes to the development of OA[2].

Next to the superficial zone is the middle zone, which has a lower cell density and in contrast to the superficial zone, spherical shaped cells and an ECM richer in aggrecan. The collagen fibrils (mainly type II collagen) have a larger diameter (70-120 nm) and are more randomly oriented in a criss-cross manner[3]. This is the first line of resistance to the compressive forces. In the deep zone, cell density, collagen and water content are minimal, whereas aggrecan content and fibril diameter are maximal[4]. The collagen fibrils and the chondrocytes are arranged in a columnar orientation towards to the articular surface, designed to distribute the loading, thus acting as a shock absorber. In this zone the resistance against compressive forces is the highest.

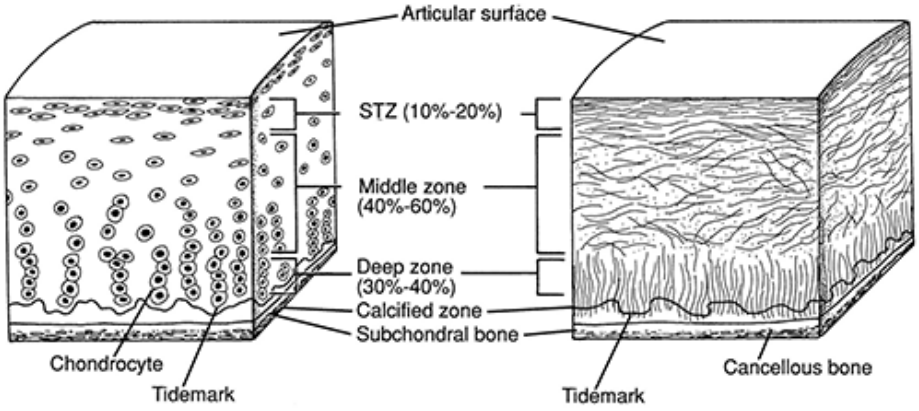

Figure 1. Cross-section of the structure of articular cartilage displaying the different characteristic regions; the superficial, the middle and the deep zones. First published in and preprinted with permission from Biomechanics of articular cartilage, in Nordin M, Frankel VH [eds]: Basic Biomechanics of the Musculoskeletal System, 2nd ed. Philadelphia: Lea & Febiger, 1989.

Closest to bone, an irregular line known as the tidemark separates the deep zone (unmineralized cartilage) from the calcified zone (mineralized cartilage)[2]. The chondrocytes in the calcified zone are hypertrophic, thus producing type X collagen instead of type II, which leads to calcification of the surrounding matrix. This calcified layer is a structural integration between the subchondral bone and the articular cartilage, securing the cartilage to bone.[3]

1.1.1 Articular cartilage formation

Knowledge about the developmental processes is of significance for the understanding of tissue homeostasis in adult tissue repair and regeneration. The cellular processes involved in cartilage formation reveal essential insights and guide us towards improved therapies. Degenerative diseases such as OA often elicit a repair response with renewed cell division and upregulated matrix synthesis similar to developmental processes, but with dysregulated signaling pathways. Chondrogenesis is a process that results in a cartilage intermediate, leading to endochondral ossification during skeletal development (see 1.2.1). The synovial joints are complex structures comprised of several tissues, including articular cartilage, bone, ligament and synovium. The joint development process during embryogenesis is divided into three different phases; mesenchymal condensation, interzone formation and cavitation (Fig. 2)[6,7].

- Chondrogenesis begins with the recruitment of undifferentiated MSCs that migrate to the areas designated to become bone. This is followed by proliferation and mesenchymal condensation, forming a cartilaginous skeletal template. Prior to the condensation, the pre-chondrocyte MSCs produce an ECM rich in hyaluronic acid and collagen type I. During the condensation the cells synthesize hyaluronidase and cell adhesion molecules, leading to decreased concentration of hyaluronic acid and thus a closer cell-cell contact. This is favorable for intracellular communication and essential for changes in cytoskeletal architecture. Transforming growth factor-beta (TGF-β) signaling induces expression of the transcription factor SRY-box containing gene 9 (SOX9), required for collagen type II and aggrecan expression during early condensation. In the end of the condensation process, expression of intracellular signaling pathways are activated, thus initiating the transition of chondroprogenitor cells to mature

committed chondrocytes. Collagen type I production is turned off, and the chondrocytes are entrapped in their ECM and obtain a characteristic round phenotype[7-10].

- At the ends of long bones, i.e. at the sites for the developing joints, the cells exit their chondrogenic pathway and dedifferentiate. The chondrocytes lose their rounded phenotype, become elongated and begin to express type I collagen, resulting in the formation of the interzone, followed by cavitation and finally formation of joint structures. The cells within this area are responsible for the generation of structures such as ligaments, synovial and joint capsule and expression of TGF-β, growth differentiation factor-5 (GDF-5) and Wnts (wingless-Type MMTV integration site family). The zone acts as a signaling center that control morphogenesis of adjacent skeletal elements. During cavitation, the interzonal cells secrete large amounts of hyaluronic acid, resulting in less cell-cell contacts and separation of the intermediate layer to form the fluid-filled synovial cavity and articular cartilage. Cells originating from the interzone condensate, differentiate and produce matrix components such as collagen type II, resulting in the growth of the articular cartilage. Tissue maturation into a fully functional joint proceeds and the development of a long bone continues with endochondral ossification, where the cartilage template is replaced by bone, as described in section 1.2.1.[7,11,12].

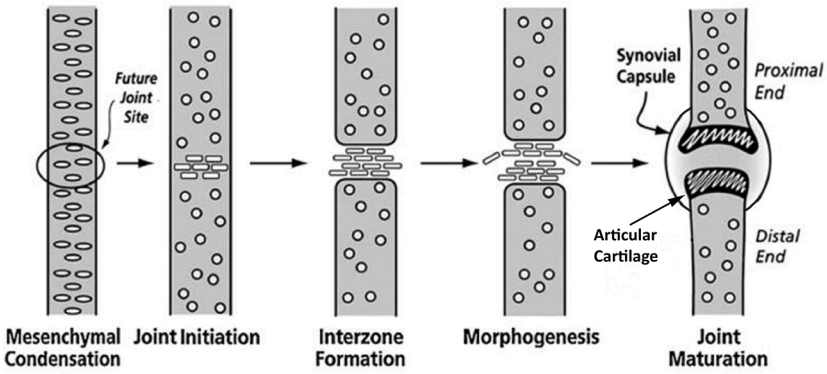

Figure 2. Illustration of the major steps of synovial joint formation. Mesenchymal condensation is followed by joint initiation and interzone formation. The interzone initiates morphogenetic processes resulting in the formation of a mature joint including articular cartilage and synovial capsule. Modified from[12].

1.1.2 Cartilage regeneration

A balance between anabolic and catabolic mechanisms maintains a healthy ECM homeostasis in articular cartilage, and a shift towards degeneration is associated with OA. The overall articular regeneration capacity is considered as limited and one reason for this is the avascular nature, thus the chondrocytes depend mainly on nutrition by diffusion from synovial fluid. Due to this, no MSCs from the blood can be recruited to repair a lesion, which is the case in the self-regenerating bone tissue (see 1.2.2). In addition to this, adult articular chondrocytes become entrapped in the dense ECM and have a very low or non-existing cellular turnover. This results in a low capacity to repopulate and repair cartilage injuries[2].

Cartilage injuries can be separated into different categories and the ability of chondrocytes to repair a defect is influenced by several factors such as defect size, depth, patient age and type of trauma to mention a few. Today, several surgical treatments of cartilage defects are available since untreated lesions induce cartilage degradation, and may lead to OA[2]. However, it has been suggested that the articular cartilage contains a progenitor cell population[13] and that their frequency is increased in human OA[14], thus opening up the possibility of further development of cartilage tissue engineering.

1.1.3 Osteoarthritis (OA)

OA is the most common musculoskeletal disease in the world, affecting around 75 % of the elderly population and the most frequently affected sites are hands, knees and hips. It is a multifactorial degenerative joint disease characterized by pain, stiffness and reduced mobility due to imbalance in mechanical loading of the articular cartilage and the lack of ability of chondrocytes to resist and respond to this stress. Besides articular cartilage, multiple components of the joint are affected by OA, e.g. synovial joint lining, adjacent supportive connective tissue and peri-articular bone. This can ultimately result in a chronic disability and a need for joint replacement, giving rise to significant psychological, social and economic burden for the patient and the society as whole. Several factors play crucial roles in the pathogenesis of OA, including repetitive trauma and genetic, aging, metabolic and developmental factors, although the mechanisms are incompletely understood[15,16].

OA involves an uneven or gradual loss of articular cartilage, inflammation, formation of new bone at the joint margins (osteophytes), alterations in the underlying subchondral bone and a variety of associated abnormalities of the synovial membrane and peri-articular structures. As described above, the adult articular chondrocytes possess a restricted capacity to regenerate the original cartilage matrix architecture. During the normal aging process, cartilage becomes increasingly mineralized, collagens and proteoglycans undergo structural changes, the cartilage flexibility is loosened and the capacity of the chondrocytes to remodel and repair the ECM is diminished[15]. Adult articular chondrocytes often may attempt, but most often fail to recapitulate phenotypes of early stages of cartilage development.

The initial stages of OA involve increased cell proliferation, synthesis of matrix proteins, proteinases, growth factors, cytokines, and other inflammatory mediators by the cells. There is also an increased synthetic activity of cartilage specific components including type II collagen and aggrecan, which probably is an attempt by the cells to regenerate the matrix. However, the rigid ECM is eventually loosened due to the production of collagenases such as metalloproteinases (MMPs) and aggrecanases (ADAMTS) by the chondrocytes, resulting in a mechanical joint overload due to the weaker cartilage[17,18]. The abnormal behavior of OA chondrocytes appears as fibrillations, matrix depletion, cell clustering and changes in composition and distribution or quantity of matrix associated proteins. The presence of the hypertrophic chondrocyte marker type X collagen, as well as other chondrocyte differentiation genes, normally not found in adult articular

cartilage, is a further evidence of phenotypic modulation and recapitulation of the developmental program[15]. OA is frequently associated with signs and symptoms of inflammation and although its role in OA has been discussed[19,20], synovial inflammation has been suggested to contribute to the dysregulation of chondrocyte function. Increased levels of inflammatory mediators such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in relation to matrix degradation enzymes are well documented. These cause an imbalance between the catabolic and anabolic activities of the chondrocytes during remodeling of the cartilage ECM. It has been recognized that TNF-α has a pivotal role in the induction of a pro-inflammatory cytokine cascade[17], and that IL-1β also induces expression of other pro-inflammatory cytokines such as IL-6 and IL-8[15,21,22]. However, IL-6 has also anti-inflammatory properties[19] and a possible protective role in OA[23].

Other cells and tissues of the joint also contribute to OA pathogenesis and increased subchondral plate thickness is a characteristica of OA. OA cartilage has been reported to lose its avascularity and blood vessels and channels invade from the underlying subchondral bone. The role of the subchondral bone is currently gaining increased attention[24,25]. However, the question whether subchondral alterations precede cartilage degradation or follow on the damage caused by loss of cartilage is still controversial. Pain and structural alterations represent the most noticeable clinical appearance associated to OA. However, since these factors are not usually discovered until the late phase of the disease, it exists a lack of access to tissue samples from patients with early OA, thus hampering the understanding of the biology behind the disease mechanisms. Therefore current treatments are mostly focused on symptomatic relief of pain and inflammation, with no major possibility to reduce the ongoing joint destruction. Thus, an effective prevention of the structural modifications at an early stage is a key objective of new therapeutic approaches[19,26]. Several OA susceptibility associated polymorphism genes involved in ECM components or developmental signaling pathways such as Wnt, GDF-5 and asporin have been reported[27-29]. A single nucleotide polymorphism (SNP) in the GDF-5 gene that results in reduced mRNA levels has been shown to be linked to hip and knee OA in a range of ethnic groups[30]. But how the altered GDF-5 expression can cause OA has not been recognized, as the downstream action of GDF-5 activity has not been thoroughly investigated. Nevertheless, new potential candidate genes and molecular targets for OA therapies/diagnosis have been identified in proteomic and genomic analyses of OA cartilage and secretome[31-33].

1.2 Bone

The main function of the skeleton is to provide a strong supportive and mechanically optimal structure for soft tissues and muscles, and to allow body movement. Bone is a tissue that constantly adapts to biomechanical needs and environmental stress; growth of children and the adolescent, increased bone density observed in professional athletes, or simply during the repair of a fracture. All of these needs are served by alternations in the two opposing processes; bone resorption and bone formation. These are the predominating functions of the osteoclasts and osteoblasts respectively[34]. In addition to this, the skeleton is the anatomical site for blood cell and platelet production (hematopoiesis), calcium metabolism and endocrine regulation. This varied multitasking ability of bone tissue is possible due to its unique molecular, microscopic and macroscopic structures. Bone formation occurs in several successive phases and involves the production and maturation of the osteoid that is followed by mineralization of the matrix[35], processes that are briefly described below.

Bone cellular components

Bone is composed of cellular and non-cellular elements. The cells are derived from several cell lines and include mesenchymal osteoprogenitor cells, osteoblasts, osteocytes, bone lining cells and osteoclasts - all participating in the dynamic process of bone growth, repair and remodeling.

Osteoblasts are derived from MSCs whose major function is secretory and are responsible for ECM deposition and its subsequent mineralization[36,37]. Osteocytes are star-shaped subsets of terminally differentiated osteoblasts that have been entrapped within the calcified ECM. They act via their characteristic dendritic outgrowths as mechanosensors in bone tissue, thereby regulating bone mass and structure in response to increased or decreased mechanical stress. Osteocytes represent 90-95 % of the cells of mature bone tissue and participates actively in calcium and phosphate homeostasis[38,39]. Bone lining cells are a flattened type of relatively inactive non bone-forming osteoblasts, but the nature and precise functions of these cells are not well known[40], although a direct contact between them and mature osteoclasts have been reported[41].

Osteoclasts are large multinucleated cells derived from a hematopoietic origin and are formed by the fusion of mononuclear progenitors of the monocyte/macrophage family. They constitute less than 1% of all bone cells. They have osteolytic properties and resorb mineralized matrix efficiently by acidification of the intervening contact zone, thereby forming resorption pits

in the bone surface[42-44]. Osteoblasts colonize the pits and initiate new bone deposition and regulate osteoclast differentiation (e.g. via RANKL/OPG balance). Important signaling pathways include the action of several hormones including parathyroid hormone (PTH), vitamin D, growth hormones, steroids as well as several cytokines. In summary, the balance between osteoblast and osteoclast functions coordinate bone homeostasis during development and remodeling throughout life and is biomechanically modulated by the osteocytes[41,42].

Bone extracellular matrix

The non-cellular bone matrix is divided into inorganic and organic parts. It is known as the osteoid when first deposited but yet not mineralized. The majority of the inorganic part (60%) consists of calcium phosphate crystallites in the form of hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), which gives bone its compressive strength[45]. The organic part (40%) includes mainly type I collagen fibrils (85-90%) that are embedded in PGs (such as decorin, biglycan and asporin) and GPs. Decorin and biglycan are members of the small leucine-rich proteoglycans (SLRPs) that play important roles in matrix structure and cell metabolism, regulating the storage of growth factors. The collagen type I give the bone its tensile strength and flexibility.

The dominant non-collagenous matrix proteins are GPs such as osteonectin (ON), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN) and alkaline phosphatase (ALP)[46,47]. ON binds collagen, HA and growth factors, regulating cell proliferation and production of MMPs. OPN acts within the ECM promoting osteoclast attachment to mineralized surfaces, and mRNA levels are upregulated upon bone mechanical loading. BSP is restricted to mineralized tissues with affinity to type I collagen as well as HA. The expression of the Ca^{2+}-regulator OCN, one of the most abundant non-collagenous proteins in bone, is restricted to bone and dentin. This protein is involved in remodeling and the control of bone density, and is considered as a late marker for osteogenesis and bone formation. Further, it is present in hard but not in soft callus and shows a peak in expression after two weeks of fracture healing[45,48]. ALP is on the other hand regarded as an early osteogenic differentiation marker, and is a hydrolase enzyme removing phosphate groups from various types of molecules, although the mechanisms are incompletely understood. It is among the first functional genes expressed during the calcification process and the enzyme increases the local concentration of phosphate whilst decreases phosphatase concentrations, thus acting as a mineralization promotor[49].

Types and structures of bones

Histologically, two different types of bone exist depending on the pattern of collagen organization. Woven bone (or primary bone tissue) is immature bone with unorganized randomly directed collagen fibers. It is mechanically weak but rich in cells and possesses high flexibility. This kind of bone is found in fetal bone and during adult fracture repair. It is rapidly formed and subsequently replaced via the remodeling process during which the mature lamellar bone is formed. The lamellar bone displays regular parallel alignment of collagen fibers and is mechanically strong.

Bone is further divided into two structurally different forms; compact and trabecular bone (Fig. 3). The compact (cortical) bone is the rigid outer layer that is strong, dense and protective, whereas the inner layer is called trabecular (cancellous) bone and has a spongy structure. It is lighter and less dense than the compact bone. The trabecular bone which is found at the ends of long bones and proximal to joints is more vascularized and has a larger surface area than compact bone, thus making it ideal for metabolic activity e.g. exchange of calcium ions[35,45].

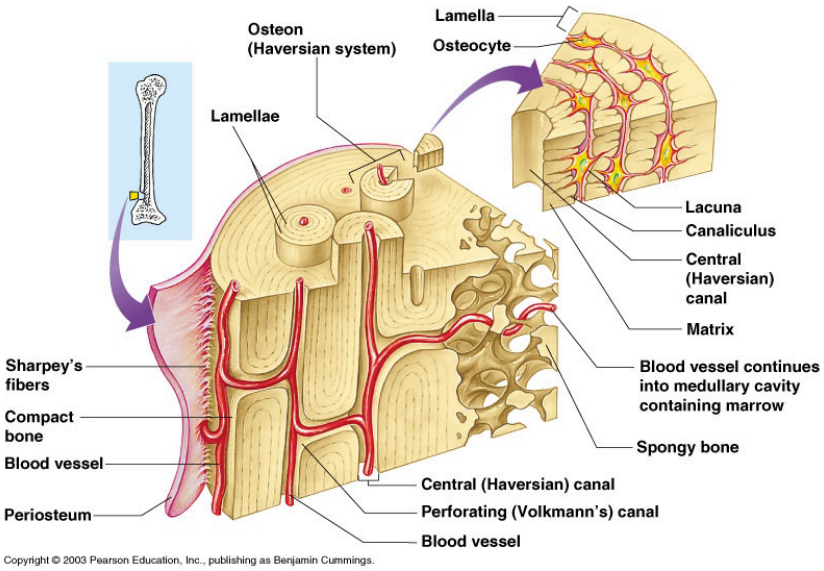

Figure 3. Internal bone structure showing periosteum, compact and trabecular (spongy) bone, as well as Haversian systems.

Cortical bone is composed of osteons (or Haversian systems) that are circular structures containing blood vessels and nerves, surrounded by concentric lamellae. In between the lamellae, osteocytes are laid down and intercommunicate via microscopic channels called canaliculi. Each osteon is in direct contact with the periosteoum, the bone marrow and other osteons through Volkmann's canals. The cavities of the cortical bone are filled by bone marrow containing blood vessels.

In contrast to compact bone, trabecular bone consists of a series of small rod-shaped structures (trabeculae) forming a lattice-shaped network of bone tissue, which contains lamellae with osteocytes. Bone marrow fills up the open spaces between the trabeculae giving rise to its rich vascularization.

The bone surface is covered by periosteum (outer) and endosteum (inner) membranes of connective tissues. These membranes contain osteoprogenitor cells that differentiate into osteoblasts and acts as a continuous supply of cells involved in and supporting bone growth, remodeling and repair[50].

1.2.1 Bone development

Osteogenesis (bone tissue formation) may occur via two different processes; intramembranous and endochondral ossification, which in most cases occur simultaneously[38,45].

- Intramembranous ossification involves a process where MSCs differentiate directly into functional osteoblasts (Fig. 4). This occurs during the formation of flat skull bones and during regeneration of bone in mechanically stabilized regions, such as when using bone-anchored implants. The cells of the mesenchymal lineage, which are embedded within a membrane of connective tissue directly differentiate into the osteogenic lineage and then produce the unmineralized matrix (osteoid) that eventually mineralize. During this process, the transcription factor runt-related protein 2 (RUNX2)[51] is indispensable for osteoblast differentiation. Other important regulating factors such as bone morphogenetic proteins (BMPs) and Wnts are also involved[52].

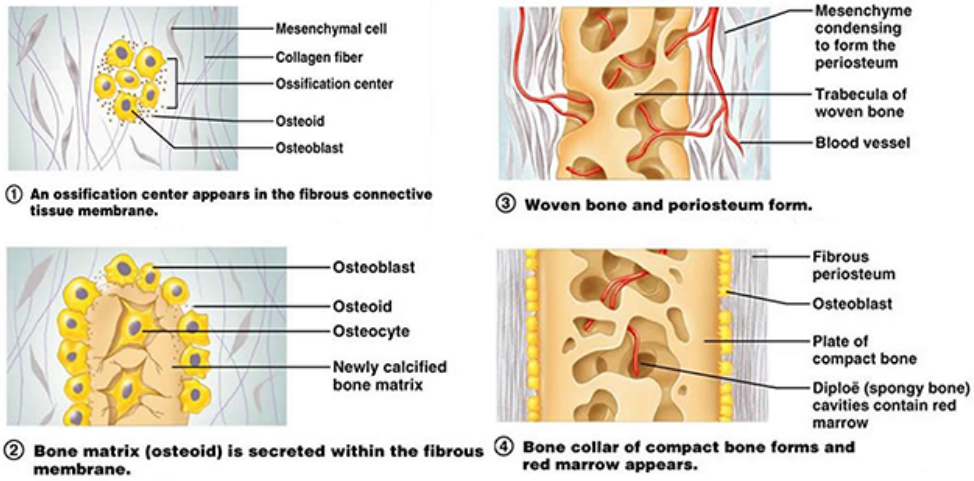

Figure 4. Intramembranous ossification. Mesenchymal cell clusters differentiate into osteoblasts that start to secrete osteoid, which is mineralized within a few days. Accumulating osteoid is laid down between blood vessels, resulting in woven bone formation that is later replaced by mature lamellar bone.

- Endochondral ossification is a complex process of which short and long bones are developed, and occurs in mechanically unstable regions during fracture repair. MSCs condensate and differentiate into a soft cartilage model (as described in section 1.1.1), followed by hypertrophic differentiation of the chondrocytes that calcify the surrounding matrix. The transcription factor SOX9 is expressed during the early events, whereas RUNX2 expression is initiated during the hypertrophic differentiation[53]. Also other regulating factors such as BMPs and Wnts are involved in this process[45,52,54]. After calcification, the chondrocytes die and blood vessels penetrate the area, bringing osteoprogenitors to the site, which finally lead to the replacement of the cartilaginous matrix by trabecular bone. During this process two ossification centers are formed; the cartilage template is first invaded at its center and later at each end by a mixture of cells that establish the primary and secondary centers of ossification. These centers are subsequently and gradually encroaching on the remaining cartilage, replacing it completely with bone (except at the articular surfaces) and by time the skeletal maturity is reached (Fig. 5)[54].

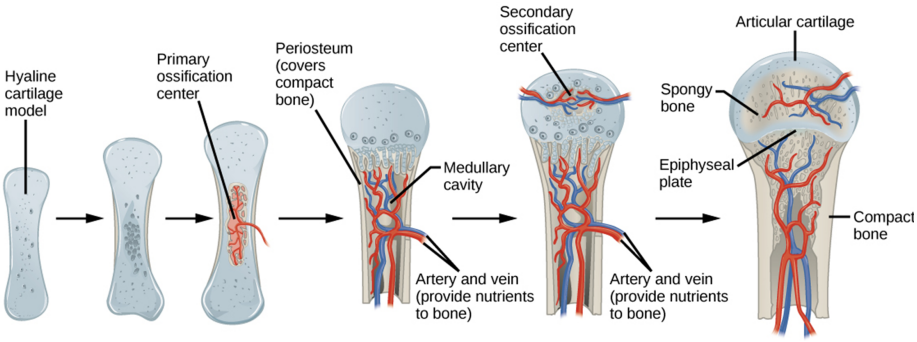

Figure 5. Endochondral ossification. Initiation of the primary ossification center occurs in the center of the hyaline cartilage template as the chondrocytes undergo hypertrophy, which is followed by invasion of blood vessels. The secondary ossification center is later formed and is separated from the primary center by the growth plate (epiphyseal plate) that is responsible for the longitudinal growth until young adulthood. In the human adult bone, the growth plate is closed and the only remaining cartilage is the articular cartilage at each end of the long bones.

1.2.2 Bone remodeling and fracture repair

Bone is a highly dynamic tissue and remodels throughout our lifetime. The remodeling process is governed by the mechanical forces acting upon it, or by self-repair of small structural defects such as fractures or micro cracks due to mechanical stress. Osteoblasts, osteocytes and osteoclasts exert a highly complex interplay between each other, coordinating bone resorption and deposition which renewals several percentages of the bone every year via the OPG/RANKL/RANK system. This process is regulated by several transcription factors and signaling pathways such as TGF-β, BMP, Notch, fibroblast growth factor (FGF) and Wnts. Abnormalities in the regulatory system results in an unbalanced formation/resorption which is associated with increased or decreased bone strength, the latter case is considered as an underlying cause of osteoporosis[35].

Fracture repair is a remarkably complex process that recapitulates certain aspects of skeletal embryogenesis, and is roughly divided into the closely linked phases of hematoma formation, acute inflammation, repair and remodeling[55]. Bleeding from the fracture, periosteum and surrounding soft tissues results in a blood clot formation (hematoma), which acts as a source of the hematopoietic cells that initiate the inflammatory cascade and subsequently reorganize to granulation tissue. This process is associated with various signaling molecules such as FGF, BMP, vascular endothelial growth

factor (VEGF) and platelet-derived growth factor (PDGF), which are important for chemotaxis, angiogenesis and MSC invasion. The bone fracture leads to disruption of normal blood supply and necrotic areas displaying osteocyte death and hypoxia appears. During the repair phase the tissue debris is cleared by macrophages and the dead bone is subsequently resorbed by osteoclasts. The fracture site is then invaded by fibroblasts and progenitor cells from the bone marrow, endosteum and periosteum. The periosteum has been recognized as one of the most important response sites during healing[56]. These cells differentiate both towards the chondrogenic and osteoblastic lineages, resulting in the formation of a heterogeneous tissue called soft callus that replaces the dead bone and provide mechanical support.

The soft callus is subsequently and gradually ossified resulting in a woven bone hard callus, which occurs via a combination of intramembranous and endochondral ossification. In the final stage of repair, the woven bone is slowly replaced by lamellar bone through the remodeling stage in which the healing bone is restored to its original shape, structure, and mechanical strength[57].

1.3 Wnt signaling pathways

The Wnt signaling pathways play essential roles in the above mentioned processes and the rest of the introduction will primarily focus on these multifaceted signaling pathways in detail, although mainly the canonical Wnt pathway.

Overview of the Wnt signaling pathways

Wnt ligands (Wnts) are a group of evolutionarily well conserved secreted cysteine-rich glycoproteins that are essential for embryonic and post-natal development and tissue homeostasis. Currently, 19 Wnt ligands that associate with receptors, leading to activation of the Wnt signaling pathways have been described. Three different Wnt pathways are known today; the canonical Wnt pathway, also called the β-catenin pathway as β-catenin is the transducer of the signal, whereas the planar cell polarity (PCP) pathway and the Wnt5a/Ca^{2+} pathway are referred to as the non-canonical Wnt pathways and function independently of β-catenin. The non-canonical Wnt pathways are less studied, but are gaining more and more attention[58,59].

The large repertoire of Wnt ligands can interact with numerous receptors, antagonists and activators in a multitude of ways - inducing a huge variety of responses, which also includes other signaling pathways. This gives rise to a

series of complex interactions that, thirty years after their initial identification still are not completely understood[60].

This thesis will mainly focus on the canonical Wnt pathway owing to its crucial involvement in embryonic skeletal development i.e. the regulation of chondrocyte and osteoblast differentiation and proliferation, and tissue regeneration/healing, but also due to its consequential association with the degenerative joint disease OA[7,61-63].

The canonical Wnt signaling pathway

Canonical Wnt ligands, such as Wnt3a, trigger signaling activation by binding to the receptors Frizzled (FZD). The FZDs are 7-transmembrane-spanning proteins and constitute a family of at least 10 different receptors. In the canonical Wnt pathway, also the co-receptors low density lipoprotein related proteins 5 and 6 (LRP5/6) are required for transduction of the signal. In the absence of Wnt ligands, the key intracellular protein β-catenin associates with cadherins at the plasma membrane, and any excess of the protein is phosphorylated by a destruction complex, consisting minimally of glycogen synthase kinase-3β (GSK-3β), adenomatosis polyposis coil (APC) and AXIN. This leads to a rapid ubiquitin-mediated degradation of β-catenin in the proteasomes.

On the other hand, in the presence of Wnt ligands, the formed ligand-receptor complex will subsequently bind to the destruction complex, which effectively reduces the activity of GSK-3β. This results in a reduced phosphorylation of β-catenin and an accumulation of the protein in the cell cytosol, leading to the subsequent translocation of the protein into the cell nucleus[62,64]. Nuclear β-catenin interacts with DNA bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins which induces expression of Wnt/β-catenin downstream target genes such as AXIN2, Wnt-1 inducible signaling pathway protein 1 (WISP1) and FOS-like antigen 1 (FOSL1)[65-69].

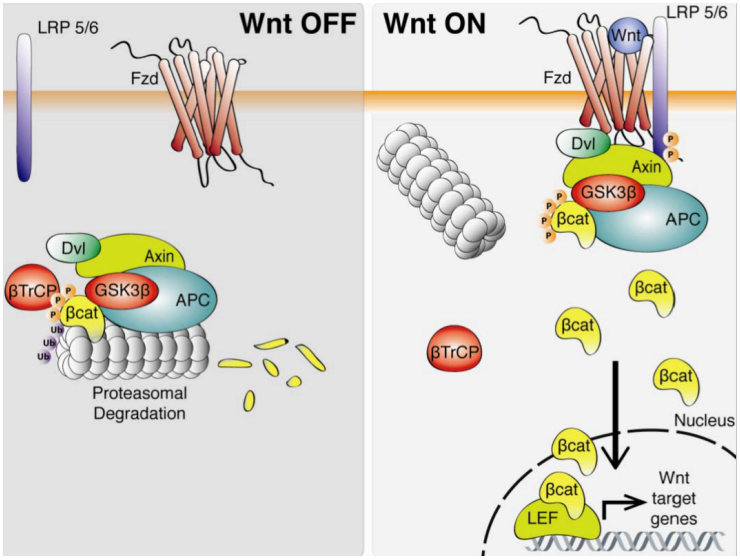

Figure 6. Overview of the canonical Wnt signaling pathway. In the absence of Wnt ligands, GSK-3β phosphorylates β-catenin that is targeted for ubiquitination and degradation in the proteasome (left panel). In the presence of a Wnt ligand, it binds to the FZD receptor and LRP5/6 co-receptors that trigger association of the destruction complex. In this condition, the ubiquitination is blocked and β-catenin is stabilized in the cytoplasm, leading to transduction of β-catenin into the nucleus and transcription of Wnt target genes (right panel). © 2013 Nibaldo C. Inestrosa and Lorena Varela-Nallar. Originally published in: http://dx.doi.org/10.5772/54606 under CC BY 3.0 license.

Several antagonists such as Dickkopfs (DKKs), secreted frizzled-related proteins (SFRPs), sclerostin and Wnt inhibitory factors (WIFs) regulate the canonical Wnt signaling pathway. The inhibitors of the Wnt pathway generally exert their function via two distinct mechanisms; SFRPs and WIFs bind to secreted Wnts in the extracellular space and can interfere with both canonical and non-canonical pathways. In contrast, DKKs and sclerostin bind to the LRP5/6 co-receptor and block Wnts from associating with the FZD/LRP5/6 complex, thus are considered as specific to the canonical cascade[62,64].

β-catenin

β-catenin is a multitasking and evolutionary conserved protein that plays a crucial role in several developmental and homeostatic processes. Besides being the key effector of canonical Wnt signaling in the nucleus, β-catenin is also a structural component of cadherin-based junctions. Hence, there are generally two pools of β-catenin in cells; one pool that is tightly associated

with cadherins at cell-cell junctions, whereas the other is a "free" fraction in the cytosol/nucleus, participating in regulation of gene transcription. Imbalances in the structural and signaling properties of β-catenin often results in disease and dysregulated cell growth associated to cancer and metastasis[67,70]. The answer to how β-catenin is capable to mediate both adhesive and signaling activities seems to lie within its structural composition and conformational changes[71]. Further, in its role as regulator of the canonical Wnt pathway it appears as fold change (FC) rather than absolute levels of β-catenin is critical, indicating that even low levels of the protein may be sufficient for inducing transcriptional changes[72]. This also seems to be the case for the signaling cascade as a whole, since it is apparent that even subtle changes in the intensity, amplitude, location, and duration of the Wnt signaling pathway affects skeletal development, bone remodeling, regeneration, and repair[62].

GSK-3β

GSK-3β is a serine/threonine kinase that unlike most other signaling mediators plays central roles in a diverse range of signaling pathways, and is hence regulated by multiple mechanisms. Besides its critical role in regulating the canonical Wnt pathway, the enzyme phosphorylates over 100 substrates and is a core component in pathways that regulate cell fate determination and morphology, such as Hedgehog[73,74]. The dysregulated form of the pathways in which GSK-3β function as a crucial regulator, have all been implicated in the development of severe diseases such as diabetes, Alzheimer's disease, bipolar disorders and tumors. Thus, its involvement in many pathophysiological processes and diseases makes it a tempting therapeutic target. As described above, in the resting state of the canonical Wnt signaling pathway, the cystolic β-catenin must be maintained at a very low level through a rapid turnover of free β-catenin, and this is regulated by GSK-3β and the other components in the destruction complex via several phosphorylation steps[73-75].

1.3.1 The yin-yang relation of the Wnt signaling pathway

Skeletal stem cells ascend from a common osteochondroprogenitor cell that produces both cartilage and bone. The levels of SOX9 relative to RUNX2 regulate the decision of the progenitors to differentiation into a chondrocyte or an osteoblast. If the expression of SOX9 remains high relative to RUNX2, the osteochondroprogenitor cell differentiates into the chondrogenic lineage; whereas when SOX9 levels are lower relative to RUNX2 the cell adopt an osteoblastic fate instead. Hence, it seems that SOX9 serves as a master

regulator of the skeletogenic cell fate. Further, the expression of SOX9 in osteochondroprogenitor cells is in turn directly regulated by Wnts, and by repressing the expression of SOX9, Wnts pushes the cells into the osteogenic lineage. Conversely, if the expression of the Wnt signaling is inhibited, SOX9 levels remain high and cells then assume a chondrogenic fate[64,76,77].

The fetal perichondrium arises from cells surrounding a chondrogenic condensation (as described earlier). It is at this stage that the regulation by the Wnt signaling pathway influences whether perichondrial cells acquire a chondrogenic or osteogenic costume. The general destiny of these cells is to adopt the osteogenic lineage; however, if the Wnt signaling is repressed, the perichondrial cells continue to express SOX9 and hence differentiate into chondrocytes[76].

1.3.2 Role in cartilage generation

The canonical Wnt signaling pathway is required for embryonic joint specification, formation and chondrogenesis. One of its roles in the early phase of the skeletogenesis is to maintain the chondroprogenitor cells in a proliferative state and to prevent maturation into chondrocytes, whereas its role in the final stages of endochondral ossification pushes osteoprogenitor cells into osteoblast maturation[64,78].

β-catenin is highly expressed in prechondrogenic MSCs commited to the chondrogenic lineage, but decreased in differentiated chondrocytes, possibly implicating it as a negative regulator of the chondrogenesis[79]. Reduced β-catenin expression is needed for chondrogenic differentiation of MSCs and maintenance of the differentiated phenotype of chondrocytes. It has been shown that the non-canonical ligand WNT5A is first expressed in the mesenchyme close to the condensation area, potentially recruiting MSCs into the chondrogenic lineage. Thereafter is the expression of WNT5A shifted to the perichondrium by further development, possibly contributing to the appositional growth[80,81]. WNT5A has been shown as a strong inhibitor of the canonical Wnt pathway, and WNT5A-null mouse embryos are dwarfs with shortened limbs, low expression of SOX9 and type X collagen, which also display lack of endochondral ossification. This suggests that WNT5A could serve as an inhibitor of the canonical Wnt signaling during cartilage development[82].

The important cartilage associated transcription factor SOX9 is continuously expressed, in the beginning in the pre-cartilaginous MSCs; reaching up to the pre-hypertrophic chondrocytes and is finally downregulated in the

hypertrophic cells[83]. It is known that SOX9 inhibits canonical Wnt signaling by competing with β-catenin binding to LEF/TCF proteins, inducing reduced expression of β-catenin target genes. In addition, experimental ablation of SOX9 in cartilage results in a similar phenotype to that of chronic activation of the canonical Wnt signaling pathway[84]. Since SOX9 expression precedes differentiation of MSCs into chondrocytes, the protein could also regulate the canonical Wnt signal from the initial start of cartilage formation up until the pre-hypertrophic stage. The terminal maturation of hypertrophic chondrocytes would then involve downregulation of SOX9 and WNT5A, hence enabling a boost of canonical Wnt signaling.

1.3.3 Role in OA pathogenesis

Given the essential role of the Wnt signaling pathway in skeletal development and cartilage and bone biology, it is also likely to be associated with OA.

Accordingly, the pathway has been implicated in the pathogenesis of the disease with increased levels of β-catenin in human degenerative/OA cartilage[85,86]. Further, increased level of β-catenin in cartilage explants has been associated with IL-1β treatment, one of the primary inflammatory cytokines involved in cartilage destruction (as described earlier)[22]. In addition, upregulation of several other Wnt related markers have been associated with IL-1β stimulation[87-89]. The localization of β-catenin in untreated articular chondrocytes was predominantly shown in cell-cell contacts, whereas IL-1β stimulation induced accumulation of the protein to the nucleus, indicating that the cytokine can induce the nuclear translocation and transcriptional activation of β-catenin[85]. Moreover, specific activation of the β-catenin gene in articular chondrocytes generated an OA-like phenotype in mice, demonstrating characteristics such as loss of articular cartilage layers and woven bone formation in the subchondral bone. Further, also the gene expression of the matrix degradation collagenase MMP13 was then increased.

In addition, gene expression of WNT1, WNT3A and WNT7A was significantly reduced whereas the gene expression of WNT5A and WNT11 was significantly increased in articular chondrocytes from β-catenin cAct mice. In contrast, the gene expression of the Wnt inhibitor secreted frizzled-related protein 2 (SFRP2) and the Wnt target gene WISP1 was significantly increased[86]. Activation of β-catenin in mature chondrocytes stimulates hypertrophy, matrix mineralization, and expression of MMP13 and VEGF. In line with this, overexpression of β-catenin strongly induces expression of

matrix degradation enzymes such as MMPs and ADAMTS5[78]. Thus, this implicates that the canonical Wnt signaling might activate the cartilage matrix catabolism, having a crucial and baneful role in cartilage destruction such as that in OA[61,78].

Whole genome single nucleotide polymorphism (SNP) screenings have revealed several Wnt related markers as candidate genes associated with OA. Loughlin *et al*. found a functional polymorphism in the secreted frizzled-related protein (FRZB) gene that is associated with hip OA in females and links the Wnt signaling pathway further to OA pathogenesis[90]. The association between FRZB and OA has been subsequently confirmed in other studies[6]. The FRZB gene encodes the secreted Wnt related inhibitor secreted frizzled-related protein 3 (SFRP3), which during the influence of Wnt signaling regulates chondrocyte maturation. Further, knockout mice deficient in this gene showed cartilage damage linked to the Wnt signaling pathway and MMP13 expression, potentially contributing to OA development[91]. OA susceptibility has also been suggested in the LRP5 gene, and LRP5 knockout mice display mild instability induced OA, which increased cartilage destruction[92].

Elevated serum levels of DKK1 are associated with increased disease activity in patients with rheumatoid arthritis (RA), and DKK1 has further been associated with chondrocyte apoptosis in OA joints[88]. Blom *et al.* reported increased expression of synovium-localized β-catenin in experimentally induced OA. Further, increased expression of WISP1 in the synovium and cartilage have been revealed, and a similar expression was observed in human OA[93], implicating WISP1 as regulator of MMPs and aggrecanase expression. Additionally, polymorphisms of the WISP1 gene, whose protein product is shown to inhibit the differentiation of precursors into chondrocytes and may also affect the chondrocyte phenotype, has been associated to the occurrence of spinal OA[28]. Finally, overexpression of Wnt related genes have also been reported in OA bone[94].

1.3.4 Role in bone generation

As mentioned before, the Wnt signaling pathway possesses multiple functions during osteogenesis and the canonical Wnt signaling is in particular regarded to be of crucial importance in bone biology. In addition to its role in pushing skeletal stem cells into the osteogenic lineage, Wnts also stimulate osteoblast proliferation and support osteoblast maturation[62,64,76,95].

As mentioned earlier, the Wnt signaling pathway is involved in both intramembranous and endochondral ossification. During direct bone formation, Wnts and β-catenin accumulation directs osteoblast precursors into mature osteoblasts, and they deviate away from the adipogenic or chondrogenic lineage. Regarding the endochondral ossification, the Wnt signaling is crucial in the final differentiation steps pushing the chondrocytes toward hypertrophy (as described in section 1.2.1)[96]. Several studies have confirmed that the presence of β-catenin is necessary for complete osteoprogenitor differentiation, but its role in mature osteoblasts is less well known. However, activation of β-catenin has been recognized as a strong signal contributing to osteoclastogenesis by regulating the expression of RANKL and osteoclastogenesis inhibitory factor (OPG) in osteoblasts[95-97]. Mechanical loading has been reported to directly and indirectly activate β-catenin signaling in osteoblasts, and the extracellular canonical Wnt inhibitors sclerostin (strongly expressed by osteocytes[98]) and DKK1 have been implicated in bone response upon mechanical stimulation[96,99].

Mutation in the Wnt signaling cascade can lead to excessive bone growth or resorption, and the first indication of linkage between bone biology and the canonical Wnt signaling pathway was discovered over a decade ago[100]. Loss of function mutation of the co-receptor LRP5 causes syndrome characterized by low bone mass, accompanied with frequent bone fractures[100,101], whereas a gain of function mutation of LRP5 leads to high bone mass[102,103]. The essential role of LRP5 in the regulation of bone mass in humans is further underscored with the association of SNPs of the LRP5 gene with decreased bone mineral density and an increased risk of osteoporotic fractures[104-106]. The mechanism by which LRP5 regulates bone mass is not fully understood, but LRP5 and LRP6 are known to transduce Wnt signaling *in vitro* and indicated overlapping roles during *in vivo* skeletal patterning[107]. Furthermore, gene variation in WNT16 has recently been associated with bone mineral density and osteoporotic fractures, whereas WNT16 knockout mice showed a substantial decrease in bone thickness and strength[108-110], indicating crucial roles of WNT16 in bone biology.

1.3.5 Role in fracture healing

The initial phase of skeletal tissue repair or active bone remodeling is similar to that occurring during skeletal embryogenesis – once aging the skeletal stem cells have to decide between the osteogenic or chondrogenic route of action[76].

One report of Wnt involvement in fracture repair identified upregulation of WNT5A, β-catenin, FZD, and numerous target genes during the process[55]. A later follow-up study demonstrated upregulation of additional Wnt related markers such as WNT4, WNT5B, LRP5, dishevelled (DVL), TCF1 and peroxisome proliferator-activated receptor delta (PPARD). In contrast, the transcription factor LEF1 was repressed during the initial phases of bone repair and the subsequent maximal bone formation. However, since it is recognized that LEF1 inhibits RUNX2-dependent activation of OCN in osteoblasts, and the fact that RUNX2 is the transcription factor required for osteoblast development, it seems likely that decreased LEF1 expression is necessary for bone repair to occur[111].

As described above, β-catenin appears to have various roles at different stages of bone repair; early in the process β-catenin regulates the ratio of osteoblast versus chondrocytes that arises from pluripotent MSCs. Later on β-catenin induces the differentiation of osteoblasts and boosts their matrix production[99]. Upregulation of LRP5 gene expression is shown in fracture callus, and β-catenin expression has been observed in callus and in proliferating chondrocytes, osteoblasts and periosteal osteoprogenitor cells. This indicates that the canonical Wnt signaling pathway is active both in endochondral and intramembranous ossification[112]. The involvement of the Wnt signaling pathway during intramembranous ossification has also been shown in recent studies[113,114]. Fractured femurs of LRP5 knockout mice have been reported to be smaller, less mineralized, and biomechanically inferior to those from wild-type littermates. The study further showed that DKK1 Ab administration increased the size, mineralization and biomechanical properties of the fractured tissue, demonstrating that deletion of LRP5 delays the reestablishment of biomechanical integrity during fracture repair[112]. The LRP5-mediated canonical Wnt signaling seems to be less important to mineral accumulation at the fracture site than for the restoration of proper tissue structural arrangement. This identifies LRP5 and the canonical Wnt pathway as key components of fracture repair, although the non-canonical Wnt pathways have recently been implicated in bone formation during both intramembranous and endochondral fracture healing[59].

1.4 Implants

Orthopedic and dental implants are medical devices used in order to replace a missing joint, bone or a tooth, or to mechanically or structurally support damaged bone tissue. Biomaterials are artificial or natural materials from which we can make devices that interact with biological systems in order to co-exist for a long time with minimal failure risk. Each implant is designed to correct or reinforce the affected body part to improve function, to withstand compressive forces, enhance mobility and reduce pain, thus improving the quality of life for the patient. In case of fractures, the majority heals without complications although around 10% of the patients experience a healing delay and/or non-union. This is associated with many contributing factors and often results in socioeconomic consequences. Fracture location, infection, poor fixation, patient age and sex, diseases and use of drugs such as nicotine, alcohol and chemotherapeutics are all factors that contribute to failure or problems related to implants. Osteoporosis is one of the most significant clinical problems, since it is also associated with increased fracture risk upon mechanical loading of bone[56,115]. Consequently, there is a need for effective fracture healing therapies, and a potential use of modulators of the Wnt signaling pathway may be helpful.

In this thesis, HA coated screw-shaped titanium implants and poly(lactic-co-glycolic acid) (PLGA) plug-shaped implants were used with and without incorporation of the canonical Wnt signaling activator Li^+ (see below). Both HA and PLGA are well known and characterized biomaterials within the biomaterials field, often designed as a solid body or used as a scaffold or delivery system[116-118].

Hydroxyapatite (HA) coated implants

HA is a widely used biomaterial due to its excellent compatibility with bone and has a mineral composition similar to that of natural bone. HA is considered as biocompatible, osteoconductive and osteoinductive[118,119]. Several different methods for coating metallic implants with HA exists[115]. One of these is a precipitation method which results in a HA coating by soaking the substrate into a calcium and phosphate containing solution. This method chemically mimic certain steps involved in bone formation during normal bone growth in the body as described by Kokubo[120].

Poly(lactic-co-glycolic acid) (PLGA) implants

PLGA is a co-polymer that is often used in controlled drug release delivery systems. It is biodegradable and biocompatible, and degrades into lactic and glycolic acids in the presence of water, compounds that are naturally

occurring in the body[121-123]. PLGA devices can be manufactured with various techniques[124], and give rise to a variety of complex drug release and degradation mechanisms[122,125].

1.4.1 Bone healing around implants

The use of implants requires a surgical procedure that inevitably leads to a surgical trauma. This is then followed by insertion of a foreign material that alters the wound healing process, although the detailed changes are not well understood. Nevertheless, it is recognized that inflammation is prolonged and infection of the wound may occur more often than without implants. The provoked biological response will depend on the features of the implant surface and the surgical procedure. Bone healing around titanium implants is often regarded as similar to the healing process observed at site of injury or fracture in terms of inflammation, regeneration and remodeling as descried earlier. However, different surface characteristics such as charge, hydrophilicity and topography will largely affect the healing process at molecular level and determine the kinds of cells, proteins, molecules and salts that will bind or absorb to surfaces[115,126-130]. Hence, a biodegradable compared to chemically inert implant will give rise to various cellular and molecular responses during fracture healing. In addition, the molecular mechanism by which the topographical cues affect cells and tissues is still poorly understood and has hampered the optimization of biomaterials development.

Intramembranous ossification seems to be the principal mechanism of bone healing of bone-anchored implants, and the interface cellular picture of early bone regeneration around Ti implants has recently been reviewed by Palmquist *et al*.[118,131]. This ossification process occurs only within a few days to a week after fracture, whereas endochondral ossification is a slower process that continues for weeks. However, several factors may influence which type of ossification that occurs e.g. defect size, localization, blood supply and mechanical stability at the fracture site.

1.4.2 Wnt signaling pathways and implants

The clinical success of an endosseous (placed in bone) implants depend on their functional integration into bone, i.e. osseointegration[132] and, hence on the cell response to materials- and surface characteristics. As described above, it is known that surface characteristics influence the biological outcome of the implant. In addition, it has become clear in recent years that surface micro- and nano topography and chemistry also affects the Wnt

signaling pathways, although the underlying mechanisms are still poorly understood.

Both *in vitro* and *in vivo* global genome analyses have identified the Wnt signaling pathways as differentially regulated by surface topography and wettability[133-135]. *In vitro* analysis of hMSCs demonstrated WNT5A as upregulated on rough surfaces, which was further correlated to increased expression of RUNX2 and BSP[135]. An *in vivo* rat evaluation showed that members of both the canonical and the non-canonical Wnt pathways were upregulated on rough compared to smooth surfaces, indicating that multiple Wnt pathways are in fact involved in fracture healing around implants[134]. In line with this, *in vitro* studies with MG63 cells, human osteoblasts and MSCs have demonstrated that mRNA expression of several Wnt ligands such as WNT5A was modulated by titanium surface roughness and energy. Furthermore, the canonical ligands WNT1, WNT3A and WNT7B were downregulated on rough surfaces, thus indicating a suppression of the canonical Wnt pathway and activation of the non-canonical Wnt5a/Ca^{2+} signaling pathway on rough surfaces[136,137]. On the contrary, rough and nanostructured titanium surfaces displayed enhanced expression of Wnt3a and β-catenin, but did not all affect the WNT5A expression[138]. This variety in results arises most likely from differences in sample topography and would have implications for the osseointegration *in vivo*.

Moreover, the addition of the well known Wnt signaling activator LiCl (see below) to cells cultured on rough surfaces has been demonstrated to activate the canonical Wnt pathway, displaying increased expression of osteogenic markers such as ALP, OCN and Wnt related markers such as WISP2. The signaling pathway was more potently activated on hydrophilic surfaces[139]. Canonical Wnt signaling inhibitors such as DKK1 and DKK2 have also been demonstrated to display modified expression and activities in the context of rough microstructure surfaces[140].

1.5 Modulation of the canonical Wnt signaling pathway

Since imbalances in the structural and signaling properties of the Wnt signaling pathway contribute to numerous diseases in human, extensive attention has been paid to small molecules that target the Wnt pathways[67]. In order to block Wnt signaling pathway during cancer, the most effective target would be the TCF/β-catenin complex, due to its critical function in transduction of the signal to downstream action. However it has proven to be

an elusive target due to its complexity. Modulation via such an essential player in a well conserved pathway as the Wnt cascade brings about undeniable risks to induce negative effects in other integrated systems.

Since the canonical Wnt pathway is regarded as a dominant mechanism regulating bone metabolism and fracture healing, could activation of the pathway or blocking inhibitors of it be potentially targets for modulation of fracture repair. Due to this, targeting of the Wnt pathway has emerged as a promising strategy during the latest years[141].

1.5.1 DKK1 and sclerostin neutralizing antibodies

Efforts have been made to influence fracture healing by blocking the inhibitors of the Wnt signaling pathway. In particular, neutralizing antibodies against the canonical Wnt inhibitors DKK1 and sclerostin have been studied more extensively[142]. Several recent studies showed that sclerostin-blocking antibodies improved screw fixation, increased bone formation and bone mineral density[143-145]. Studies with DKK1-blocking antibodies indicated a similar effect on implant fixation, bone regeneration as well as increased bone volume and density[146,147]. A recent clinical trial, the first in humans, indicated that systemic administration of a sclerostin antibody was safe and induced increased expression of bone-formation markers and reduced the expression of the serum bone-resorption marker C-telopeptide (sCTx). Also the bone mineral density was increased in patients receiving the antibody[148]. Thus, DKK1 and sclerostin are emerging as new targets for anabolic therapies and have potential for managing skeletal disorders[142].

1.5.2 Lithium (Li^+) modulation of the Wnt pathway

The simplest canonical Wnt activator is lithium chloride (LiCl) that has been used for decades in bipolar disorders[149], presumably influencing a variety of neurotransmitters[150]. In addition to its psychotropic effects, Li ions (Li^+) activate the canonical Wnt signaling pathway by inhibiting GSK-3β, thereby securing for accumulation of cytoplasmatic β-catenin. Li^+ is the best-characterized inhibitor of GSK-3β, but is fairly specific for this enzyme compared to other protein kinases. Also and as described earlier, since GSK-3β is not specific for participation in the Wnt signaling cascade only, a relatively high dose is required to achieve the inhibitory action of Li^+.

Li^+ is a naturally occurring element and water-soluble, but does not bind to plasma protein and is able to cross the blood-brain barrier. The mode of Li^+ inhibition is via competition inhibition of Mg^{2+} and when orally

administrated it is predominantly absorbed through the stomach without undergoing metabolism. It is subsequently excreted almost exclusively via the kidneys[73,150]. The therapeutic effect in humans become evident after a few weeks of treatment with a plasma concentration near 1 mM[149-151]. Animal studies have been performed with daily doses of 200 mg/kg, and resulted in plasma levels comparable to those of treated humans[152,153].

Preclinical studies have demonstrated that systemic administration of Li^+ can increase bone mass, bone formation, and improve fracture healing in mice. Further, retrospective clinical analyses have shown a reduced risk of fractures in patients on Li^+ treatment[141,151,152,154,155]. Li^+ in itself is generally safe, although the therapeutic index is narrow and the long-term systemically administrated therapy is associated with several potential side effects such as weight gain, tremor and impaired renal function. When Li^+ is found to be effective and administrated for years or decades it can gradually accumulate within the brain and lead to chronic toxicity. This is also subject to other factors including Li^+ dose, individual tolerance, renal function and age[149].

Due to this, it would be more beneficial in biomaterial applications to enhance bone healing via local administration of Li^+ from e.g. implant coatings or biodegradable implants. Earlier studies have incorporated Li^+ into various biomaterials or added it directly to cell culture medium, investigating the Wnt signaling activation and potential improved osteogenic differentiation[139,156-158]. However, summarizing the current knowledge within this field it is clear that a proper timing is important for Wnt targeting in clinical fracture repair[95,112,113,154].

2 AIMS

The general objective of this thesis was to increase the understanding of the Wnt signaling pathway during bone and cartilage regeneration, and to gain improved insights into its involvement in functional impaired cartilage healing and to explore its modulation capability in bone fracture repair mechanisms.

The more specific aims of the included studies were:

- To study the underlying cellular alterations leading to OA cartilage degeneration in relation to the skeletal developmental essential Wnt signaling pathway (*Papers I and II*).

- To study the effects of OA related factors such as the pro-inflammatory cytokines IL-1β and IL-6 with respect to the Wnt signaling pathway and its coupling to GDF5 (*Paper I, mainly Paper II*).

- To study whether the Wnt signaling pathway can be modified by locally released Li^+ from HA coated bone-anchoraged implants, and if this results in improved bone regeneration (*Paper III*).

- To study how the global gene expression profile was affected by local Li^+ release from biodegradable PLGA implants, and if the Wnt signaling pathway was activated. (*Paper IV*).

3 MATERIALS AND METHODS

For a more detailed description of Material and Methods used in this thesis, the reader is referred to Papers I-IV. A more general description of the methods is presented below.

3.1 Isolation and culturing of chondrocytes (Papers I - II)

3.1.1 Sources of chondrocytes

In Paper I, human cartilage biopsies from OA patients undergoing total knee replacement and control patients with macroscopically healthy cartilage were collected (24-48 hours, post mortem). Donors with OA were selected for the study if they fulfilled four criteria: 1) displayed symptoms of severe OA, 2) underwent total knee replacement, 3) showed radiological evidence of OA, 4) displayed OA grade 2–3 according to Ahlbäck criteria, and exhibited a Mankin score ≥ 4. Articular cartilage from five donors (62–81 years) was collected based upon these criteria. Control patients with no pre-existing history of OA symptoms and macroscopically and microscopically healthy cartilage were selected (exhibiting a Mankin score ≤ 1, these donors are referred to as normal donors (ND)). Healthy articular cartilage biopsies were obtained from eight donors (40–84 years).

In Paper II, macroscopically normal equine articular cartilage (shavings for 3D pellet culture) was collected from middle carpal joints (the weight bearing area), within 24 hours after euthanasia. The horses had no known clinical history of disease, and were euthanized because of reasons unrelated to the study. Further, osteochondral samples were collected from adjacent sites (including sections of articular cartilage that comprised all zones of hyaline articular cartilage and calcified cartilage, separated by the tidemark, and subchondral bone) for histological examination. Osteochondral fragments/fractures (OCF) were collected from horses (carpal and fetlock joints) undergoing arthroscopy due to joint problems that caused lameness. All joints had additional articular cartilage lesions, such as fibrillation, that indicated OA (determined by means of light microscopy). The OCFs included articular cartilage with areas of moderate surface fibrillation. Because horses have naturally occurring osteoarthritis that is similar to that of humans, the horse is a suitable model for investigating this issue and the results are applicable between species[159,160].

3.1.2 Isolation of chondrocytes

In Paper I, chondrocytes were isolated from normal donors (ND) and OA cartilage biopsies as described previously for handling of cartilage biopsies used for transplantation[161]. Equine cartilage used in Paper II was handled in a similar way. Briefly, the biopsies were transported to the cell culture lab in sterile saline solution supplemented with antibiotics and fungizide. After removal of subchondral bone, isolated chondrocytes were obtained by mechanical mincing of the cartilage followed by overnight collagenase digestion, which degraded the collagen fibers. After the enzymatic digestion the cells were released from the matrix and single cells or small clusters of cells were observed in the suspension.

3.1.3 Expansion of chondrocytes

In this thesis, chondrocytes were expanded *in vitro* in order to reach a sufficient number of cells for further investigation or culture in pellet mass culture (see below). The cells were expanded using specific culture conditions using medium with ascorbic acid, L-glutamine and serum. If necessary, cells were expanded by passage to new culture bottles upon 80% confluence. The cells were released from cell culture bottles using trypsin-EDTA solution diluted in phosphate buffered saline (PBS).

3.2 *In vitro* chondrocyte culture model

3.2.1 Monolayer chondrocyte culture

In monolayer culture, the cells grew adherently to plastic culture plates/bottles. Single cells were seeded at a density about 3000-8000 cells/cm^2. A defined cell culture medium without serum for chondrogenic differentiation containing TGF-β1 was used[162,163].

3.2.2 Three dimensional chondrocyte culture

In Paper II, passage 1 equine chondrocytes were cultured in a 3D pellet mass culture system (also called pellets). This culture system provides important conditions for recovering of a differentiated phenotype from *in vitro* expanded cells - a defined medium containing certain growth factors for chondrogenic differentiation and a 3D environment at a high cell density. It was previously shown that the 3D pellet mass culture acts as a good differentiation system for chondrocytes and the cells share similarities with native cartilage regarding cellular distribution, matrix composition and density, as well as tissue ultrastructure[164,165].

3.2.3 Cytokine stimulation

In Paper I, monolayer chondrocytes isolated from ND and OA donors (passage 1) were stimulated with human recombinant IL-1β (10 ng/mL) for 24 hours or left unstimulated. Gene expression of Wnt related markers was then studied using qPCR, as described below. In Paper II, equine pellets were stimulated with either recombinant equine IL-1β (5ng/mL), recombinant equine IL-6 (5ng/mL) or left unstimulated. The pellets were stimulated from 1 hour (short-term) up to 48 hours (long-term), and subsequently analyzed with histology, microarray analysis or qPCR.

3.3 *In vivo* experiments (Papers III - IV)

The schematics of the animal experiments can be seen in Figure 7 below, details can be found in Materials and Methods sections of Papers III and IV.

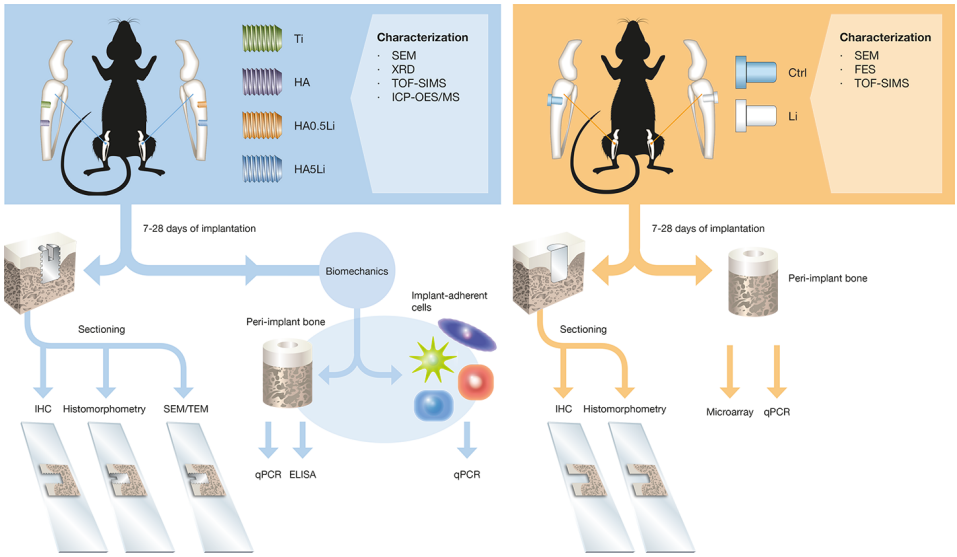

Figure 7. Schematic illustration of implant characterization, in vivo trials and subsequently performed analyses. In vivo rat tibia model with HA-coated titanium implants (with or without Li^+) indicated in blue. Implantation with Li^+-containing PLGA and Ctrl implants without Li^+ indicated in orange.

3.3.1 Implants

In Paper III, titanium (Ti) screw-shaped HA-coated (with/without LiCl) implants were prepared by soaking in a calcium and phosphate buffer solution, and sterilized by an ultraviolet ozone (UVO) treatment. In Paper IV, lithium salt (Li_2CO_3) containing PLGA plug-shaped samples and control samples containing sodium salt (Na_2CO_3) were prepared by a hot-melt procedure, and subsequently sterilized by an ultraviolet (UV) treatment.

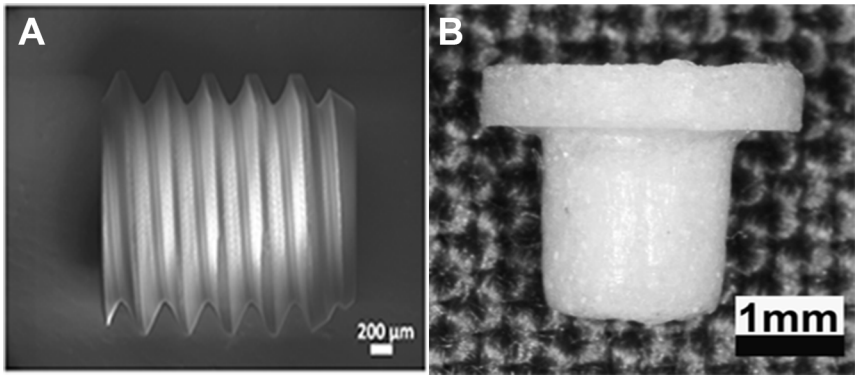

Figure 8. Implants used in this thesis. Ti screws coated with HA with or without Li^+ were inserted into rat tibia in Paper III (A). In Paper IV, plug-formed PLGA implants with Li_2CO_3 or Na_2CO_3 were used and inserted into rat tibia (B).

Endotoxin test

All implants were tested for endotoxin in order to exclude contamination. The endotoxin analyses were performed with *Limulus amebocyte* lysate, and all implants showed values below the recommended maximum level.

3.3.2 Surface characterizations

Electron microscopy

Different types of electron microscopy such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are widely used in material science[131]. The surface morphology of implants used in Papers III and IV was examined using SEM (LEO Electron Microscopy Ldt). In order to study the bone response to the HA coating (Paper III) back scattered SEM and TEM (FEI Company) was used. TEM samples, ultrathin sections were prepared by focused ion beam (FIB, FEI Company) milling, and subsequent

analyzed by bright field and high-angle annular dark field, in conjunction with elemental analysis to evaluate the micro and nano scale interaction between implant and bone surfaces.

TOF-SIMS Imaging

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a surface sensitive spectroscopy, and uses a pulsed ion beam to remove molecules from the very outermost surface of the sample. The particles are removed from atomic monolayers on the surface (secondary ions) and subsequently accelerated into a "flight tube". Their mass is then determined by measuring the exact time at which they reach the detector (i.e time-of-flight), and the charge is also measured in the detector. TOF-SIMS is widely used in material science[166], and was used in Papers III (data not shown) and IV in order to monitor the Li^+ distribution in the coating/implants using a TOF-SIMS IV instrument (ION-TOF).

XRD

In order to analyze the crystallinity of the HA coatings in Paper III, a thin-film X-ray diffractometer (TF-XRD, Siemens) with an incidence angle of 2° was used. XRD is applied to determine the atomic and molecular structure of crystals, in which the crystalline structures cause the x-ray beam to diffract into many specific directions. The technique is widely used in material science[167].

Chemical composition

In Paper III, coatings were dissolved in HCl/HNO_3 solution and inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300DV) was used for the analysis of Ca, Mg, and P contents. The content of the smaller Li ions was analyzed using inductively coupled plasma mass spectrometry (ICP-MS, Elan 600). In ICP-OES, plasma energy is given to the sample, whose component elements (atoms) are excited. When these excited atoms return to low energy position, emission rays (spectrum rays) are released at wavelength characteristic of a particular element. The intensity of the emisson relates to the concentration of the element within the sample. ICP-MS is based on the same principle but uses a mass spectrometer in order to separate and quantify the ions. These techniques are widely used in the medical and forensic fields, and in material science[168].

Li^+ release profile

In Papers III and IV, coated HA+Li^+ discs or PLGA implants with included Li_2CO_3 were submerged in PBS buffer. Samples were collected at specified time points and the amounts of released Li^+ into the buffer was determined

using ICP-MS (described above) or flame emission spectrometry (FES, iCE 3300AA Spectrometer) with a detection wavelength of 670.8 nm. FES detects the emitted energy when excited atoms return to their ground state. The analysis is preceded of an aerosolization step of the sample that is performed by exposing the samples to a flame. FES is commonly used for the determination of trace metals, especially in liquid samples such as clinical analyses of body fluids[169].

3.3.3 Animal model and surgical procedures

In thesis, the animal studies in Papers III and IV were performed using the well documented rat tibia model of osseointegration[170,171]. Male Sprague–Dawley rats (360-400 g), fed on a standard pellet diet and water were used in Papers III and IV. Male rats that are bigger than female rats were used to facilitate the surgical procedures. The implantation procedures were performed under isoflurane inhalation, and each rat received analgesic (Temgesic 0.03 mg/kg) subcutaneously prior to the implantation, and daily postoperatively. Prior to implantation, rats were shaved and cleaned (5 mg/mL chlorhexidine in 70% ethanol) and the surgery was performed under sterile conditions.

In Paper III, Ti screw-shaped implants uncoated or coated with HA (with or without Li^+) were installed in the left and right tibia (two implants/tibia). In Papers IV, PLGA plug-shaped implants (with or without Li^+) were inserted in left and right tibia (one implant/tibia). The locations of test and reference implants were decided using a predetermined schedule, ensuring alteration between the legs and sites. After bone preparation with dental guide drill under profuse irrigation with NaCl 0.9%, the implants were inserted manually in each tibia. After suturing, the animals were allowed free postoperative movements with food and water ad libitum, and sample retrieval was performed after 7 and 28 days. The rats were sacrificed by an intraperitoneal overdose of sodium pentobarbital under anesthesia, with a 0.5 mL mixture of pentobarbital (60 mg/mL), NaCl, and diazepam (1:1:2).

Different retrieval procedures were performed depending on the subsequent analyses (see Fig. 7). In Paper III, implants were exposed for biomechanical analysis before complete removal. Subsequently, the implants with adherent biological material were unscrewed by a hexagonal screw driver and placed in RNAlater®. The peri-implant bone was then harvested using a trephine burr and was immediately placed in RNAlater®, stored at 4°C until analysis. For histology and immunohistochemical analyses, implants with surrounding bone were dissected *en bloc* using a dental disc and fixed for subsequently

analyses. For transcript profiling analyses in Paper IV, peri-implant bone was carefully collected with the use of a trephine burr and was immediately placed in RNAlater®, stored at 4°C until analysis. In addition, bone-implant blocks were harvested and fixated for subsequent microscopic examination, as illustrated in Figure 7.

3.3.4 Biomechanical analysis

The biomechanical evaluation of bone implant integration was evaluated by the removal torque (RTQ) analysis, which measures the torque (unit: Nm) needed to loosen the implant from bone, thus gives a quantitative measurement of the implant stability. In Paper III, the peak removal torque was measured on the implants placed in the tibia after 7 and 28 days of healing with an upgraded version of equipment as described elsewhere[172].

3.4 Histological techniques

Histology is the study of microscopic structures and composition of cells and tissues. Prior to histological examination, samples must be fixated in order to preserve the biological material and to maintain the cellular and molecular structure. Biological material used in this thesis was fixated in formaldehyde solutions, containing different amounts of formaldehydes that cross-link proteins and prevents enzymatic degradation. The tissues were subsequently embedded in paraffin or plastic resin allowing sectioning and generation of thin sections placed on glass slides. These were later stained with appropriate histological stains or used for antibody detection (immunohistochemistry (IHC) or histomorphometry, as described below).

3.4.1 Histological staining

Alcian Blue van Gieson

In order to characterize cartilage depositions in the biopsies, Alician Blue van Gieson was used. This is a well known combination dye that stains negatively charged proteoglycans such as glycosaminoglycans (GAGs, a characteristica of mature hyaline cartilage). Alcian Blue is a cationic dye carrying up to four cationic groups that bind to GAGs (presumably bind more firmly to GAGs than Safranin-O, see below) whereas the van Gieson dye stains collagens and connective tissue. Alician Blue van Gieson stains cartilage blue, connective tissue red, muscle and cytoplasm yellow[173].

Safranin-O

Safranin-O is a monovalent cationic dye that binds to the negatively charged proteoglycans. The dye binds to GAGs on chondroitin-6-sulphate or keratin sulphate, components of mature articular cartilage. This dye stains the sulphated proteglycans organe to red, cytoplasm blue-green and nuclei black[174].

Heamatoxylin and eosin staining (HE)

Heamatoxylin and eosin staining is one of the most widely used stains in histology and is a combination of a basic and an acidic dye. Hematoxylin, the basic dye, stains acid structures (ribonucleic acids) such as nuclei and ribosomes blue-violet. Eosin, the acidic dye, colors non-specifically the basic parts of the cell (most cytoplasmatic proteins and ECM), resulting in a pale pink staining[174].

Toluidine Blue

Toludine blue is a basic dye widely used in histology. In alkaline solutions, the dye binds to nuclei acids, which then appear blue. Toludine blue is utilized for optimal demonstration of mineralized bone, osteoid seams, osteoblasts and osteoclasts. It is also possible to distinguish between the old and the newly formed bone (new bone give rise to darker shade than the old bone)[174].

3.4.2 Histological scoring systems

Mankin scoring

The often used microscopic method for OA histopathological assessment was developed in 1971 by Mankin *et al*[175]. The method was used as an inclusion/exclusion criteria for the cartilage biopsies used in Paper I. The Mankin system is based on a 14 point score system for the evaluation of architectual/structural changes (surface irregularities, vessel penetration), cellular changes (proliferation, cloning) and the histochemical presence of Safranin-O staining. A modified Mankin system[176], not including inspection of the tidemark, was used in this thesis since the biopsies obtained did not include the subchondral bone. The modified Mankin scale has a maximum score of 13.

Alhbäck scoring

Ahlbäck scoring is used as a macroscopic classification of knee OA and is widely used in clinical practice, first published in 1968[177]. Cartilage biopsies in Paper I were classified according to Ahlbäck as an inclusion/exclusion criteria.

3.4.3 Histomorphometry

Histomorphometry was used to quantify bone growth outside and in contact with the implants. In Paper III, both bone-implant contact (BIC) and the bone area (BA) within the threads were evaluated as data representing the osseointegration. High BIC- and BA values indicate good osseointegration. In Paper IV, only the BA was measured due to the form of the degrading PLGA implant. All specimens were evaluated by light microscopy (Nikon Eclipse E600, Japan).

3.4.4 Immunohistochemistry (IHC)

In order to specifically visualize proteins in cartilage and bone immunohistochemistry (IHC) was performed. The expression of Wnt and osteogenic related markers was analyzed with the technique first introduced by Coons *et al.* [178], based on the binding of a primary antibody to a target epitope. The primary antibody is subsequently visualized using secondary antibodies with reporter molecules such as fluorescent compounds, enzymes or metals attached[179].

In this thesis, both fluorescent and non-fluorescent secondary antibodies were used to visualize the antigens. To unmask the target site for the specific antibody, antigen retrieval was performed. Since horse-radish peroxidase (HRP) conjugated secondary antibodies were used, the endogenous peroxidase activity was quenched with 3% H_2O_2. To further prevent non-specific binding, the sections were blocked with 2-3% bovine serum albumin (BSA) in PBS. In Papers I and II, the secondary antibodies were visualized using the tyramide signal amplification (TSA)-Direct Cy3 kit, and the nuclei were subsequently stained with the DNA-binding molecule 4',6-diamidino-2-phenylindol (DAPI). In Papers III and IV, the secondary antibodies were visualized using Betazoid 3,30-diaminobenzidine (DAB) Chromogen kit, and all slides were counterstained with Mayer's hematoxylin. In all papers, isotype specific controls and sections without the primary antibody were used as negative controls. The sections were examined with Nikon fluorescence or light microscopes, and digital pictures were captured with the NIS Elements D (Nikon).

3.5 Gene expression analysis

Gene expression is the initial event leading to alteration of the information stored in the deoxyribonucleic acid (DNA) molecules into functional proteins, which in general are the specific players in cell responses. Gene expression is a very dynamic process that allows cells to respond and adjust to external and internal stimuli. The expression profile of a cell portrays its molecular state and is used as a signature to distinguish between different cell types and to detect changes upon external stimuli. In this thesis, gene expression was examined using global gene microarrays and quantitative real-time polymerase chain reaction (referred to as qPCR[180]).

3.5.1 RNA isolation

In Papers I and II, total ribonucleic acid (RNA) for qPCR and microarray analysis was extracted from cartilage biopsies, chondrocytes cultured in monolayer or in 3D pellets using Qiagen's RNeasy Mini Kit according to the manufacturer's protocol for animal cells. Prior to RNA isolation, monolayer cultured chondrocytes were harvested in RLT lysis buffer, whereas the structure of biopsies and pellets was homogenized with stainless steel beads and RNA precipitation was performed using a TissueLyser. Total RNA was extracted using QIAzol lysis reagent and chloroform. QIAzol is a phenol/guanidine-based lysis reagent that allows cell disruption and DNA/RNA extraction; after addition of chloroform the homogenate is separated into an aqueous phase containing RNA, and an organic phase containing both protein and DNA. Any contaminating genomic DNA was removed from the isolated RNA using DNase.

In the animal studies, the retrieved unscrewed implants (Paper III) and corresponding surrounding bone (Papers III and IV) were placed individually in RNAlater®, and were homogenized in QIAzol lysis reagent and chloroform as described above. In Paper III, the organic phase containing DNA and proteins was collected for subsequent protein isolation (see below). Total RNA from implants and surrounding bone was extracted using the RNeasy Micro or Mini kit, according to the manufacturer's instructions. To prevent any contamination from genomic DNA, DNase was used.

In all papers included in this thesis, the concentration of RNA was measured by a Nanodrop device at 260 and 280 nm. The ratio of 260/280 nm is considered to indicate the purity of the sample. We considered values between 1.8 and 2.1 to be acceptable[181]. For RNA samples used in the microarray analysis, the integrity of the RNA (28S to 18S rRNA ratio) was

evaluated using an Agilent Bioanalyzer. The values were between 8-10, thus the RNA was considered to be intact[182].

3.5.2 cDNA synthesis

Total RNA was transcribed to complementary DNA (cDNA) using random hexamers primers and TaqMan Reverse Transcription (RT) reagents, according to the manufacturer's instructions. This step, often referred to as the RT step, is critical for accurate quantification since the amount cDNA produced must correctly reflect the input amounts of the mRNA. It is recognized that the RT step contributes with most of the introduced variation of experimental accuracy. Random hexamer primers, i.e. short oligomers of all possible sequences, were used since they anneal throughout the target molecule and will copy all RNA (tRNA, rRNA and mRNA), thus synthesizing large pools of cDNA. This is also the primary strategy of choice if rRNA (such as 18S) shall be used as reference gene (see below)[183].

3.5.3 Quantitative real-time PCR analysis

Real-time PCR is a quantitative and very sensitive gene expression analysis, first described by Holland *et al.*[184] and is based on the 5' nuclease chemistry.

- When starting the real-time PCR reaction, the temperature is raised in order to denature the double-stranded cDNA. An oligonucleotide probe, labeled with a fluorescent reporter dye in the 5' end and a non-fluorescent quencher in the 3' end, is designed to anneal between two primers and to their specific target sequences. At this point, the quencher absorbs the fluorescence from the reporter dye as long as the probe is intact.

- In the following step, the reaction temperature is lowered to allow the primers and probe to anneal to their specific target sequences. Then temperature is increased leading to Taq polymerase synthesizing a complementary DNA strand using the unlabeled primers and template as a guide. When the polymerase reaches the probe, its endogenous 5' nuclease activity separates the reporter from the quencher, resulting in emission of fluorescence from the reporter dye.

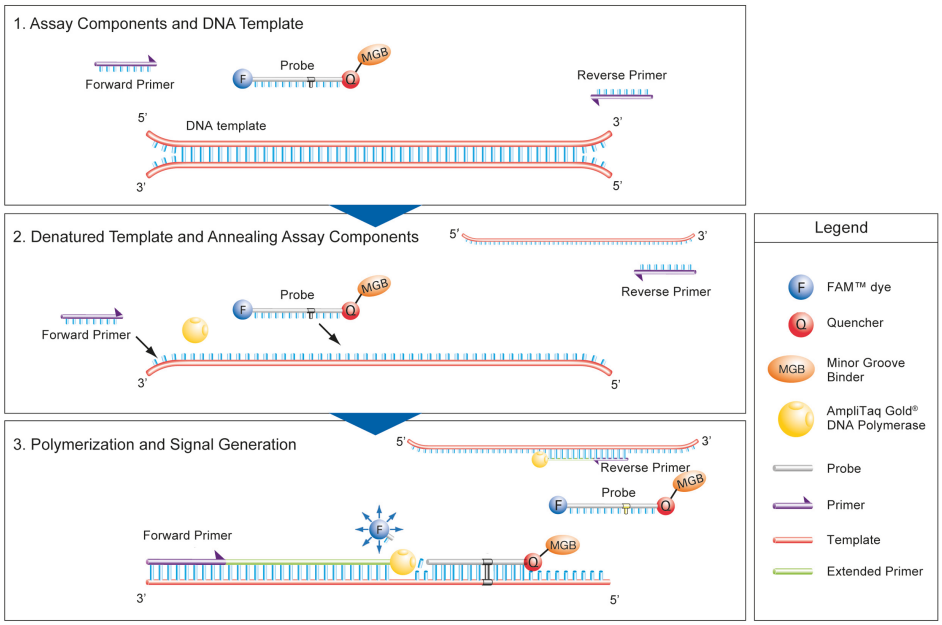

Figure 9. Overview of the real-time PCR showing annealing, polymerization and signal generation (http://www.lifetechnologies.com).

With each PCR cycle, more dye molecules become released from their respective probes, resulting in increased fluorescence that is proportional to the amount of probe target sequences. The higher the gene expression of the specific gene of interest is, the sooner will a significant increase in fluorescence be detected. The fluorescence is measured throughout the whole reaction, but the amount of amplified products is calculated in the exponential phase while the reagents are still in excess and are compared to a reference. When the fluorescence reaches a certain level in the exponential phase, a cycle number (Cq value) is obtained for each sample. This Cq value relates to the number of cycles required to reach a certain fluorescence threshold – if a gene has a high expression fewer cycles are needed in order to reach the threshold compared to a gene with a low expression.

Commercially available assay-on-demand mixes of primers and TaqMan® MGB (minor groove binding) FAM (6-carboxyfluorescein) dye labeled probes were used in this thesis. In Paper II, Custom TaqMan® Gene Expression Assays was used for the design of glucuronidase, beta (GUSB). Inconsistencies in RNA isolation and in the commonly used RNA analysis procedures listed above can introduce errors into the analysis process. In order to reduce the non-biological variation in all papers included in this

thesis, endogenous references were chosen for the normalization. An endogenous reference is recognized as expressed relatively stable in different tissues and should not vary due to external stimuli. Depending on the various biological materials as well as study set-ups, different internal references were used in this thesis. In all papers included in this thesis, analysis was done using a 7900HT instrument (Life Technologies). The relative gene expression was evaluated by the $2^{-\Delta\Delta Ct}$ method[185], and in Papers III-IV the GenEx Enterprise 5.2.3.13 was used for this purpose.

3.5.4 Microarray analysis

With the development of microarrays, researchers have obtained the opportunity to monitor the expression of thousands of genes simultaneously. This is achievable by the principle of base pairing and allows e.g. for identification of up- and downregulated genes or groups with similar expression profiles. The general procedure of microarray experiments include several steps[186]. The microarrays consist of a surface (chip) onto which millions of probes are immobilized. When two complementary sequences recognize each other, such as the probe on the array and the mobile target in the sample, they will lock together (hybridize). Firstly, the RNA is reverse transcribed, usually to cDNA and then labeled with a fluorochrome and the solution is subsequently hybridized onto the array. Unhybridized and excess cDNA is washed away and the chip is subsequently scanned to measure the fluorescence intensities that are translated into expression values. These intensities are directly proportional to the number of transcripts corresponding to each gene, thus the expression level for the gene.

However, when working with microarrays, there are several issues to be aware of, and which can introduce significant biases in the final results[187]. Each of the above-mentioned steps is a source of variability; non-biological variations such as chip, probe and experimental design to mention a few. Further, sample preparation, image acquisition, data normalization and analysis can also introduce errors. There is also a risk that the mobile probes wrongfully cross-hybridize to non-complementary immobilized ones on the chip. Given the huge number of genes detectable simultaneously, there is an inevitable risk that genes with low FC disappears in the large mass, thus displaying poor sensitivity for low expressed genes. Lastly, microarray analyses sense mRNA levels and not the translated protein levels, thus microarray techniques do not take the complex picture of mRNA translation and post-translational modifications into account. Data normalization is essential and removes the above-mentioned non-biological variations,

ensuring that the difference intensities actually are due to differential expression and not due to e.g. hybridization or scanning artifacts.

Undeniably, microarray techniques rely heavily on bioinformatics; the application of statistics and computer science to the field of molecular biology. Thus, bioinformatics solve the need of interpretation of the immense amounts of data generated within the genomic and proteomic fields[188]. However, dealing with bioinformatics in the language of biology is challenging, as biological processes are extremely complex and involve numerous components. To facilitate this, functional classification of the generated microarray data can be performed with annotations from for example Gene Ontology[189] (http://www.geneontology.org/) and the DAVID software tools[190,191] (http://david.abcc.ncifcrf.gov/). DAVID uses several databases such as KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg), GO and BioCarta (http://www.biocarta.com) as sources for the analyses.

Nevertheless, since these databases for obviously reasons include only already recognized annotations, the interpretation of microarray data containing unknown transcripts (lacking annotations of function and gene affiliations) become therefore complex and requires extensive follow-up studies. For this reason, most researcher dealing with microarray data focuses on the already annotated genes. In addition, the utilizers have to be aware of the substantial biological questions and issues since although we search for significant gene expression changes, even low expressed genes can induce a significant change in the biological outcome[192]. Thus, biologically relevant expression changes may not always be captured within a statistical test. Due to the statistical issues raised in the microarray technology, it is indispensable that the results are verified by using other methods. In this thesis, the microarray analyses were used as a screening tool in order to define trends within expression profiles and were subsequently confirmed with other methods such as qPCR and IHC, assessing both gene and protein expression. Within this thesis, total RNA was extracted from the cells and tissues using the above described protocols and subjected to microarray analyses according to the manufacturer's recommendations.

3.6 Protein expression analysis

3.6.1 Protein extraction

Extraction and preparation of total protein for a subsequent analysis by enzyme-linked immunosorbent assay (ELISA) was performed in Paper III. The total protein fraction was isolated from the organic phase of the QIAzol reagent-treated peri-implant bone samples undergoing total RNA isolation (described in 3.5.1). Briefly, 100% ethanol was added to the samples that were centrifuged to sediment DNA and the supernatants containing protein were transferred to new reaction tubes. Isopropanol was added to the samples in order to precipitate the protein. After centrifugation, the supernatant was removed and the protein pellets were homogenized in lysis buffer (provided with the ELISA kit) using stainless steel beads and TissueLyser. The lysis buffer was supplemented with a protease inhibitor cocktail to reduce possible proteolysis.

3.6.2 Determination of protein concentration

Total protein concentration was determined using bicinchoninic acid (BCA) assay according to the manufacturer's instructions, using BSA as a standard. This method is based on the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium and a subsequent detection of Cu^{1+} by BCA. The first step involves chelation of copper with proteins in an alkaline environment to form a light blue complex. In the second step of the color development reaction, BCA reacts with the reduced cation (Cu^{1+}) that was formed in step one. This leads to an intense purple-colored reaction product due to the chelation of two molecules of BCA to one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear concentration dependent absorbance at 562 nm. The BCA reagent is approximately 100 times more sensitive than the pale blue color of the first reaction.

3.6.3 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assays (ELISAs) are highly sensitive, robust and cost-effective methods for the detection and quantification of proteins within basic research[193]. The method uses antibodies and color changes to identify the protein of interest and is based on antigen capture by an antibody coated on the ELISA plate. A primary antibody is coated with the antigen bindings site(s) upwards onto the plate, which catch antigens in the added samples. This bound antigen is subsequently recognized by an added enzyme-conjugated secondary antibody, thus creating an antibody

sandwich. Unbound antibody is rinsed away, and a substrate for the conjugated enzyme is added, thus initiating a colorimetric reaction and the developed color is subsequently read using a spectrophotometer. The optical density (OD) measured is in proportion to the amount antigen of interest, bound in the initial step.

In Paper III, a two-site sandwich human total β-catenin ELISA kit was used according to the manufacturer's instructions. Briefly, tissue lysate (for protein extraction see section 3.6.1) was exposed to the pre-coated plate. After incubation at room temperature, the plate was washed several times and the detection antibody and the streptavidin (SA)-HRP conjugate were added. Finally, the substrate solution was added and the absorbance measured at 450 nm.

3.7 Bioinformatics

3.7.1 Comparative and statistical analyses of microarray data

In Paper I, the gene expression profiling was performed using the Affymetrix oligonucleotide microarray HG-U133plus2.0. Raw gene expression data were normalized and analyzed with GeneChip Operating Software 1.4 (GCOS, Affymetrix). Comparative and statistical analyses were performed with the BIORETIS web tool (http://www.bioretis-analysis.de). Further, the functional classification of Wnt related genes was performed with annotations from the Gene Ontology Annotation Database (http://www.ebi.ac.uk/GOA)[194]. In this study, the limit was set to at least ≥75% possible significant change calls. Downstream T-test statistics applying the Welch test on log2–transformed signal values were performed to obtain a significance level. Expression differences were given as FC, and only significantly altered genes that displayed a mean FC of FC ≥ 2 or ≤− 2 were selected for further analysis.

In Paper II, the Affymetrix® Human Gene 1.1 ST Array, was used for global transcriptome quantification. Expression signals were extracted and normalized by applying the Robust Multichip Average (RMA) normalization method. The probe match tool on NetAffx™ Analysis Center was used to compare that the equine nucleotide sequences of the added samples matched the human probe sets on the array, thus confirming sequences of equine and human corresponding genes. To minimize the risk of filtering out potentially relevant genes in the pathway a FC > 1.1 on mean values was used.

In Paper IV, the gene expression analysis was performed using Affymetrix® Rat Gene 2.0 ST Array. Expression data were normalized and summarized using the RMA algorithm implemented in the Affymetrix Expression Console version 1.1.2 software[195]. T-test analyses were performed on log2-transformed signal values to identify significantly differentially expressed genes between groups, using the TMEV v4.0 software. Expression differences were given as FC, and only significantly altered genes that displayed a mean FC of FC ≥ 1.5 or ≤ −1.5 were selected for further analysis.

3.7.2 Hierarchical clustering

In order to reduce the dimensionality and facilitate the interpretation of the microarray data, one can use different clustering systems to group samples with similar expression profiles. The purpose of clustering is to identify co-regulated and functionally related genes in large datasets. In paper I, the Genesis 1.7.2[196] software was applied to perform a hierarchical cluster analysis of genes related to the Wnt signaling pathway. The analysis was performed with log2-transformed signals normalized by genes and a Pearson correlation was used as distance measure.

3.7.3 Protein-protein interaction analysis

Protein-protein interactions (PPI) are fundamental to every biological process within the cells and the visualization of PPI networks helps to reveal vital biological processes. Signal transduction, i.e. the process where mechanical and/or chemical stimuli to a cell become converted into a defined cellular response, plays a fundamental role in many biological and disease mechanisms.

In order to explore possible interactions among proteins coded by differentially regulated genes and to identify hub proteins, the search tool for the retrieval of interacting genes/proteins (STRING) can be applied (http://string-db.org/). STRING is a database of known and predicted interactions[197], that include direct (physical) and indirect (functional) associations. These are derived from four sources: Genomic context, High-throughput experiments, Co-expression and Previous knowledge. In Paper I, the analysis was restricted to only experimentally determined protein interactions, excluding for example text mining for increasing the validity of the results. The PPI network were derived from differentially expressed genes retrieved from the entire data set displaying a mean FC ≥3 or ≤− 3 and as classified as signal transducers. A gene or protein of interest was classified as a hub if it displayed at least 5 interactions with other genes/proteins[198].

3.7.4 Pathway analysis

In Papers I and IV, the KEGG pathway database was searched using the DAVID software tools[190,191] in order to identify significant differently expressed pathways. All genes on the array fulfilling the specific criteria used in respective study were included in the gene list.

3.7.5 Functional annotations of differentially expressed genes

To further explore the functional properties of a group of differentially expressed genes, information from Gene Ontology[189] (GO) can be applied. A gene functional classification analysis evaluates the enrichment of GO annotations (i.e. terms describing the genes or gene products) and consists of three categories of annotation terms; Biological processes, Molecular functions and Cellular components. In Paper IV, the DAVID software tool was used for GO annotation enrichment analysis in order to understand more about the biological properties according to biological process of the differentially expressed genes.

3.8 Statistics

Different statistical tests were used in the present thesis for the assessment of significant differences between data. Specific tests were selected depending on the type of the data and the number of groups in the investigation. Non-parametric tests were used when a normal (Gauss) distribution of the data could not be guaranteed.

In Paper I, the significance level of the microarray data was determined using the Welch's T-test on log2-transformed signals. Statistical analysis of gene expression was performed using the Mann-Whitney U test (cartilage biopsies) and Wilcoxon paired signed-rank test (monolayer cultured cells).

In Paper II, data were first analyzed by two-way ANOVA with the variables time, stimulation and the interaction between the two variables. The observed statically significant variables (from ANOVA) were then analyzed using the post hoc Holm-Sidak method (multiple comparisons) to further identify group(s) that differed significantly within the variable. To further evaluate the effect of the cytokines, the logarithmic gene expression values for short and long-time stimulations were compared to unstimulated using pairwise T-test.

In Paper III, statistical significance was determined using one-way ANOVA followed by the post hoc Tukey's test. When normal distribution of the data

could not be guaranteed, the Mann-Whitney U test or Wilcoxon paired signed rank test was performed. Finally, the significance level of the microarray data in Paper IV was determined using Student's t-test (Paired; Li^+ vs. Ctrl and Un-paired; Li^+ day 28 vs. 7, Ctrl day 28 vs. 7). When normal distribution of the data could not be guaranteed, the Wilcoxon paired signed-rank test was used.

In all papers, logarithmic values of the gene expression data were used for the statistical calculations. In all studies, the statistical analyses were performed with SPSS v19 (IBM Corp., Armonk, NY, USA) or SigmaPlot 12.5 software and further also with GenEx (MultiD analyses) in Papers III and IV. Unless otherwise stated, data are expressed as mean ± standard deviation. P values < 0.05 (*) indicate statistically significant differences.

3.9 Ethical approvals

In this thesis, the donation of cartilage was approved by the ethical committees at the Medical Faculties at Gothenburg University (Dnr 040-01, Dnr 367-02) and Charité-Universitätsmedizin Berlin.

Ethical approval to conduct equine studies was given by the Ethical Committee on Animal experiments, Stockholm, Sweden (N283/10, N348/10).

Animal studies were performed with a prior received ethical approval by the University of Gothenburg Local Ethical Committee for Laboratory Animals (Dnr: 259-2011, 279-2011).

4 SUMMARY OF RESULTS

4.1 Paper I

In order to study the expression of Wnt related markers in human OA, articular cartilage biopsies from OA (graded by Ahlbäck and Mankin scores, as described earlier[199]) and healthy control donors (ND) were subjected to genome wide microarray analysis. Using this approach, we showed that several genes in the Wnt signaling pathway displayed a significantly altered expression in OA compared to normal cartilage. Both the KEGG pathway analysis and the hierarchical cluster analysis demonstrated that the Wnt signaling pathway was significantly dysregulated in OA compared to ND articular cartilage, see Table 1 and Figure 1 in Paper I. The gene expression of several inhibitors of both the canonical and the non-canonical PCP Wnt signaling pathways were significantly increased in OA cartilage, whereas the non-canonical Wnt5a/Ca^{2+} pathway was activated.

More in detail, the expression of the canonical extracellular inhibitors DKK1, DKK3, WIF1, SFRP1, and SFRP4 were significantly upregulated in OA. Other signs revealing the canonical Wnt pathway as negatively affected at several levels of the transducing cascade were the increased expression of intracellular inhibitors involved in phosphorylation and destruction of β-catenin, such as AXIN2, SOX17, and TAXIBP3. In addition, also intranuclear inhibitors of the canonical pathway displayed increased expression in OA, e.g. TLE4. Further, the expression of the transcription factor TCF7L2 was decreased. With regard to markers of the PCP pathway, Paper I demonstrated increased expression in OA cartilage of the gene encoding the transmembrane protein VANGL1 and decreased expression of DAAM2, PRICKLE2, and DVL2, encoding cytoplasmatic proteins. Increased expression of WNT5A, CAMKII, NFAT5 and NFATC2 involved in the Wnt5a/Ca^{2+} pathway were revealed in OA cartilage, as seen in Figure 2 and Table 2, Paper I. The microarray results were verified by qPCR and IHC, demonstrating abundant protein expression in OA cartilage of AXIN2, DKK1, DKK3 and WNT5A (Table 3 and Figure 3). Finally, when investigating the effect of IL-1β stimulation in relation to Wnt related markers, an increased expression of WNT5A, FOSL1, SFRP4 was induced by the cytokine in chondrocytes obtained from both OA and ND cartilage, as shown in Figure 5 in Paper I.

4.2 Paper II

The effect of IL-1β and IL-6 stimulation on GDF-5 in equine chondrocytes was investigated in Paper II, and demonstrated a significantly lower GDF-5 gene expression upon stimulation with IL-1β compared to unstimulated controls. In addition, the GDF-5 expression in IL-6 stimulated pellets was significantly increased compared to IL-1β treatment. The effect of cytokine stimulation was further investigated at each time point and showed a significantly higher GDF-5 gene expression in chondrocyte pellets stimulated with IL-6 for 2 hours than control, as seen in Figure 1, Paper II. Microarray analysis was subsequently used to examine possible affected signaling pathways during short-time IL-6 stimulation (1-2 h). We revealed increased expression of GSK-3β and coiled-coil domain containing 88C (CCDC88C) after 1 hour and a decreased expression of β-catenin at the later time point, for more general information, see Table 1 in Paper II.

IHC staining of GDF-5 in macroscopically normal articular equine cartilage was observed in the superficial and upper middle zones, whereas the GDF-5 staining pattern in OCFs (osteochondral fragments) was observed in the middle and deep parts of the articular cartilage (Figure 2, Paper II). IHC reaction for nuclear dephosphorylated β-catenin was prominent in pellets stimulated with IL-1β, but only few β-catenin positive cells were observed in IL-6 stimulated pellets, similar to unstimulated control. The cellular immunostaining for GSK-3β was positive in IL-6 simulated pellets, visual only in a few cells of IL-1β treated or non-treated pellets (Figures 3 and 4, Paper II).

4.3 Paper III

HA-coated Ti screws with or without Li^+ (designated HA, HA0.5Li, and HA5Li according to the LiCl content of the soaking buffer) were inserted into rat tibia for 7 or 28 days, and the effect on the Wnt signaling pathway was investigated. The implants displayed a sustained release of Li^+ *in vitro*, with an initial burst release (Supplementary Figure S2C in Paper III).

Using qualitative histology *ex vivo* we revealed an early surface-specific induced bone response with deposits of newly formed bone, that showed a slightly increased contact-osteogenesis around HA-coated implants (irrespective of Li^+ content), as seen in Figures 1 and 2, Paper III. The presence of periostin positive cells was observed throughout the regenerated bone, especially in bone lining cells, whereas immunoreactivity for calcitonin receptor was observed both on mononuclear and multinucleated cells

(Supplementary Figure S3). The HA-coated implants showed, irrespective of Li^+ content, a significantly higher BIC and RTQ values than uncoated Ti control. On the contrary, the uncoated Ti demonstrated significantly increased larger BA than HA5Li after 28 days, as seen in Figures 3 and 4 in Paper III. SEM analysis demonstrated larger direct bone-HA implant contact than compared to that between bone and uncoated Ti, and TEM analysis revealed a well osseointegrated HA implant (Figure 5 and Supplementary Figure S4, Paper III).

At the early time point, the gene expression of OCN, CTSK, COL1A1, LRP5/6 and WISP1 were significantly higher in implant-adherent cells from all HA coatings (with and without Li^+), than compared to Ti. OCN and LRP5 showed the same expression profile at the later time point. COL2A1 displayed a significantly decreased expression in peri-implant bone from HA0.5Li compared to Ti, 7 days after implantation (Figures 6 and 7, Paper III). The protein expression of β-catenin was significantly higher in HA0.5Li peri-implant bone than in Ti at the first time point, and similar expression was revealed after 28 days, as seen in Figure 8 in Paper III. In summary, the results revealed an affected canonical Wnt pathway by HA coated implants.

4.4 Paper IV

Using an approach similar to that in Paper III, Li^+ was incorporated into PLGA plug-shaped implants and subsequently inserted in rat tibia for 7 or 28 days. We used global gene expression profiling by microarray analysis, with a special attention given to the Wnt signaling pathway. *In vitro* Li^+ release profile analysis showed a sustained, almost linear release pattern, as seen in Figure 3 in Paper IV.

The microarray analysis revealed a large number of significantly differentially regulated genes over time, within the two implant groups (Li^+ and Ctrl), see Table 1, Paper IV. Functional cluster analysis based on biological process showed that both Li^+ and Ctrl implants were related to e.g. skeletal system and blood vessel development. Further, Ctrl implants displayed clusters related to bone formation and ossification, whereas the Li^+ group was associated with cartilage development and condensation (Supporting information Tables S5 and S7, Paper IV). KEGG pathway analysis showed that the Wnt signaling pathway was associated with the Li^+ containing implants, but not with the Ctrl group, as seen in Tables 4 and 6 in Paper IV. Further analysis revealed that the Wnt signaling pathway was significantly affected in the bone retrieved around Li^+ containing implants, with ~ 34% of all known Wnt-associated markers regulated over time,

compared to ~ 22% for Ctrl implants (Table 7, Paper IV). The result obtained from the microarray analysis was verified by qPCR analysis as seen in Table 8, Paper IV. The mRNA corresponding to FOSL1 and ASPN was significantly increased in cells retrieved from the Li^+ group compared to the Ctrl (Figure 4, Paper IV).

Additional IHC verification demonstrated the expression of FOSL1 and ASPN in the presence both Li^+ and Ctrl implants. Positive stainings were observed in periosteum, bone marrow cavities and on cells at the bone-implant interface. Also the spatial localization of β-catenin was monitored and demonstrated similar expression patterns, as seen in Figure 5 in Paper IV. Both implant groups displayed significantly decreased bone area over time (Figure 6, Paper IV), and both periostin and calcitonin receptor positive cells were observed throughout the tissue as well at the bone-implant interface, Figure 7 in Paper IV. Multinucleated cells that were occasionally negative for all the above mentioned markers were observed at the bone-implant interface, seen in Figures 5 and 7 in Paper IV.

5 GENERAL DISCUSSION

This section provides a general discussion on how the results from the studies within this thesis compares to results from similar studies, and possible implications thereof.

5.1 Wnt related markers expressed in OA cartilage

The canonical Wnt pathway has been implicated in the pathogenesis of OA[61,85,86], and expression profiling of OA affected subchondral bone has revealed an altered Wnt signaling pathway[94]. However, the relative shortage of knowledge regarding cellular expression profiles of Wnt associated markers in OA prompted us to more comprehensively investigate the expression profile of Wnt markers in human OA cartilage, using genome-wide microarray. We have earlier reported the results from a broad gene expression comparison of ND and OA cartilage, and focused here on the Wnt related markers[199]. The approach used in Paper I resulted in the discovery of a group of Wnt related genes, not previously associated with OA, displaying significantly altered expression in OA compared to ND human articular cartilage. The main finding of Paper I was the discovery of a panel of canonical inhibitors displaying significantly increased expression in OA cartilage. Similar results were shown for the PCP pathway.

Regarding the canonical Wnt pathway the expression of several extracellular, intracellular and intranuclear inhibitors[64,200,201] were increased in OA cartilage, suggesting that this pathway was negatively affected at continuous stages of the signaling transduction. This may be somewhat contradictory when comparing several other studies implicating increased levels of β-catenin, i.e. an activated canonical pathway in OA cartilage[22,61,85,86]. Nevertheless, the expression of β-catenin was not further elucidated within Paper I, but our findings demonstrated increased expression in OA cartilage of genes encoding proteins involved in β-catenin phosphorylation and destruction[200-204]. This suggests that the activity/stability of β-catenin was affected in OA cartilage. In addition, the expression of the transcription factor TCF7L2[67,70] was decreased in OA cartilage, further indicating a suppressed transcription of downstream canonical Wnt genes.

We also demonstrated increased expression of the non-canonical ligand WNT5A and several of its downstream mediators[64,205-209], suggesting an

active Wnt5a/Ca^{2+} pathway. These findings are in line with a study performed by Zhu *et al.*[86] demonstrating increased expression of the ligand WNT5A in human OA cartilage (and increased protein expression of β-catenin). The authors also suggested that the Wnt5a/Ca^{2+} pathway was upregulated via β-catenin. It is also recognized that WNT5A has dual roles *in vivo*. WNT5A can both activate or inhibit the canonical pathway, depending on factors such as available receptors, time and site of expression[210]. Hence, this could to some extent explain our findings, though we cannot rule out the possibility that the signaling downstream β-catenin was affected by other compensation mechanism than those revealed within the present microarray analysis, thus suggesting that the canonical Wnt pathway is partly inhibited in OA cartilage.

WNT5A has earlier been shown to negatively regulate type II collagen expression and is also involved in the regulation of MMPs[87,89]. Further in line with the dual roles of WNT5A, a recent study by Hosseini-Farahabadi *et al.* revealed that WNT5A seems to keep the activity of the canonical Wnt pathway low during the development, thus promoting chondrogenesis, but in conditions of excess, WNT5A seemed rather to induce an unexpected rapid loss of the cartilage matrix by induction of metalloproteinase and aggrecanase enzymes[211]. On the other hand, our analysis revealed increased expression of the Wnt/β-catenin target gene FOSL1[212,213] in OA cartilage, thus implicating increased canonical Wnt signaling. This is not in accordance with our results obtained from the microarray analysis demonstrating increased expression of canonical Wnt signaling inhibitors at several levels along the signal pathway. However, this could hence indicate that the canonical Wnt pathway was partly inhibited. Moreover, FOSL1 (also referred to as FRA1) is known to interact with NFATC2, a transcriptional target of WNT5A, to form the transcriptional AP-1/NFAT complex[206,214]. Hence, functioning also as a target gene of the non-canonical Wnt5a/Ca^{2+} pathway, and definitely introducing even more complexity to the Wnt signaling pathways network.

The Wnt5a/Ca^{2+} pathway was activated supported by our results with increased expression of e.g. WNT5A, NFATC2, CAMKII in OA cartilage, and this might explain the increased expression of FOSL1. Finally, the FOSL1/AP-1 complex is further implicated in MMP1 regulation[215] and increased bone mass[216], both of which are characteristics of OA pathogenesis. However, the AP-1 genes is also involved in tumors and inflammatory response[217,218], thus the increased FOSL1 expression in OA cartilage does not unanimous indicate Wnt signaling association.

5.2 Wnt markers affected by IL-1β and IL-6 stimulation

In Papers I and II, we stimulated chondrocytes with IL-1β and/or IL-6. The IL-1β stimulation led to increased expression of several Wnt related markers, partly explaining their increased expression in OA. Previously, other members within the Wnt signaling pathways have been shown to display an upregulated expression associated with IL-1β stimulation[87-89], and this cytokine may induce Wnt signaling by increasing the expression of β-catenin[219].

In Paper I, the gene expression of IL-1β was significantly increased in OA compared to healthy cartilage (microarray analysis), in line with several previous studies[21,22,220]. Further, the pro-inflammatory IL-6 is shown to be overexpressed in OA tissue and would therefore be considered as a potential contributing factor in the pathogenesis of the disease. Nevertheless, IL-6 has also showed anti-inflammatory properties, thus proposing a protective role in OA cartilage[19,23]. With the recognized activation of the canonical Wnt signaling pathway in OA cartilage in mind[61,66], similar conclusions can be drawn from our analysis demonstrating reduced expression of several Wnt related markers after short-time stimulation with IL-6. Finally, the IL-6 receptor was downregulated in OA cartilage (Paper I), and other unpublished data from our group indicate that IL-6 stimulation not only reduced the expression of canonical Wnt associated genes (Paper II), but also the expression of non-canonical Wnt5a/Ca^{2+} mediators such as CAMKII (data not shown). Together this could indicate an impaired cartilage healing in severe OA due to a decreased IL-6 responsiveness, but also an attempt to repair by suppressing, not only the canonical but also the Wnt5a/Ca^{2+} pathway (as seen activated in OA cartilage, Paper I) via IL-6 mediated mechanisms.

5.3 Cytokine stimulation regulating GDF-5 – a possible Wnt association

GDF-5 (also known as BMP14) is expressed in the early developing joint interzone, and it has multiple functions during cartilage and joint development. Mutations in this gene can lead to defects in the developing skeleton[221]. GDF-5 is further recognized for a role in bone formation during embryogenesis as well as joint maintenance and repair[222-227]. In addition, GDF-5 stimulates chondrocyte metabolic activities and chondrogenesis, and it has been shown to be expressed in both healthy and OA joints as well as

bone[222,228,229]. However, what role GDF-5 plays in OA and how GDF-5 affects other signaling pathways in cartilage is still unknown.

In Paper II, we demonstrated that *in vitro* IL-1β stimulated pellets displayed a significantly decreased expression of GDF-5 compared to the unstimulated control, as well as an increased β-catenin protein expression. These results are in line with other findings demonstrating a strong downregulation of GDF-5 in RA human chondrocytes stimulated with IL-1β[230]. Taking the many studies that link IL-1β to OA as well as the joint associated GDF-5 into account; it is not unlikely that the degenerative features in OA pathogenesis could be linked to decreased expression of GDF-5. In addition, SOX9 expression has been shown to be decreased by IL-1β stimulation and in contrast, to be increased by IL-6 stimulation[165]. This indicates anti-chondrogenic effects of IL-1β and strengthens the indications about a possible pro-chondrogenic role of IL-6 (as discussed above). As showed in Paper II, IL-6 stimulation significantly increased the expression of GDF-5.

To continue the discussion regarding the proposed cartilage protective role of IL-6 by reducing Wnt signaling; contradictory roles of the Wnt signaling associated regulation of GDF-5 in the joint interzone have been reported in literature[63,231], and SMAD and canonical Wnt signaling have been suggested to inhibit each other[232]. Further reports on SMAD/BMP pathway cross-talk with Wnt signaling exists[233,234], and was also indicated in Paper II. Hence, it is possible that enhanced GDF-5 expression attributes to downregulation of the canonical Wnt signaling pathway as induced by IL-6. A recent finding within our group has revealed that GDF-5 stimulation increased gene expression of the canonical inhibitors DKK1 and FRZB (*in manuscript*). This was verified by increased protein expression of DKK1 and subsequently decreased protein expression of β-catenin, as well as decreased gene expression of the canonical Wnt targets FOSL1 and PPARD. Thus, in the light of Paper II, this strengthens the suggested cartilage protective role of IL-6 in the context of increased GDF-5 expression.

5.4 Potential drug targets/pharmacological treatments for OA

There exist presently no approved therapy that can modify the structural degradation of the cartilage associated with the progression of OA. Consequently, there is a need for effective prevention at an early stage but since the pathogenesis of the disease is multifaceted and incompletely understood, this complicates matters[19,26]. As described in the introduction,

several OA susceptibility associated polymorphism markers such as Wnt-related genes and GDF-5 have been extensively evaluated in order to discover new potential candidate genes and molecular targets[27-29,31-33]. Given the large increase in of WNT5A expression in human articular OA cartilage (Paper I) together with its recognized regulation of type II collagen and MMPs[87,89], it may serve as an attractive target. In line with this, conditions with excess WNT5A have been reported to induce a rapid loss of the cartilage matrix by induction of MMPs and aggrecanase enzymes[211].

Further, mutations in the GDF-5 gene resulted in abnormal joint development and is associated with development of OA[235]. We showed in Paper II that IL-6 might inhibit the canonical Wnt signaling via GDF-5. Summarizing with these results in mind, WNT5A and its effector molecules could serve as potential drug targets in order to inhibit cartilage structural degradation. For the same purpose, GDF-5 and/or IL-6 could be considered as pharmacological treatments supporting anabolic cartilage healing in a disturbed homeostasis such as seen in OA.

5.5 Affected Wnt genes during bone regeneration around HA implants

In contrast to the recognized negative effects of canonical Wnt signaling in degenerative cartilage, an active canonical Wnt signaling cascade would be beneficial for improvement of new bone regeneration. The osteoinductive properties of HA are well documented[118,119], although scientists still lack a fundamental understanding of the biological mechanisms underlying this phenomena[118]. Several *in vitro* studies have evaluated the osteoinductivity of CaP minerals by monitoring the expression of osteogenic differentiation markers such as ALP, OCN and COL1A2[236-239]. *In vitro* gene expression profiling of MSCs cultured on natural HA was reported by Lu *et al.* demonstrating that Wnt signaling was influenced by the presence of the material[240]. *In vivo* evaluations at gene level are less prevalent, although microarray analysis has demonstrated that signaling pathways related to β-catenin are indeed affected by HA/Collagen scaffolds after implantation in mice[241]. Yang *et al.* described recently enhanced β-catenin staining around HA scaffolds over time *in vivo* (although not to the same extent as HA-Sr (strontium) scaffolds)[242]. Further, given the essential involvement of the Wnt signaling pathway during bone development, repair and regeneration, it would be most likely to assume that the pathway was affected by the HA coatings, since it is so similar to natural HA in bone.

A more specific analysis of Wnt related markers in Paper III revealed increased protein expression of β-catenin in combination with increased expression of LRP5/6 and WISP1, indicating that the canonical Wnt signaling pathway was significantly affected by HA coatings (at several stages along with the signal transduction cascade). Given the osteogenic potentiating role of these markers[100-103,107,243] this may at least to some extent explain the osteoinductive properties of HA and may somewhat clarify clues involved in the phenomena by which they induce bone formation. Of course, we cannot rule out the possibility of other interacting or compensatory mechanisms.

In paper III, the non-canonical transcription factor NFATC2[207] showed an reduced gene expression around HA implants. NFATC2 has also been implicated as an inhibitor of osteoblast function[244], and since the canonical and non-canonical Wnt pathways are believed to cross-react with each other[245], it is possible that the expression of the non-canonical transcription factor was reduced due to an active canonical signaling, which is in line with the increased expression of the LRP5/6 and WISP1 genes as mentioned above.

5.6 Translational effects of Wnt related genes

The transcriptional effects of IL-6 or Li^+ stimulation in Papers II and IV on Wnt related genes were small in magnitude. When evaluating the gene expression profile during fracture healing with different implant surfaces, it was revealed in one study that the largest number of significantly differentially regulated genes was more connected to time of implantation than different topographies[246]. However, given the complex picture of bone regeneration this is not a surprise[113,114,247], and minor alterations induced by various stimuli are most likely concealed within the powerful processes of bone healing, growth and remodeling. It would be fatal to neglect low transcriptional changes since one can not rule out the possibility that gene expression differences demonstrating low FC, in fact lead to a significant biological outcome when they occur for set of related genes that participate in the same biological process[192].

In accordance with this, our findings demonstrated considerable proportions of affected genes within the same biological process. The Wnt signaling cascade is a well conserved, essential pathway in osteogenesis and it is recognized that even small changes in amplitude and duration of numerous

Wnt-related markers can regulate the entire pathway[62]. As nicely showed by Goentoro *et al*., FC rather than absolute levels of β-catenin is essential for its regulatory function, indicating that even low levels of this protein can be sufficient for the induction of transcriptional changes[72]. Taken as a whole, this also exemplifies the importance of subtle alterations in gene expression. On the contrary, some of the differentially expressed Wnt genes in OA compared to ND in Paper I, displayed relatively large magnitudes. Perhaps this is a reflection of the more powerful dysregulated processes leading to macroscopic observed cartilage degeneration as seen in OA.

5.7 Li^+ in implant surfaces

Oral Li^+ treatment for mood disorders has been widely used in humans for over a half-century, although the adverse effect of systemic Li^+ intake continues to be a major concern. Earlier studies have showed the positive effects of a systemic Li^+ administration in relation to enhanced bone mass, reduced fracture risk and increased fracture repair[141,151,152,154,155]. Due to Li^+ narrow therapeutic window[149], trials with local administration could be useful to improve the local bone regeneration via modulation of the Wnt signaling pathway.

In line with this, incorporation of Li^+ into various biomaterials or added directly to cell culture media have been performed, analyzing the Wnt signaling activation and osteogenic differentiation[139,156-158]. Further *in vitro* evaluation with cells cultured on Li^+ containing mesoporous bioglass (MBGS) scaffolds and electrolytically deposited CaP/Li have been reported[156,157]. The amount of released Li^+ was reported to depend on the amount of Li^+ incorporated in the first place, displaying a burst release and further confirmed in Paper III. Overall, high Li^+ concentration can result in potential cytoxicity, and hence low concentrations of Li^+ were used in several studies[156,157]. Also we used relatively low Li^+ concentrations. The chemical incorporation of Li^+ into the CaP lattice was discussed earlier[157], and the observed burst release is most likely explained by the fact that Li^+ is not a part of the crystal lattice. Moreover, it has been shown that relatively low amounts of Li^+ can inhibit apatite mineralization of biomaterials[248]. With these issues in mind, the Li^+ content was limited by the present coating-generating medium in Paper III. Hence, increased ionic concentration in the solution would most likely have generated lithium phosphate instead of HA.

Various Li^+ containing coatings or implant materials will give rise to different release profiles and subsequent biological output. PLGA is frequently used as a controlled drug release delivery matrix within

pharmaceutics and increasingly within the field of bone regeneration. Reports have demonstrated encapsulation of drugs, low molecular weight compounds and recombinant proteins into the polymer[121,249], whereas ionic incorporation seems to be less prevalent. In terms of small ions such as Li^+, the release profile from PLGA can be more controlled than from HA; much depending on an overall increased incorporation into the delivery system, and thus not restricted to the implant surface. Further, in Paper IV the rate-limiting step for Li^+ release seemed to be the water penetration rate, hence different from that observed in Paper III. When comparing Papers III and IV in detail, the amount of *in vitro* released Li^+ was approximately 1000 times higher from PLGA implants than from HA coated implants. With respect to the uniqueness of the bone environment, the mode of Li^+ release *in vivo* is expected to diverge from that of a fully submerged implant in buffer. However, assuming for a moment of an equal *in vitro* and *in vivo* release rate, this would of course introduce major different impacts on the biological outcome.

5.8 Modulation of Wnt pathway by Li^+ release

Previous reports have focused on the *in vitro* evaluation of the biological effects induced by Li^+ in biomaterials, whereas the *in vivo* aspects of Li^+ administration have been through systemic delivery[141,151,152,154,155]. In Papers III and IV, the expression of Wnt related markers was evaluated in Li^+-containing-implant-adherent cells and/or cells retrieved from per-implant bone tissue *ex vivo*. Further, Han *et al.* found that incorporation of 5% Li^+ into MBGS (Li/Ca/P/Si = 5/10/5/80, molar ratio) enhanced cell proliferation and differentiation with increased expression of bone markers such ALP, OPN and OCN *in vitro*[156]. Wnt activation was shown by increased gene expression of AXIN2 and β-catenin. However, this study did not further elucidate the protein expression of β-catenin that is the factual regulator of transducing the canonical Wnt cascade signal.

In Papers III and IV, we demonstrated protein expression of β-catenin expression in the context of bone-anchored implants. A significantly increased β-catenin expression was in our study induced by Li^+-containing HA implants compared to Ti, but no specific effect of Li^+ was observed. One possible explanation to the non-significant effects of Li^+ was the low amount of Li^+ in the HA coatings as discussed earlier. In addition, the superior osteoindictivity displayed by HA most likely overshadowed the Wnt modulating effect and potential subsequent effect on improved bone

regeneration by Li^+. Hence, HA is not optimal as a Li^+ delivery system when monitoring the Li^+/Wnt pathway in relation to the field of bone healing around bone anchored implants.

In Paper IV, the IHC spatial staining pattern of β-catenin positive cells was observed in tissue sections harboring osteoblast progenitor cells and in the bone-implant interface. In this study, the gene expression profile of Wnt associated markers was more deeply investigated and showed that the Wnt signaling pathway was significantly affected by the Li-containing implants, demonstrating 34% of all Wnt genes as affected (compared to 22% for Ctrl implants without Li^+). Li^+ in the implants increased gene expression of the FOSL1 and ASPN, genes that have been recognized in regulation of bone formation and osteoblast biomineralization[216,250,251]. Both FOSL1 and ASPN have been associated with OA (Paper I and[32,252]), perhaps in relation to the increased subchondral thickness seen in the disease pathogenesis, and underlines their essential functions in bone regeneration. FOSL1 is mostly regarded as a canonical Wnt target gene, participating in the AP-1 complex and also involved in non-canonical signaling[206,214], as discussed earlier. Thus, the complexity of the Wnt signaling pathways is intriguing. However, given the use of Li^+ in the rat fracture model and the role of FOSL1 in fracture healing, it is likely to assume a relation between FOSL1 expression in Paper IV and the canonical Wnt signaling. In line with our study, Heo *et al.* reported an increase in FRA1 gene expression in cells stimulated with Li^+[253]. In addition, links exist between the extracellular canonical Wnt inhibitor sclerostin, secreted by osteocytes[98,254,255], and ASPN[256]. Hence, it is possible that increased ASPN expression induced by Li^+ is related to active Wnt signaling caused by the local Li^+ administration, although an extended evaluation of this assumption is needed.

Nevertheless, although an activation of the Wnt pathway by Li^+ in implants was observed, no effect of this in relation to increased bone regeneration/healing was seen. However, concluding the current understanding within the field, it is clear that a proper timing of Li^+ administration is tantamount for the analysis of Wnt targeted therapeutics and possibilities of Wnt regulation to become of clinical relevance in fracture repair[95,112,113,154].

6 CONCLUSIONS

In Paper I, the genome wide expression profiling of human articular OA cartilage revealed a partly inhibited canonical Wnt pathway, whereas the expression of several members within the non-canonical Wnt5a/Ca^{2+} pathway was increased. The increased expression of several Wnt related markers in OA was mediated by the pro-inflammatory cytokine IL-1β. These results shed further light on WNT5A and its downstream effector molecules during the degenerative processes of OA, indicating their putative roles as drug targets. In Paper II, the effect of the pro-inflammatory cytokines IL-1β and IL-6 was monitored in the context of GDF-5 and the canonical Wnt signaling pathway in articular cartilage. IL-1β stimulation confirmed the recognized activation of the canonical Wnt pathway, whereas the suggested cartilage protective function of IL-6 was indicated by reduced expression of canonical Wnt related markers. IL-6 stimulation also resulted in increased GDF-5 expression, enabling a potential regulatory role of IL-6 via inhibition of the canonical Wnt cascade with subsequent upregulation of GDF-5. These results further enhance the suggested role of IL-6/GDF5 in cartilage degeneration as a prospective disease modifying treatment.

In Paper III, Li^+ was included into HA coated implants and the expression of Wnt related markers was monitored in a rat tibia model. The results showed no significant effect of Li^+ alone, although the osteoinductive properties of HA were mediated through increased expression of the Wnt related markers β-catenin, LRP5/6 and WISP1. The superior bone inducing ability of HA likely overshadowed the Wnt activation effect of Li^+. In Paper IV, Li^+ was delivered to rat tibia by biodegradable PLGA, displaying a controlled Li^+ release that activated the canonical Wnt pathway. 34% of all Wnt related genes were affected by Li^+, and increased expression of FOSL1 and ASPN was shown. In spite of the significant effect of Li^+ on Wnt signaling, no improved bone regeneration around the implants was observed.

To conclude, this thesis tackles the involvement of the Wnt signaling pathway in cartilage and bone regeneration, and reflects upon its double-edged impact in various tissues and homeostasis. The OA pathogenesis is multifactorial and there is no single marker to target that prevents the degenerative processes, thus instead a combined understanding is indispensable. However, modifications of evolutionarily conserved systems such as Wnt signaling is not straightforward, exhibiting issues such as proper delivery systems to be resolved prior to becoming of therapeutic significance. This thesis has hopefully, through the discovered results, contributed to increased knowledge that could help improving Wnt signaling associated osteochondral therapies.

7 FUTURE PERSPECTIVES

The increasing knowledge of the molecular mechanisms that underlie structural cartilage degeneration is fundamental for the improvement of existing therapies and to develop new ones. The same is true for bone regeneration and healing around bone-anchored implants; without basic knowledge progression is not possible. This thesis has shed some light on the involvement of the Wnt signaling pathway in the unbalanced homeostasis in cartilage resulting in OA. However, the knowledge is still incomplete and improved therapies is an inevitable must to tackle this multifactorial disease that affects the health in especially elderly people worldwide. Tissue regeneration mimics largely embryonic processes, and this thesis has showed that the well conserved Wnt pathways were dysregulated in OA cartilage.

WNT5A has emerged as a putative drug target, although the clinical safety needs to be addressed to avoid negative effects in other integrated signaling systems. WNT5A gene knockout or gene silencing in OA animal models would be a first step to further investigate its potential role in the disease pathogenesis. Alternatively, the use of scaffolds with slow release of WNT5A neutralizing antibodies could be one option to suppress the excess of WNT5A locally in the OA joint.

Further, the same could be applied for a local delivery of IL-6, possibly acting as an inhibitor of increased expression of β-catenin recognized in OA and simultaneously exerting its cartilage protective anabolic properties. In this thesis, a special attention was given to the canonical Wnt pathway in relation to IL-6 stimulation. More specific investigations regarding its effect on the non-canonical Wnt5a/Ca^{2+} pathway could reveal additional roles applicable in OA therapies.

In order to be able to use Li^{+} as a positive Wnt modulator to enhance implant-bone integration and bone fracture repair, the choice of delivery systems need to be thoroughly optimized. Due to its cost-effectiveness and wide usage in human therapies, Li^{+} has the potential to develop into a fracture treatment, although a proper timing and Li^{+} dose are crucial for clinical use. We have showed a local activation of the canonical Wnt pathway by Li^{+} releasing implants. But in order to transfer the beneficial effects on bone regeneration seen in systemic administration to the locally applied delivery, a delivery system that does not lead to substantial positive or negative effects on the bone healing by itself would be ideal. This could be achieved by study the effects of Li^{+} in a fracture model without bone-anchored implants such as critical-size calvarial or long bone defects perhaps using e.g. Ca/P granules with or without the combination of PLGA.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone who has contributed to this thesis. Special thanks to:

Pentti Tengvall, my main supervisor**,** for giving me the opportunity and guiding me through the world of biomaterials. For your encouragement and always being available, giving me quick feedback throughout the years. For giving me the creative freedom to evolve - I have truly learnt a lot!

Peter Thomsen, co-supervisor, for your encouragement, fruitful discussions and sharing your histological expertise at the microscope.

Anders Lindahl, co-supervisor, for your always positive and encouraging personality. For valuable suggestions, fruitful discussions and sharing your biological expertise.

Karin Ekström, co-supervisor, for your encouragement, fruitful and valuable input. For carefully proofreading of my thesis.

All my co-authors, and all former and present colleagues at the Department of Biomaterials, Department of Clinical Chemistry and Transfusion Medicine, and the BIOMATCELL project. Thanks for sharing your knowledge - giving me the opportunity improving mine, especially **Camilla Karlsson**, for your guidance and help introducing me to the scientific world in the beginning of my doctoral studies, **Anders Palmquist**, for encouragement, sharing your knowledge and introducing me to the mysterious field of electron microscopy, **Wei Xia** for a nice collaboration and explaining hydroxyapatite to me, **Carl Lindahl** for helping me with the coating process and always being enthusiastic about our project, **Lena Emanuelsson**, **Birgitta Norlindh** and **Kazyou** for sharing your surgical excellent skills, teaching me to handle our little friends at EBM, **Taka** for excellent skills in photography and your friendly personality, **Anna Johansson** for valuable help in the cell culture room, **Mats Brittberg** for sharing your Wnt knowledge, being available and giving me quick feedback, **Eva Skiöldebrand** for sharing your expertise about horses and inflammation, for fruitful discussions and encouragement, **Stina Ekman** for a nice collaboration and sharing your histological expertise, **Per Borchardt** for excellent TOF-SIMS analyses, **Maria Lennerås** for sharing your qPCR knowledge and laughs, **Omar Omar** for interesting discussions, **Camilla Brantsing** for sharing your knowledge about qPCR and molecular biology, **Narmin Bigdeli** for help with antibodies and great music in the cell culture room, **Helena Barreto Henriksson**

for sharing antibodies and IHC protocols, **Marianne Jonsson** for always being available and sharing your qPCR expertise, **Pia Andersson**, **Catherine Concaro** and **Magret Hagman** for fun discussions in the lunch room and showing interest in my work, **Vivien Lettry** for interesting discussions and always being happy, **Josefin van der Lee** for sharing your cell culturing knowledge, being a great conference companion with lots of energy, **Kristina Vukušić** for nice discussions and help with cells, **Henrik Svensson** for being a nice room- and classmate, making statistics quite fun actually, **Julia Asp**, **Stina Simonsson** and **Ruth Wickelgren** for scientific input and discussions, **Joakim Sandstedt** for fun discussions and your excellent homemade meatballs, **Johan Stenberg** and **Cecilia Granéli** for your encouragement and company in the cell culture room throughout the years, **Lars Enochson** for your encouragement, shared laughs and cinnamon buns, your 24/7 online support.

Thanks to **Emilia Svala** and **Marie Leander** for your dear friendship, invaluable encouragement and support - it would not have been the same without you!

Thanks to **Jane Synnergren** at the System Biology Research Center, University of Skövde, for your truly endless support and advice regarding the complex microarrays. Thanks to **Anette Larsson** and **Anna Bergstrand** at Department of Chemical and Biological Engineering, Chalmers, for a nice collaboration, providing me with implants and for sharing your time and expertise.

Thanks to **Tom Nielsen,** principal teacher Hornavan gymnasium, for your encouragement and faith in me as a young student, **Björn Eriksson,** biology teacher Hornavan gymnasium, for opening the amazing world of biology to me.

Above all, my most humble thanks to my beloved family and all my dear friends, especially **Anette** and **Olle,** for your excellent dinners and hospitality, giving me the opportunity to relax in Skummeslövstrand.

My godmother **Eta,** for your support and encouragement, good advice and teaching me about Buddha. My dear sister **Caroline**, for just being you, your sisterly patronage and letting me be part of your lovely family; **Tom**, **Theo** and **Valle**.

Mina älskade föräldrar, för allt Ert stöd och uppmuntran genom åren - Mamma **Marianne** för din avsmittande imponerande kämparglöd, Pappa **Jan** för din guidning i livet så väl som på fjället.

Johan, for all your love, patience and understanding - coaching me through my life's toughest wrestling game.

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