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THE ROLE OF THE MICROENVIRONMENT IN GROWTH PLATE AND ARTICULAR CHONDROCYTE DIFFERENTIATION

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The Role of the Microenvironment in Growth Plate and Articular Chondrocyte Differentiation

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“I've spent more time than many will believe [making microscopic observations], but I've done them with joy, and I've taken no notice those who have said why take so much trouble and what good is it?”

- Antonie van Leeuwenhoek

To my beloved Family

POPULAR SCIENCE SUMMARY

Degeneration of articular cartilage from disease or trauma is a common cause of pain and physical disability and therefore cause a lot of suffering in the affected individuals, which also leads to a high burden to the society and global health. Currently, there is no efficacious therapy that can reverse the degenerative process or to engineer new articular cartilage that can replace the degenerated cartilage. The main treatment option is to replace the degenerated joint with a matching prosthesis. Some recent findings indicate that endogenous skeletal stem cells have the potential to regenerate high-quality articular cartilage when the local microenvironment is specially modified. Therefore, a more comprehensive understanding about the regulation of articular cartilage formation and maintenance is needed. Specific questions this thesis trying to answer are (i) What are the optimal conditions for the methods used to study mRNA and protein expression on bone sections? (ii) Does the synovial joint microenvironment inhibit endochondral bone formation and promote articular cartilage formation? (iii) Finally, if and how much do the transplanted perichondrium cells contribute to the regenerated articular surface after reconstruction of injured articular cartilage with perichondrium transplants?

To address the first question, based on previous literature reports and our own experience with the methods, we designed a study to systematically test a selected set of critical parameters for each of the methods. The findings helped us establish optimized protocols that consistently detected the targeted mRNAs and proteins with high sensitivity and specificity. This was critical to subsequent studies.

In order to address the role of the local microenvironment for the formation and maintenance of articular cartilage, we developed an animal surgery model to exchange the locations of articular and growth plate cartilage and traced the transplanted cells to see how they behave. We found that, in growth plate cartilage ectopically placed at the articular surface the most superficial layers of cells gradually become similar to those in their neighboring native articular cartilage, while the articular chondrocytes ectopically put into epiphysis gradually lose their "articular" appearance. This indicated that certain factor(s) present in synovial fluid might help to maintain the "articular" character. What is the factor behind promoting such a cellular fate shift? With the aim to find the possible answer we cultured cartilage cell pellets with conditioned media fractions some of which may contain what we want, to see how these cell pellets behave. With the information revealed by these pellets we tried to secure promising fractions and subsequently explored the involved factor properties. Our results indicated that a large sized (> 35kDa) protein secreted by synovium cells may be or contain the bioactive factor(s) important for differentiation/maintenance of articular cartilage and therefore holds a tremendous clinical implication for articular cartilage repair strategies in the future. In the second study which was done in goat, articular cartilage biopsies were turned upside down, thereby switching their respective microenvironments. Similarly, with time, the shape of surface cells at the flipped graft resembled those of the neighboring native articular cartilage, supporting the hypothesis that synovial fluid contains the surface hypertrophy inhibiting

factor(s). However, bony structure developed in the center of the transplant preventing the formation of an intact articular cartilage.

Finally, we modified the rat transplantation model which allows the tracing of transplanted cells and used it to address the third question. In this study, we used rib perichondrium or tibial periosteum grafts to repair articular cartilage defects at the distal femur in recipients and found that perichondrium, but not periosteum transplants, were able to regenerate high-quality, hyaline articular cartilage surfaces that were maintained during the remainder of the study.

ABSTRACT

One crucial process during skeletal development is joint formation. It starts with the interzone appearance characterized by high expression of *Gdf5*, *Gli3*, *Wnt4* and *Wnt9a*, low expression of *Col2a1* of the local compact elongated cells which subsequently give rise to most joint elements including articular cartilage as the ongoing joint cavitation. PRG4 cannot be detected in superficial chondrocytes covering the joint surface until the cavitation is complete. Lineage tracing study has shown that these flattened *Prg4* $^{+}$ chondrocytes play the role of progenitors giving rise to the deeper-layer chondrocytes. The joint cavity is filled up by lubricant synovial fluid the blood-ultrafiltrate of which contains abundant hyaluronic acid, and together with the action of lubricin, the friction at the joint surface is minimized.

Growth plate cartilage has a similar layer characterized structure yet distinctive function and fate from articular cartilage. Longitudinal bone growth is realized by a stepwise chondrocyte proliferation and differentiation program in the growth plate. Slowly dividing resting zone chondrocytes differentiate into the underlying proliferative chondrocytes that undergo clonal expansion and orient along the long axis of the bone, forming columns of cells. Post-proliferative chondrocytes undergo hypertrophic differentiation, mineralize the matrix, attract invading vessels and bone cells and are replaced by bone. In spite of a common origin, articular and growth plate chondrocytes are believed belonging to different lineages. But the related cellular and molecular mechanism responsible for such a divergence has not been clear.

Though several clinical articular cartilage repair practices such as autologous chondrocyte implantation and allograft transplantation have been continuously refined, the lack of a clear mechanistic landscape about articular cartilage homeostasis and knowledge of the actual contribution of transplanted cells always lead to variant outcomes.

In **study I**, we first systematically tested and optimized critical steps of the techniques required to assess chondrocyte differentiation in the subsequent studies. For skeletal tissues, proteolytic-induced epitope retrieval (PIER) was superior to heat-induced epitope retrieval (HIER) as it unmasks the antigens with preserved morphology and it was useful for *in situ* hybridization (ISH) as well as for immunohistochemistry (IHC) and immunofluorescence (IF). However, experimental parameters including enzyme concentration, its incubation time, temperature and time of DNA hydrolysis, must be carefully optimized to ensure high assay sensitivity and specificity.

In **study II,** with the optimized toolbox above, we tested our hypothesis that the synovial microenvironment inhibits chondrocyte hypertrophy and promotes articular cartilage differentiation. We ectopically transplanted growth plate into the distal femur surface in recipient animals and found that no hypertrophic changes occurred in the transplanted growth plate cartilage anymore at the joint surface, instead, an articular like cartilage was formed. We then used the *in vitro* model of cell pellet cultures with the synoviocyte conditioned or the control chondrogenic media to validate the *in vivo* findings. Likewise, it was found that the expression of hypertrophic differentiation markers was down regulated whereas articular

surface marker *Prg4* was effectively induced in pellets exposed to the conditioned media. Further exploratory studies suggested that the synoviocyte conditioned media factor is a large-sized (> 35kDa) and heat resistant protein mainly secreted by synoviocytes. In conclusion, we found a novel mechanism by which the local synovial microenvironment suppresses chondrocyte hypertrophy at the articular surface and promote articular cartilage differentiation.

In **study III**, we hypothesized whether the prenatally programmed fate and function of epiphyseal and articular chondrocytes can be influenced by postnatal local cellular microenvironment. We transplanted the donor articular-epiphyseal cartilage complex (AECC) autografts in inverted orientation to recipient sites with different weight-bearing properties in goats. Macroscopic, histological and differentiation status of grafts were followed up by the end of postoperative week 1, 2, 3, 6, 12, and 24. It was found that grafts localized at the trochlea and lateral condyle were with more cartilaginous appearance through 6 weeks post-surgery compared to the medial condyle, and epiphyseal chondrocytes transplanted onto the articular surface differentiate into superficial articular like chondrocytes which are densely packed with smaller and flattened morphology. In conclusion, these findings suggested that the synovial local microenvironment may contain biochemical and or biomechanical factors promoting articular cartilage differentiation while inhibiting endochondral ossification, despite the articular and epiphyseal growth chondrocytes displayed resilience to postnatal transdifferentiation within the surgically switched local cellular environment.

In **study IV**, the role of the transplanted perichondrial cells in healing the resurfaced joint surface was investigated. We harvested perichondrium and periosteum allografts from the *eGFP* $^{+}$ donor rats and ectopically transplanted them to the full thickness articular cartilage defect at the distal femur in wild-type littermates. Surgery femurs were collected at postoperative day 3, 14, 56, and 112 for transplanted cells tracing, microscopic, histological and differentiation analysis. It was shown that almost all cells at the defect are transplant derived. Perichondrium initially has abundant SOX9 + cells that with time differentiate into the expanding hyaline cartilage positive to *Col2a1* and negative to *Col1a1*, and the proteoglycan rich matrix at the injury sites. Notably, at latter time points, the perichondrium derived cartilage actively remodeled into bone at its borderline next to the underlying subchondral bone, and at day 112 post-surgery Prg4 expression was detected in perichondrium derived superficial chondrocytes. Periosteum initially lack SOX9 expression which later underwent a transient increase however, with time it formed fibrous layer with thinning thickness, and it provided the derived cells into the subchondral bone. To conclude, the transplanted perichondrium and periosteum transplants did not only stimulate regeneration responses but also by themselves transformed into the regenerated tissue. Perichondrium grafts differentiated into hyaline cartilaginous structure resembling articular cartilage, whereas periosteum gradually formed a thinning fibrous layer.

In summary, this project systematically optimized skeletal tissue-based targeting assays, provided evidence of protective factors existing in the synovial microenvironment and explored the potential properties of this factor. Therefore, it sheds light on a novel mechanistic signaling

transduced by the interaction between the bioactive synovial factor(s) and articular cartilage superficial chondrocytes which are protected from hypertrophy and ossification. Besides, a reference was given for the chondrogenesis of perichondrium and periosteum transplants when used to reconstruct the full thickness defected articular cartilage.

LIST OF SCIENTIFIC PAPERS

I. **Dou Z**, Chau M, Muder D, Vedung T, Nilsson O. Optimized protocols for in situ hybridization, immunohistochemistry, and immunofluorescence on skeletal tissue. Acta Histochem. 2021 Jul;123(5):151747. doi: 10.1016/j.acthis.2021.151747. Epub 2021 Jun 30. PMID: 34217048.

II. Michael Chau, **Zelong Dou**, Marta Baroncelli, Masaru Kanekiyo, Lars Ottosson, Ameya Bendre, Ellie Landman, Alexandra Gourogianni, Kevin Barnes, John D. Bacher, and Ola Nilsson. Synovial cells secrete a temperature-stable protein that inhibits hypertrophic differentiation and induces articular cartilage differentiation of chondrocytes. *Manuscript.*

III. Michael Chau, Ferenc Toth, Ameya Bendre, Alexandra Armstrong, **Zelong Dou**, Jeffrey Macalena, Marc Tompkins, Denis Clohisy, Ola Nilsson, Cathy Carlson. Microenvironmental Influences on Articular Chondrocyte Differentiation: An In Vivo Study in Juvenile Goat. *Manuscript.*

IV. **Dou Z**, Muder D, Baroncelli M, Bendre A, Gkourogianni A, Ottosson L, Vedung T, Nilsson O. Rat perichondrium transplanted to articular cartilage defects forms articular-like, hyaline cartilage. Bone. 2021 Oct;151:116035. doi: 10.1016/j.bone.2021.116035. Epub 2021 Jun 8. PMID: 34111644.

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LIST OF ABBREVIATIONS

AC	Articular cartilage
ACI	Autologous chondrocyte implantation
AECC	Articular-epiphyseal cartilage complex
Alp	Alkaline phosphatase
a-SMA	Actin alpha 2, smooth muscle, aorta
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	Bromodeoxyuridine
CM	Conditioned media
CREB	cAMP response element-binding protein
CSPCs	Cartilage stem/progenitor cells
DZ	Deep zone
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
Erg	ETS-related gene
Erk	Extracellular signal-regulated kinases
ESR	Estrogen receptor
ETS	Erythroblast transformation-specific
FFPE	Formalin-fixed, paraffin-embedded
FGF	Fibroblast growth factors
FGFR	Fibroblast growth factor receptors
FPLC	Fast protein liquid chromatography
Fzd	Frizzled
GDF	Growth differentiation factor
GFP	Green fluorescent protein
Gli	Glioma-associated oncogene family zinc finger
GP	Growth plate
GTP	Guanosine triphosphate
Hh	Hedgehog

HIER	Heat induced epitope retrieval
Hip	Hedgehog interacting protein
Hox	Homeobox
IDZ	Intermediate zone
IF	Immunofluorescence
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IHH	Indian hedgehog
ISH	*In situ* hybridization
JNK	c-Jun N-terminal kinase
LRP	Low-density lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MSCs	Mesenchymal stromal/stem cells
NICD	Notch intracellular domain
OA	Osteoarthritis
OCT	Optimal cutting temperature
Osx	Osterix
PI3K	Phosphatidylinositol-3-kinase
PIER	Proteolytic-induced epitope retrieval
Ppr	Parathyroid hormone 1 receptor
Prg4	Proteoglycan 4
PSCs	Periosteal stem cells
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
Runx2	Runt-related transcription factor 2
SEM	Standard error of the mean
Shh	Sonic Hedgehog
Smo	Smoothened
Sox	SRY-related high mobility group box
SSC	Skeletal Stem Cell

SZ	Superficial zone
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1

1 INTRODUCTION

During embryogenesis, bone formation is initiated with the condensation of mesenchymal stem cells that subsequently differentiate into chondrocytes to form the cartilaginous anlagen. Within this structure the central chondrocytes withdraw from the cell cycle and start their hypertrophic differentiation. After that, bone collar is formed around the center of the anlagen and blood vessels are attracted with the coupling of VEGF secreted by the hypertrophic chondrocytes, giving rise to the vascularized primary ossification center. In parallel, an interzone structure is formed at the site of future joints. Thereafter, joint cavitation initiates, allowing for the appearance of joint cavity which is then filled by synovial fluid. As an ultrafiltrate of plasma, synovial fluid is additionally rich in hyaluronic acid and lubricin. After the occurrence of the joint cavitation *Prg4* expression can be detected at the most superficial layers of the developing articular surface.

During the postnatal phase, apart from trauma and injuries, the articular cartilage health is challenged by its degenerative changes which may lead to osteoarthritis, a pathological condition that results from the destruction of articular cartilage and affects nearly 10% of the population worldwide and is one of the leading causes of disability in the elderly. Currently, therapeutic options for degenerative cartilage disease are limited. Although some progress has been made with cell-based therapies, the native morphology and function of articular cartilage cannot be restored yet. Cartilage regeneration using autologous chondrocytes or mesenchymal cells generally results in hypertrophy, indicating that the chondrogenic differentiation is steered towards hypertrophic differentiation as seen in endochondral ossification in the growth plate rather than permanent articular cartilage (1). In addition, hypertrophy-like changes are found in osteoarthritic articular chondrocytes, as cells lose their articular phenotype and show similarities to terminally differentiating chondrocytes as found in the growth plate (2).

Moreover, the perichondrial transplantation technique has been clinically used by hand surgeons for joint repair and sometimes with excellent long-term outcomes; however, the contribution of the transplanted cells and their progenies during the healing phase and the quality of the reconstructed articular surface has not been thoroughly studied. The main objective of this thesis is to expand the current knowledge about bone development and chondrocyte biology, which may provide novel implications to treat osteoarthritis and other cartilage-related disorders in the future.

2 LITERATURE REVIEW

2.1 THE SKELETON

2.1.1 Overview

One of the hallmarks that distinguishes the vertebrate (i.e., birds and mammals) from the invertebrate is the formation of a skeletal system composed of cartilage and bone. During embryogenesis, the mesoderm-derived cartilage and bone are molded by chondrocytes and osteoblasts which are derived from osteochondral progenitors. Skeletal formation is initiated by a process termed as mesenchymal condensation during which mesenchymal progenitor cells aggregate at locations of the future skeletal elements. Further skeletal formation continues via two major routes. One is intramembranous bone formation, in which osteochondral progenitors directly differentiate into osteoblasts without a chondrogenesis phase to form the membranous bone. In the other way, mesenchymal osteochondral progenitors firstly differentiate into chondrocytes to form a pre-cartilage anlagen, and with time some chondrocytes continuously mature by being hypertrophic differentiated and finally replaced by osteoblasts resulting into bone, and such a process is termed as endochondral ossification (3). Among the diverse skeletal structures, this thesis focuses on the limb joint and growth plate which allows for various degrees of mobility and the longitudinal bone growth respectively. In the underlying part of limb and synovial joint formation, I tried to summarize representative studies illustrating how different parts of the condensed mesenchyme are committed towards future skeletal elements, the structure and gene expression profile of the interzone, and subsequently how cavitation happens to form a normal synovial joint. In the part of longitudinal bone growth, the attention was given to studies deciphering how cells behave from the step of mesenchymal condensation till the formation of the secondary ossification center, and studies recognizing the importance of the resting zone as a pool of skeletal stem cells within the layer structured growth plate.

2.1.2 Limb and Synovial Joint Formation

This process begins during the embryonic stage, with mesenchymal cells migration and condensation from the lateral plate mesoderm to the outgrowing limb buds. *Hox* genes specify segment identity along the primary body axis in vertebrates by encoding highly conserved transcription factors to affect the skeletal patterning process (4). A group of uniform mesenchymal cells experience uninterrupted Y-shaped condensation and differentiate into type II collagen expressing chondrocytes (5-7). This transformation process is characterized by a cooperative activation on *Col2* enhancer by SOX5, SOX6, SOX9 (8). In that Y-shaped condensation template, proximal arms correspond to the future humerus or femurs, whereas distal arms give rise to radius/ulna or tibia/fibula. The first sign for joint development is the appearance of three cell-layered interzone at the presumptive joint sites, breaking that uninterrupted condensation (9, 10). This interzone comprises a flattened cell layer lying in between two endochondral layers (7). Within this structure, expression of chondrogenesis related genes such as *Col2a1* is down regulated, whereas another set of genes including *Gdf5*, *Wnt4* and *Wnt9a* are expressed (11-14). Linage tracing studies by *Gdf5-Cre/CreER mouse*

stains revealed that the major joint component derives from *Gdf5* positive lineages, thus indicating that interzone cells are the joint progenitors (15-18). In addition, genes such as *Gli, Wnt16, Erg*, and doublecortin get expressed within the interzone (19).

Joint cavitation is an important process during synovial joint formation that separates two adjacent cartilaginous elements. This process starts at around the time when hypertrophic cells are found at the center of neighboring cartilaginous elements (20). Some studies tried to explain the mechanism of this spacing event by the apoptosis of central cells within the interzone. However, rare cell death could be detected in this area (20, 21). Instead, the joint cavity develops with the filling up of fluidic hyaluronan, which is supported by the upregulation of hyaluronan synthases, the binding proteins and the uridine diphosphoglucose dehydrogenase activity in the intermediate interzone before and during the cavitation progression (20, 21). The cavitation progression is so crucial that the disruption of it leads to severe skeletal deformity. In a loss of function study where the gene locus of hyaluronan synthases *2* (*Has2*) was conditionally knocked out in mice, synovial joints were defective in elbow and proximal shoulder with more compact cells which did not give any distinctive cavitation space in certain embryonic stage when cavitation is expected. Apart from being delayed, the emerging joint cavity was dramatically smaller and less extensive with more closely packed cells (20). Moreover, P38 and Erk1/2 in the mitogen-activated protein kinase (MAPK) signaling pathway may play a regulatory role in the above hyaluronan-related events during joint formation (20, 21). In addition, certain factors such as myogenesis, movement, and muscle contraction are crucial in directing the cavitation and a healthy joint development, though it is currently not well known how intracellular signaling transduction can be affected by a mechanical stimulus (20).

2.1.3 Longitudinal Bone Growth

Longitudinal bone growth occurs at the cartilaginous growth plates by endochondral bone formation (Fig.1) (22). In the condensed cartilaginous template, mesenchymal cells express SOX9, an essential transcription factor for chondrogenesis. Later, chondrocytes in the center of the template exit cell cycle and start maturation by undergoing hypertrophic differentiation. During hypertrophic differentiation, the volume of the chondrocyte increases by around 20 times making an important contribution to longitudinal growth. Hypertrophic chondrocytes also produce VEGF that attracts blood vessels and thus help couple growth plate chondrogenesis to osteogenesis (23, 24). A secondary ossification center also forms in the epiphysis, physically separating the articular cartilage from the growth plate (23, 25). During this longitudinal development the growth plate thickness remains constant because of an equal amount of produced cartilage and cartilage replaced by bone (26). Meanwhile, bone remodeling, including resorption and deposition, keeps the unique size proportions of different parts of long bones, with a preferential occurrence at the metaphyseal surface (27).

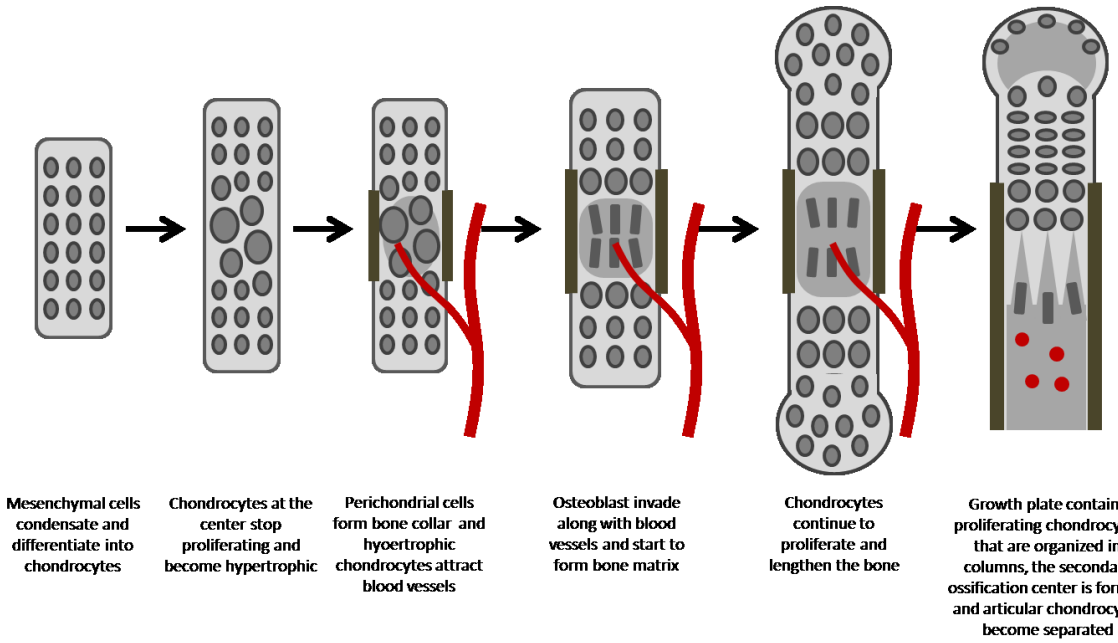


Figure 1. The stages of endochondral bone formation in the developing mouse hindlimb. (Modified from Kronenberg HM, 2003, with permission from Elsevier).

In human beings, growth occurs at the growth plate which is present in tubular bones and vertebrae (28). The growth is rapid during fetal and early postnatal life and then decreases dramatically with age and eventually comes to complete stop at the end of puberty (29), and growth plate cartilage is entirely substituted by bone, ie. epiphyseal fusion. Actually, epiphyseal fusion does not result in growth cessation, instead, it is the other way around that fusion occurs because growth has stopped (30). In such maturation process, chondrocyte proliferation declines, and growth plate thickness reduces with decrease in the number of chondrocytes, the height of hypertrophic cells and chondrocyte column density, all of which collectively are termed as growth plate senescence (31). Furthermore, it was suggested that the exhaustion of stem-like cells in the resting zone of growth plate cartilage might be responsible for such senescent changes. For example, it was observed that after surgically ablating the proliferative and hypertrophic zone of rabbit ulnar a complete zonal structure was regenerated within one week, suggesting proliferative cell clones could be derived from the speculated "stem cells" harbored in resting zone. Besides, after ectopically rotating and putting the resting zone piece alongside the proliferative zone, orientation of the proliferative columns was shifted 90 degrees, and the local hypertrophic differentiation was blocked to some extent (32). These findings suggested that stem-like cells localized in the resting zone could secret morphogens modulating the alignment of the proliferative columns parallel to the long axis of the bone while disrupting the terminal differentiation of chondrocytes within the proliferative columns (32). Different experiments from Schrier et al. indicated a qualitative and quantitative depletion of stem-like cells within the resting zone of growth plate as the senescence proceeds (33). Additionally, Nilsson et al. revealed the existence of a "stem cell" pool with the model of estrogen stimulation in rabbits, by showing it was an estrogen's transient effect rather than cell apoptosis that promotes the loss of resting zone chondrocytes, which can be maintained after stopping estrogen stimulation (34). More recently, with available genetic tracing tools, several

studies identified populations of resting-zone localized skeletal stem cells and described how they could contribute to the continuous longitudinal bone growth (35).

Moreover, being an essential part of the endocrine system, estrogen exerts a crucial regulatory effect on growth plate senescence. This could be demonstrated by two rare cases of estrogen receptor (ESR1) missense mutation, both in a male and a female patient who had phenotypical manifestations of estrogen receptor resistance (36). In the male patient, the homozygous mutation led to the translated protein truncated lacking DNA and hormone binding domains. In the younger female patient, a homozygous mutation was found in the conservative Gln residue in the domain of ligand binding (37, 38). In addition, aromatase has the important role of catalyzing the conversion from androgens into estrogens, and mutation in CYP19 could phenotypically lead to aromatase deficiency syndrome in both male and females (39). All of the above patients had dramatically delayed bone ages, growth plate fusion failure, resulting in continuous linear longitudinal growth. Conversely, treatment by extra estrogen in rabbits hastened the senescence of growth plate, with the declined growth plate height, number of proliferative chondrocytes, column densities, number and size of hypertrophic chondrocytes, thus leading to faster exhaustion of proliferative chondrocytes and an earlier growth plate fusion (40).

2.2 GROWTH PLATE CARTILAGE

2.2.1 Structure and Function

In mammals, the long bones get elongated by the growth plate, and three zones can be recognized within this cartilaginous structure (22). The formation of the postnatal growth plate is closely linked with the appearance of secondary ossification center during the fetal growth plate stage, which makes the articular cartilage (permanent cartilage) separated from the growth plate cartilage (transient cartilage). With the unique columnar structure, the disk-like postnatal growth plate cartilage sits in between the disconnected ossification centers. The most distinguishable difference from the fetal growth plate is a pool of slowly dividing chondrocytes at the top of the postnatal growth plate. This layer has been termed as resting zone where PTHrP is expressed in addition to its periarticular expression pattern in the fetal growth plate stage. This PTHrP (+) cell population is a pool of skeletal stem cells, which was evidenced by cell lineage tracing using PTHrP-Cre^{ERt} mouse strain (41). It was shown that a pool of PTHrP positive chondrocytes at the resting zone continuously proliferate and differentiate into proliferative and hypertrophic chondrocytes below and even osteoblasts and bone marrow stromal cells in subchondral bone. Moreover, these PTHrP (+) chondrocytes keep its colony-forming capability and phenotypically behave like “skeletal stem cells (SSCs)”, by showing the identical marker expression profile (Fig 2), and their downstream progenitors are capable of trilineage differentiation into cartilage, bone and stromal cells (41, 42).

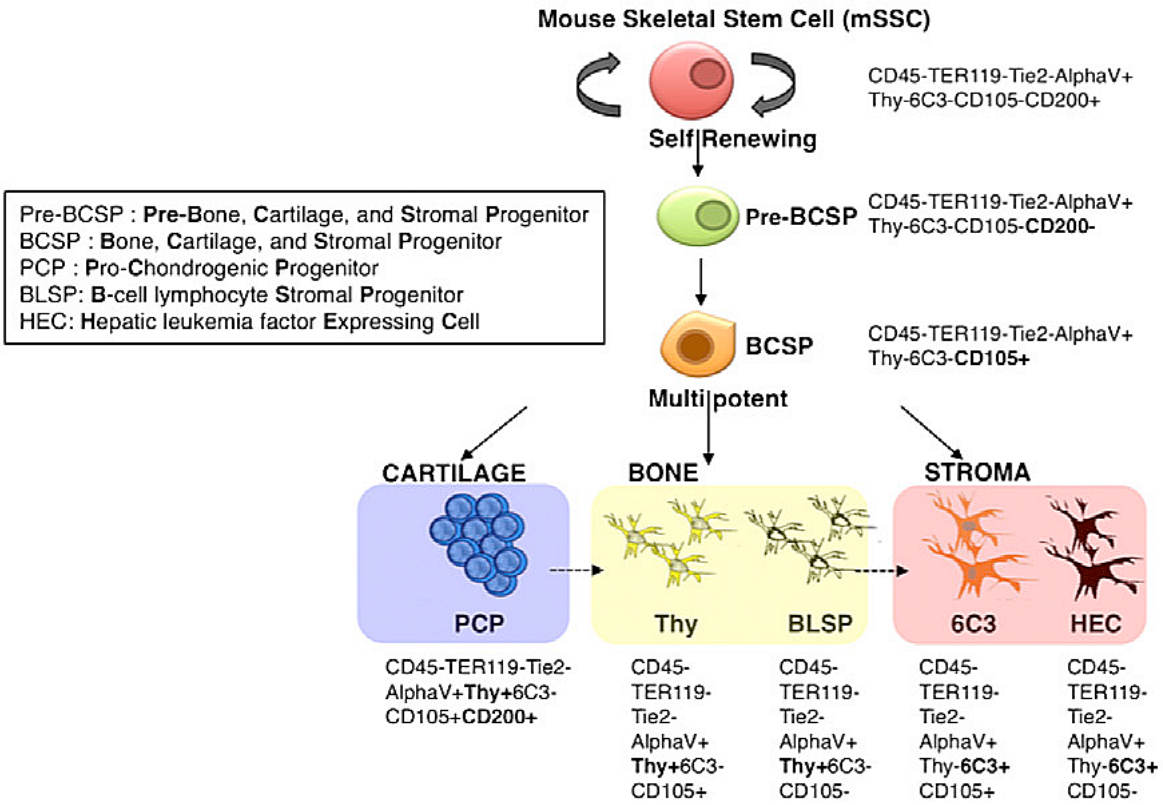


Figure 2. Schematic representation of the skeletal stem cell derived lineages (Reprinted from Chan et al., 2015, with permission from Elsevier).

Likewise, a similar population of stem cells was also confirmed within human growth plate, termed as human SSCs (hSSCs) with expression of markers such as $PDPN^{+}CD146^{-}$ $CD73^{+}CD146^{+}$ (43). At the bottom of proliferative zone chondrocytes withdraw the cell cycle and initiate their hypertrophic differentiation, at the later stage of which the height and volume of cells are increased by 4 and 10-fold respectively. Meanwhile, a 3-fold increase of extracellular matrix and 2 to 5-fold increase of certain organelles are accomplished (44). More precisely, using quantitative phase microscopy, three phases were recognized during the above cell enlargement process, and it was indicated that the dry mass increases whereas cell swelling resulting in low density during third phase is responsible for the largest variation in rapid and slow longitudinal bone lengthening. The third phase is regulated by insulin-like growth factor 1 (Igf1) (45). The genetic program of hypertrophic chondrocytes enables them to express collagen type X, MMP9, MMP13, and alkaline phosphatase (Alp) (46-49). Additionally, the angiogenic VEGF is essential to couple the invasion of blood vessels from the underlying metaphysis into the avascular mature chondrocytes contained in the cartilage (24). After that, some hypertrophic chondrocytes undergo cell apoptosis and the leftover extracellular matrix provides a frame for osteoblast invasion as well as for the capillary invasion for new bone formation.

2.2.2 Developmental Regulation

Several endocrine and paracrine regulatory elements within the growth plate cartilage are responsible for a normal longitudinal bone growth (29, 50).

2.2.2.1 Bone Morphogenic Proteins

The existence of BMPs was initially confirmed in lyophilized bone matrix by which a bone formation was ectopically induced in soft tissue (51, 52). In the following years, the isolation, purification and cloning of the coding sequences of the initial BMP family members were achieved (53-55). To date, more than 20 BMPs have been discovered and they are categorized into the signaling polypeptide under superfamily of TGF-β. Even though different BMPs within the TGF-β family are homologous to some extent in terms of gene or amino acid sequences, the induction of heterotopic bone formation by some BMPs does not apply to the other BMPs, GDFs, TGF-βs, and activins. Their biological signaling cascades in terms of osteogenesis mainly depends on their target genes, whose transcription can only be activated by certain intracellular signaling pathways (56).

BMPs bind two types of serine/threonine kinase transmembrane receptors to activate the subsequent intracellular singling pathways. Unlike TGF-βs, BMPs can elicit their effect through type I receptors (ALK1, ALK2, ALK3, and ALK6), even if type II receptors (BMPR2, ACVR2A, and ACVR2B) are absent. Comparatively, the binding affinity will be extremely intensified in the presence of both types of BMP receptors (57). Furthermore, the intracellular signals are transduced either by Smad dependent or independent mechanisms. Currently, Smad1 through to Smad8 (also termed as Smad9) have been identified. Among them, Smad1, 5, and 8 are phosphorylated by BMP type I receptor, and are so-called R-Smads two of which have been found to interact with a common partner Smad4 (Co-Smad) to form a complex moving intranuclearly (Fig 3). The formed Smad complexes then interact with coactivators (e.g. p300, CBP) and co-repressors (e.g. c-Ski, SnoN) to act upon genetic elements switching on and off the transcription of target genes (56). Apart from that, the Smad independent regulatory processes include MAPKs, Akt, JNK, PI3K, and small GTPases, which also cooperatively take part in driving cellular responses (58).

BMP signaling can be regulated throughout from extracellular to intranuclear levels. BMP signaling can firstly be inhibited by extracellular BMP antagonists (59) such as Noggin, Chordin, Gremlin, etc., which have been described in different animal species. Higher expressions of BMP antagonists could result from upregulation of BMPs, suggesting a feedback loop mechanism (60, 61). On the cell membrane, a pseudo TGF-β receptor (BAMBI) lacking the intracellular domain for further signaling transduction could trap BMPs. BMP and TGF-β signaling pathways are regulated in a similar way, as indicated by the increased BAMBI expression (62). Intracellularly, signaling could be negatively regulated by Smad6 and Smad7, Smurf1 and Smurf2 of E3 ubiquitin ligases and transcription co-inhibitors (e.g. c-Ski, SnoN) (56). BMP signaling can also be activated at extracellular levels by its potentiators, such as BMP1 and BMP1-homologous Tolloid and Xolloid, which can release the active BMPs from the inactive BMP-Chordin complexes by cleaving Chordin (63, 64). Moreover, the up regulation by heparin, heparan sulfate, Kielin/Chordin-like protein (KCP), Crossveinless-2/BMPER have been previously reported (56). Additionally, membrane anchored BMP coreceptors from the repulsive guidance molecule (RGM) family can enhance BMP signaling. In the nucleus transcription coactivators (e.g. p300, CBP, Runx2, and/or GCN5) are required

to form complexes with phosphorylated BMP-specific R-Smads for transcriptional activities (56).

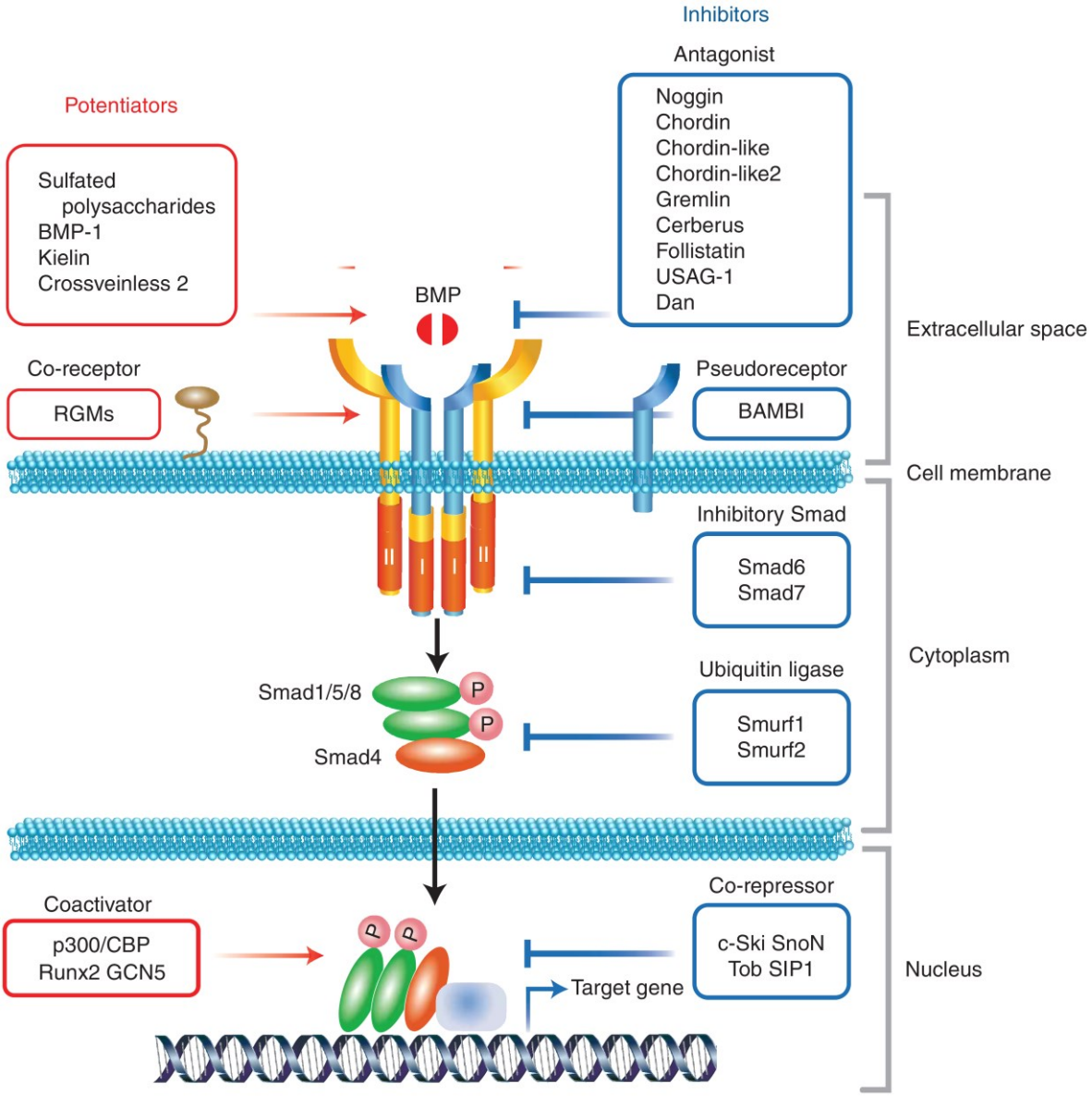


Figure 3. Schematic representation of bone morphogenetic protein (BMP) signaling with potentiators and inhibitors (Reprinted from Katagiri & Watabe, 2016, with permission from Cold Spring Harbor Laboratory Press).

In growth plate cartilage, chondrocytes and the adjacent perichondrial cells express BMPs and BMP receptors to regulate cartilage formation and development (65, 66). It was shown that overexpression of BMP2, BMP4, and GDF5 can dramatically increase cartilage element sizes and promote chondrogenesis (67, 68). During *in vitro* culturing of embryonic rat metatarsal stem cells, addition of BMP2 promoted chondrogenesis, and such stimulatory effect was reversed by adding BMP antagonist (Noggin). Noggin's inhibitory effect even in absence of BMP2 suggests an endogenous source of BMP2 during skeletal fetal development (69, 70). Mice strains lacking Bmpr1a and Bmpr1b developed generalized achondroplasia phenotypes without cartilaginous elements that formed via endochondral ossification, a dramatic absence of chondrogenesis, disorganized chondrocyte columns within growth plate cartilage, and

decreased chondrogenic protein levels in extracellular matrix. This suggested the necessity of BMP signaling in maintaining a normal chondrocyte survival and proliferation *in vivo* (71). However, overexpression of Bmpr1a accelerated hypertrophic differentiation of chondrocytes with shortened proliferative zone columns within the growth plate (66). When noggin is absent, cartilage hyperplasia (phenotypic oversized growth plate) was developed with joint formation failure, which was indicated by ectopic loss of GDF5 expressions in mutant animal strain (72, 73). Similar results were found in absence of gremlin, resulting in multiple skeletal deformities including disorder of skeletal elements patterning and altered limb bud outgrowth (73). Conversely, when the negative BMP regulator Smad6 was overexpressed *in vivo*, mutant mice displayed postnatal dwarfism with osteopenia and decreased level of R-Smads phosphorylation in chondrocytes, suggesting delayed chondrocyte maturation. In addition, BMP2-induced chondrocyte maturation was inhibited *in vitro* (74). Moreover, in the double mutant mice co-overexpressing Smad6 and Smurf1, more sever endochondral ossification delay was observed (74).

2.2.2.2 Indian Hedgehog/Parathyroid-related Protein

Indian Hedgehog (IHH) is involved in the same family as the other hedgehog family protein members such as Dessert and Sonic hedgehog, the latter of which was found in different vertebrate species controlling limb bud patterning (75). Mechanistically, IHH binds to its transmembrane receptor Patched whose normal function was interrupted and thus becomes less able to repress the transmembrane Smoothened (76). Without this inhibition Smoothened activates the downstream Gli transcription factors which translocate into the nucleus and further activate the transcription of target genes such as *PTHrP*, *Gli1*, *Hip* and *Patched* (36). PTHrP was initially identified in 1980s as the product of tumor cells with its capability of inducing hypercalcemia of malignancy by activating parathyroid hormone receptors (75, 77). It was found with structural similarity to PTH fragment of amino acid 1-34 at the amino terminal (78). Both PTH and PTHrP signal through G protein coupled receptor with similar binding affinities and activate intracellular adenylate cyclase (79).

The relation between IHH and PTHrP was initially investigated by Vortkamp et al. and Lanske et al. in 1996 (80, 81). It was demonstrated that the overexpressed IHH prevented the hypertrophic differentiation of periductular chondrocytes in the chicken limb, while PTHrP expression level was upregulated in perichondrium, which is the direct target of IHH (81). In *Pthrp* $^{-/-}$ mice, the advanced mature chondrocytes were rescued by PTHrP but not SHH treatment, suggesting that HH is functioning upstream of PTHrP (80). PTHrP and SHH treatments gave rise to thicker growth plate, while inhibiting the hypertrophic differentiation of chondrocytes in wild-type mice limbs but not in the limbs of PTH/PTHrP receptor knockout *Ppr* $^{-/-}$ mice (80). These studies indicated that the transition from proliferative towards pre-hypertrophic state of chondrocytes are regulated by the IHH-PTHrP-PPR axis and a negative feedback loop was confirmed by the IHH-induced hypertrophy effect achieved via PTHrP-PPR (82). Periarticular expressed PTHrP acts on proliferative chondrocytes which gradually move away from PTHrP and start to become pre hypertrophic and produce IHH, which then

stimulates periarticular chondrocytes to produce PTHrP in a perichondrium-dependent or independent manner (22, 83-85). This delicate regulation was also supported by the Ihh $^{-/-}$ strain, in which chondrocyte hypertrophic differentiation was accelerated, the length of the proliferative zone was shortened and the periarticular PTHrP expression was lost (85). This phenotype was consistent with the above *Pthrp* $^{-/-}$ mutant mice (80). In addition, Karp et al. found that the phenotype of compound mutant *Ihh* $^{-/-}$; *Pthrp* $^{-/-}$ mice was similar to the strain of in *Ihh* $^{-/-}$, and constitutively active PTHrP receptors in *Ihh* $^{-/-}$ mice cartilage could prevented the hypertrophy of premature chondrocytes but had no effect on either the resulted shortened limb causing dwarfism or the decreased chondrocyte proliferation (84), suggesting that PTHrP partly mediates the pre hypertrophy induced by IHH, whereas IHH promotes chondrocyte differentiation in a PTHrP independent way (84). The studies above unveiled the scenario of IHH-PTHrP negative feedback loop in growth plate development (Fig 4).

IHH signaling has a direct regulative effect on chondrocyte propagation in growth plate. For instance, in *Ihh* $^{-/-}$ and chondrocyte specific *Smo* $^{-/-}$ strains, a decreased proliferation of chondrocytes was observed (85-87), and chondrocyte hypertrophy was delayed in chondrocyte specific *Smo* $^{-/-}$ mice (88). In addition, Kobayashi et al. proposed that the periarticular chondrocytes could be differentiated into columnar proliferating chondrocytes, mechanism that was driven by IHH (89, 90). Koziel et al. further showed that downstream of IHH signaling, GLI3 negatively controls both the transition of periarticular chondrocytes towards the columnar chondrocytes and also proliferative chondrocytes to hypertrophic chondrocytes (91). Moreover, it was shown that the central zinc finger region of TRPS1 (a GATA family transcription factor) specifically binds the transactivation domain of GLI3 at transcriptional level, thus regulating chondrocyte proliferation and differentiation (92).

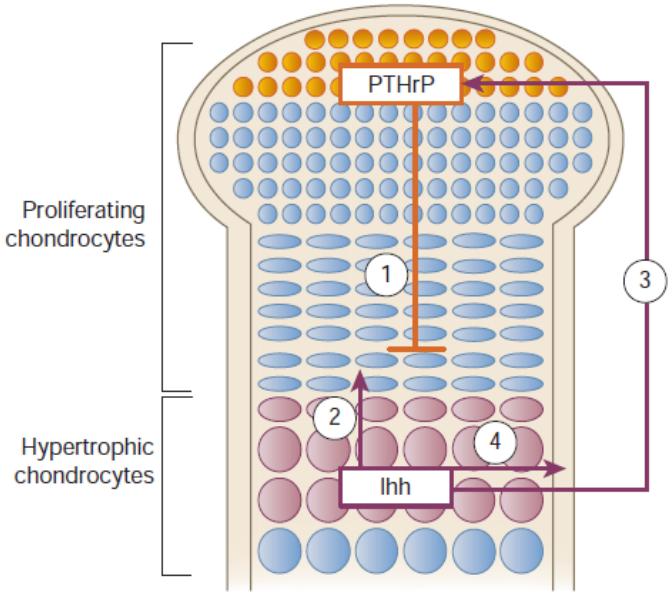


Figure 4. Indian hedgehog (IHH)/parathyroid hormone-related protein (PTHrP) negative-feedback loop (Reprinted from Kronenberg, 2003, with permission from Elsevier).

2.2.2.3 Wingless-Type MMTV Integration Site Family

Wnt proteins belong to a conserved family of cysteine-rich glycoproteins. Physiologically, they are a series of secreted growth factors participating the regulation of biological processes ranging from organ formation during the embryonic stage to the homeostasis of stem cells in

adults (93, 94). The identified 19 Wnt proteins signal through canonical and noncanonical pathways, only the former of which is β-catenin dependent (95).

The canonical cascade is triggered by Wnt proteins specifically binding to a 7-transmembrane receptor belonging to Frizzled (Fzd) family, whereas low-density lipoprotein receptor-related protein (LRP5/6) participates to the binding process as a co-receptor (96). Under a resting state, β-catenin is phosphorylated by a cytoplasmic complex containing Axin, Glycogen Synthase Kinase 3β (GSK3β), Casein Kinase I (CKI), and Adenomatous Polyposis Coli (APC), then ubiquinated and finally degraded by proteasome. The binding of Wnt and its receptors enable the phosphorylation of LRP5/6 tail. Subsequently, Axin is recruited at this complex by the scaffold protein Dishevelled (Dvl). This relocation of Axin deconstructs the above complex, so that β-catenin accumulates in the cytoplasm and is able to translocate into the nucleus to activate the downstream genes (Fig 5), by associating with transcription factors including TCF, LEF (97).

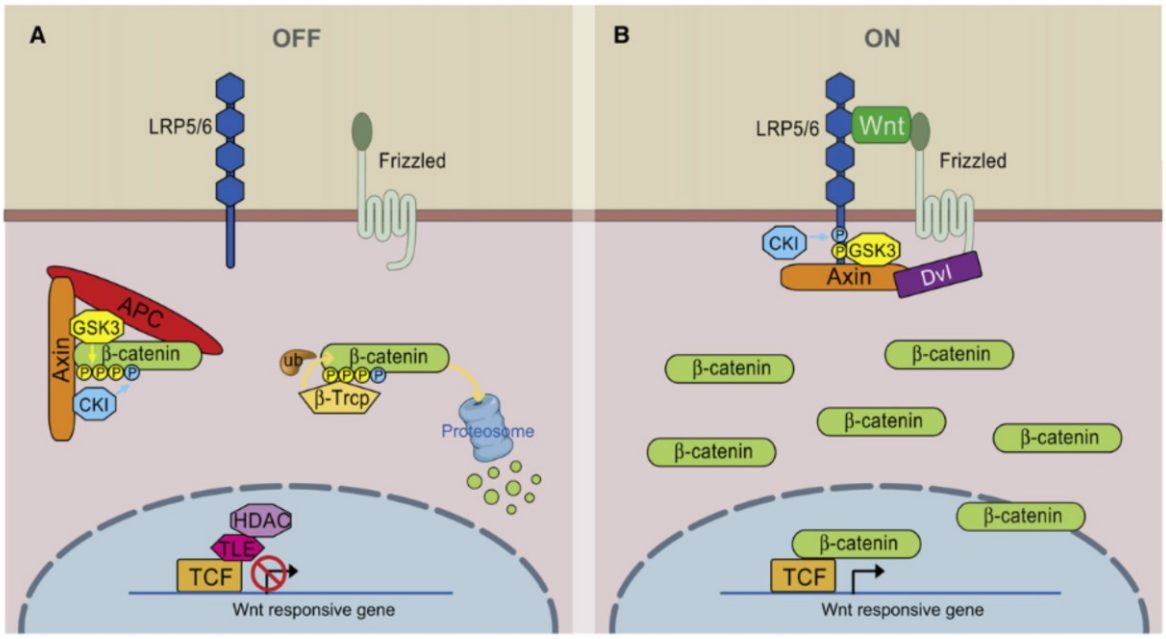


Figure 5. Overview of Wnt/b-Catenin Signaling (Reprinted from MacDonald et al, 2009, with permission from Elsevier).

Alternatively, in the noncanonical signaling pathway, Wnt ligands bind to Fzd receptor, through which small GTPase proteins, Rho and Rac are activated. These events lead to the activations of Rho-associated protein kinase (ROCK) and c-Jun N-terminal kinases (JNKs) that are responsible for the subsequent cascade phosphorylation, which triggers cytoskeleton reorganization and/or switching off the transcription of target genes. Phospholipase C (PLC), which is induced by the interaction between Wnt and Fzd, catalyzes the production of inositol triphosphate (IP3) and diacylglycerol (DAG). The accumulated IP3 leads to intracellular Ca^{2+} releasing by binding to its receptors on the ER, which further activates protein kinase C (PKC), calcineurin (CaN) and calmodulin-dependent kinase (CAMKII). As a result, the transcription of target genes is triggered by transcription factors including NF-κB, NFAT and CREB (97, 98). Collectively, the above noncanonical signal transduction process is termed as Planar Cell Polarity (Wnt/PCP) and calcium dependent (Wnt/Ca^{2+}) pathway (97).

Wnt signaling pathway plays important roles in skeletal development. Generally, it can inhibit the differentiation from mesenchymal progenitors to chondrocytes and promote bone formation (99). More specifically, the LRP5 loss-of-function mutation results in osteoporosis-pseudoglioma, which is a syndrome with low peak bone mass (97). Besides, in kindred, a gain of function mutation in LRP5 was found to reduce the dickkopf Wnt signaling pathway inhibitor 1 (DKK1) mediated antagonism, thus increasing the Wnt singling and causing an autosome dominant syndrome marked with high bone mass (100).

Postnatally, only 6 of the known 19 Wnt family members were found expressed in growth plate, being *Wnts* -2b, -4, and -10b of the canonical pathway, *Wnts* -5a, -5b, and -11 of the noncanonical pathway. Interestingly, the expression of all these *Wnt* genes was very low in the resting zone and high in the proliferative and pre hypertrophic zone, and expression decreased in the hypertrophic chondrocytes within the growth pate. This overall pattern is consistent with previous reports about Wnt regulating chondrocyte differentiation and hypertrophy during the embryonic stage. This also suggests interactions of *Wnt* genes during the growth plate chondrocyte regulation (101). Ablation of β-catenin in mouse chondrocytes lead to dwarfism with decreased chondrocyte proliferation and delayed hypertrophic differentiation and endochondral bone formation (102). Conversely, a misexpression of a stabilized form β-catenin in chick limbs could accelerate chondrogenesis by inducing advanced chondrocyte maturation and bone collar formation (103). Canonical Wnt signaling does not help maintaining the PTHrP(+) SSCs in the growth plate, which was recently revealed by genetic pulse-chase approach combined with RNA-seq analysis showing significant enrichment of Wnt inhibitors and activators in the isolated slow cycling, label-retaining chondrocytes (LRCs) and non-LRCs respectively. These findings suggested that a Wnt-inhibitory environment is needed to regulate the differentiation and maintenance of the PTHrP (+) SSCs in the postnatal growth plate (104).

2.2.2.4 Fibroblast Growth Factors

In mammalians, the FGF family members are 18 polypeptides raging in size from 15kDa to 38kDa. These members are categorized into FGF1, FGF4, FGF7, FGF8, FGF9 and FGF19 subfamilies, in terms of molecular homology and phylogeny. Furthermore, the first five subfamily polypeptides signal through a paracrine manner whereas the FGF19 subfamily members are an endocrine hormone (105, 106). FGFs share a core structure of 120 amino acids molding into a β-trefoil fold that is conventional in paracrine FGFs and contains twelve antiparallel β-strands (β1–β12) (105, 106). However, in the endocrine FGFs β11 strand is absent, which is atypical (107). This conserved core is flanked with various N and C terminals varied among different FGFs, by which determining their distinct functions about regulating cell proliferation, differentiation and migration during embryonic stage and tissue regeneration in adults (108, 109). These functions are mediated by FGF binding and dimerized to activate their receptors including FGFRs 1–4 localized at the cell surface (110, 111).

The essential roles of FGF signaling in skeletal development (Fig 6) could be indicated by achondroplasia and craniosynostosis syndromes caused by the FGFR mutations in humans, and phenotypes of genetic deficient mice mimicking these human skeletal diseases (112). Several

studies suggested that signaling of FGFR1 and FGFR3 negatively affect growth, whereas FGFR2 has a growth promoting effect (113). For example, a transient hypertrophic zone increase was observed in the FGFR1 conditional deficient mouse, and osteogenic differentiation was delayed both in mutant mice femurs and in *in vitro* cultured mutant cells, in the latter of which osteoblast proliferation was enhanced as well (114). In contrast, FGFR2 conditional knockout mice appeared to have dwarfism with significantly decreased femur length and failed in the development of some tarsal joints, as cavitation did not happen before the formation of ossification center in tarsal elements. Moreover, bone marrow density was lower than controls aging from 3 to 58 weeks, and the postnatal osteogenesis was severely impaired, with much less or even devoid of trabecular bone formation (115). In addition, a set of gain of function mutations of FGFR3 has been clinically identified leading to achondroplasia (116, 117). The missense mutations of FGFR3 led to widened resting zone but narrowed proliferative and hypertrophic zones compared to normal controls (118), suggesting a delayed chondrogenic differentiation. Conversely, inactivation of FGFR3 led to overgrowth of longitudinal bone, thickened growth plate with expanded proliferative zone, but inducing less cortical and trabecular bone formation and decreased bone mineral density. This suggested that FGFR3 has a role in growth inhibiting and regulating homeostasis of chondrogenic and osteogenic differentiation (119).

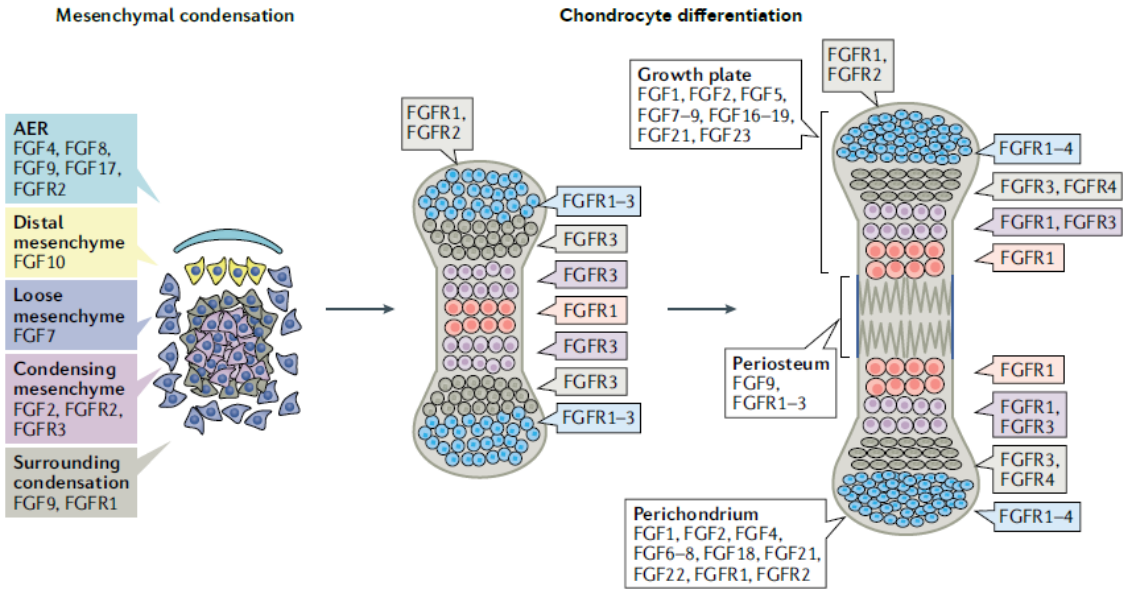


Figure 6. Expression of FGFs and FGFRs during endochondral bone formation (Reprinted from Xie et al, 2020, with permission from Elsevier).

FGFs expression studies mapped the physiological ligands of FGFRs within the growth plate (113). FGF 2, 7, 18, and 22 expression were detected within growth plate, whereas FGF1, 2, 6, 7, 9, and 18 expression was much higher in growth plate adjacent perichondrium (120). This was quantified in one-week-old rodents, by real-time RT-PCR using cDNA of perichondrium and different micro dissected zones of the growth plate. Moreover, in human growth plate, transcripts of FGF1, 2, 5, 8–14, 16–19, and 21 were detected, but only FGF1, 2, 17, and 19 could be furthered targeted on protein level (121). Furthermore, both FGF9 and FGF18 were contributing to growth plate development (113). For instance, the homozygous FGF18

knockout mouse strain displayed a similar phenotype to the mouse strain lacking FGFR3, suggesting it as a physiological ligand of FGFR3 (122). The phenotype of *Fgf9* $^{-/-}$ animal strain indicated that FGF9 promotes chondrocyte hypertrophy at the early stage and regulates growth plate vascularization and osteogenesis at the later skeletal development stage (123).

2.3 PERICHONDRIUM/PERIOSTEUM STEM CELLS

During mesenchymal condensation mesenchymal cells proliferate and differentiate into chondrocytes, which build the template for future cartilaginous element formation (124). Meanwhile, perichondrium is formed at the edge of such condensed template as fibrous layers of fibroblastic cells, and when the chondrocytes of the cartilaginous template undergo hypertrophic changes, perichondrium starts to differentiate towards osteogenic perichondrium, at bilateral sides of the hypertrophic zone within the growth plate (124). The chondrocytes and perichondrial cells proliferate and differentiate into almost all different skeletal elements such as growth plate and articular cartilage, perichondrium and periosteum, cortical bone, trabecular bone and bone marrow. This suggests that these two types of cells are sources of SSCs that give rise to various elements during postnatal phase (124).

The fibrous perichondrium and periosteum that surround growth plate cartilage and cortical bone respectively, are of importance for skeletal development and repair (124). Perichondrium originally develops at the peripheral cartilaginous template in the form of highly vascularized fibrous layers (125). Perichondrium supports bone development and supplies SSCs as wells as signals for adjacent chondrocytes (124). Deletion of FGF9 and FGF18, two abundantly expressed FGFs in perichondrium, induces a decreased chondrocyte proliferation, due to their binding failures to FGFR3 that is expressed in chondrocytes (126). After the formation of the primary ossification center, the Osx expressing perichondrial cells of the osteogenic perichondrium translocate into the marrow space (124). During the postnatal phase, perichondrium surrounds the growth plate cartilage, which contains the groove of Ranvier, which is also believed as a source of SSCs being able to migrate to the articular surface and growth plate cartilage (127). However, direct lineage tracing evidence is still absent at this moment (124).

Periosteum mainly comprises two layers, the outer fibrous layer and the inner cambium layer, which contains osteoblasts and their precursors (128, 129). Periosteal cells and osteoblasts of the bone collar are believed to derive from the fetal perichondrium, evidently shown by tissue transplantation studies (130, 131). Skeletal stem cells within periosteum show robust self-renewal ability and multipotency, and are capable of endochondral or intramembrane bone formation throughout bone fracture healing and transplantation (124). It was shown that periosteal cells displayed greater chondrogenicity, growth and differentiation ability compared to BMSCs, and contributed more to bone and cartilage formation, as shown by longer tracible integration during *in vivo* transplantation (132). Periosteal stem cells can be identified by a well-known osteoclast marker, Cathepsin K (Ctsk)-cre. These Ctsk-Cre labeled cells were found in mouse perichondrium soon after E14.5 and were continuously present in periosteum

and the groove of Ranvier during later developmental stages (133, 134). However, such a Ctsk-Cre (+) PSC population does not contribute to trabeculae and bone marrow stroma space. Moreover, it is a heterogenous population as shown by RNA-seq analysis and can be subclustered into mouse SSCs which was identified by Chan et al. Furthermore, when transplanted into the renal capsule, Ctsk-Cre (+) PSCs can only differentiate to osteoblasts, whereas a trilineage cell fate was found when it is cultured *in vitro*. However, cortical osteoblasts *in vivo* do not derive from this periosteal stem cell lineage (124). PSCs can promote bone regeneration by endochondral ossification. Lineage tracing studies showed evidence that αSMA-creER-marked cells (135, 136), Mx1-cre-marked αSMA (+) cells involved with pIpC (137) contribute to bone fracture healing differentiating towards chondrocytes and osteoblasts at the callus (133).

The relationship between fetal perichondrium and the post-natal periosteum is still currently unknown, which masks the real origin of PSCs (124). It was shown in an Osx-creER lineage tracing study that the Osx (+) fetal perichondrial cells do not contribute to the later periosteal cells (138). As a matter of fact, the existence of Osx (+) fetal perichondrial cells is supposedly transient in the bone collar without a possible contribution to perichondrial cells at the groove of Ranvier or periosteal cells at the subsequent stage (138-140). To fill this knowledge gap, an earlier cell population providing the source of these Osx positive fetal perichondrial cells thus needs to be identified (124).

2.4 ARTICULAR CARTILAGE

2.4.1 Structure and Function

Articular cartilage is a thin and translucent layer of highly specialized tissue capping the epiphysis in synovial joint cavity (20). Chondrocytes are embedded in its extracellular matrix containing collagens and proteoglycans (141). Along the axis perpendicular to the articular surface, the spanned area is subdivided into superficial zone (SZ), intermediate zone (IDZ), and the deep zone (DZ) based on the cellular morphology and collagen fiber distribution (141). The superficial zone accounts for 10% to 20% of overall articular cartilage. Chondrocytes located in superficial zone are flattened, with relatively low production of proteoglycans, with a preferential secretion of lubricating proteoglycan 4 (Prg4/Lubricin/Superficial zone protein) (142), which reduces the friction forces between the opposing synovial joint surfaces. Collagen fibrils in this zone are oriented parallel to the articular surface (141). Importantly, Prg4 has been used as a specific marker to distinguish superficial zone chondrocytes from deeper zone cells (143, 144). IDZ makes up 40%-60% articular cartilage volume. IDZ collagen fibrils are thicker and obliquely arranged to the articular surface, and chondrocytes located in this area have a rounder shape when compared to those in SZ (141). Further down, the DZ comprises 30% volume in articular cartilage, and the embedded chondrocytes are the largest compared to those in the other zones. Compressive modulus for this zone is the highest, while water concentration is the lowest. DZ chondrocytes are aligned parallel to the collagen fibers,

perpendicular to the joint line, being interspersed by radial bundles collagen type II fibers (145). In summary, from the superficial to the deep zone cell volume and the proportion of collagens and proteoglycans increase whereas cell density and water content decrease (146). Overall, articular cartilage reduces joint friction by secreting lubricin, and distribute loads with the adapted structure of intermediate and deep zones to protect the ends of the long bone (Fig 7).

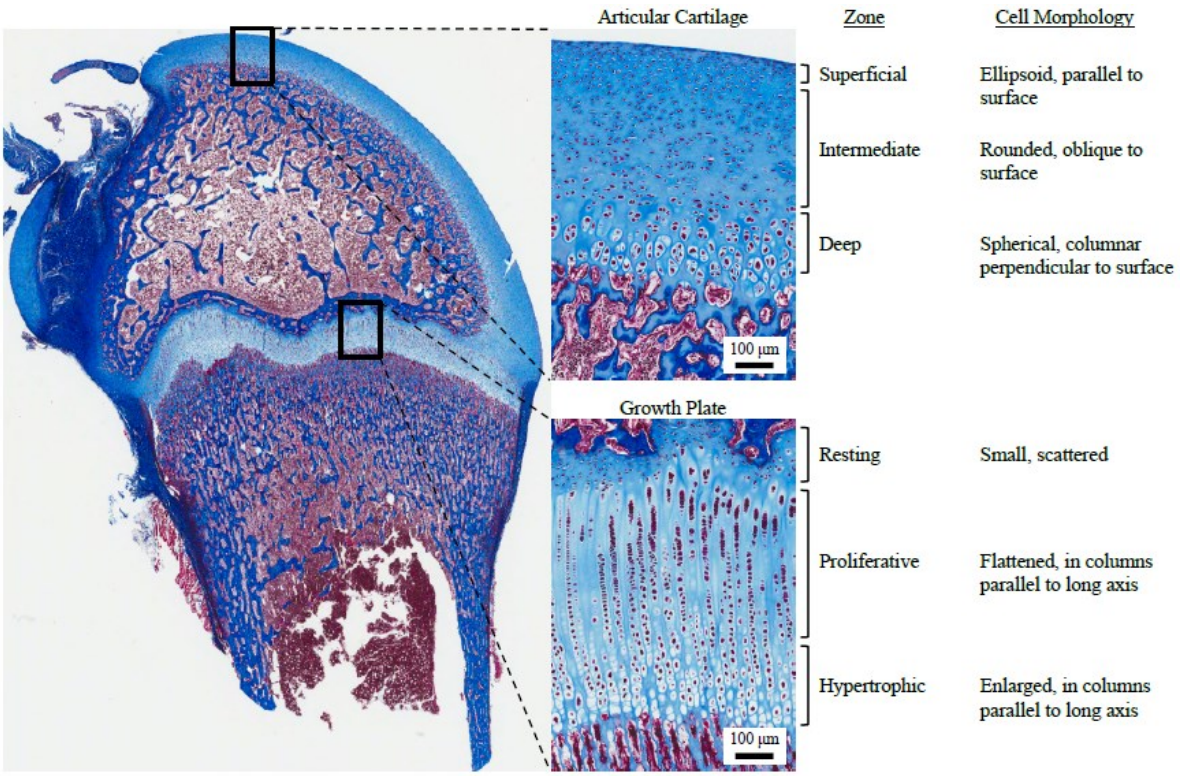


Figure 7. Morphology of growth plate and articular cartilage. Masson's Trichrome stain of a 1-month-old New Zealand white rabbit distal femur (Reprinted from Chau, 2014, with permission from the Author).

2.4.2 Developmental Regulation

2.4.2.1 Early Stage Regulators

2.4.2.1.1 Growth Differentiation Factor 5

GDF5 belongs to TGF-β superfamily, its gene was firstly found responsible for brachypodism in mutant mice with distal joint deformities (14). The importance of GDF5 in a healthy joint development could be supported by malformations such brachypodism (147) and chondrodysplasia (148-150) induced by its gene mutations, while gene mutation in its receptor also led to brachypodism (151, 152). Its interzone expression is indispensable during the cavitation in the early healthy joint development. For instance, in brachypodism mice GDF5 expression was ectopically expanded out of the interzone and distributed throughout the cartilage anlagen, which blocked cavitation and thus led to digit joint fusion (153). However, this deficient effect at proximal joints could be compensated by GDF6 (154). Using the conditional knockout GDF5-Cre^{ERT} strain, Shwartz et al. were able to trace the GDF5(+) cells during E10.5 to E18.5 (17), showing a continuous influx of GDF5(+) cells to interzone (Fig 8), whereas cells moving out to the epiphyseal cartilage lost GDF5 expression (17). Nevertheless,

most cells from the initial interzone did not give rise to articular cartilage, but they alternatively contribute to the formation of peripheral tissues such as meniscus, transient cartilage and ligaments (17). And a reciprocal cell migration between interzone and the peripheral tissues was observed up to adulthood (155, 156). Therefore, it is currently believed that the joint components form through the integration of peripheral cells (20). Though GDF5 did not directly contribute to the early development of articular cartilage, it seems it can help maintain its homeostasis, as GDF5 is a susceptible gene of osteoarthritis (157-159). Its detailed role on adult articular cartilage is still unclear (20).

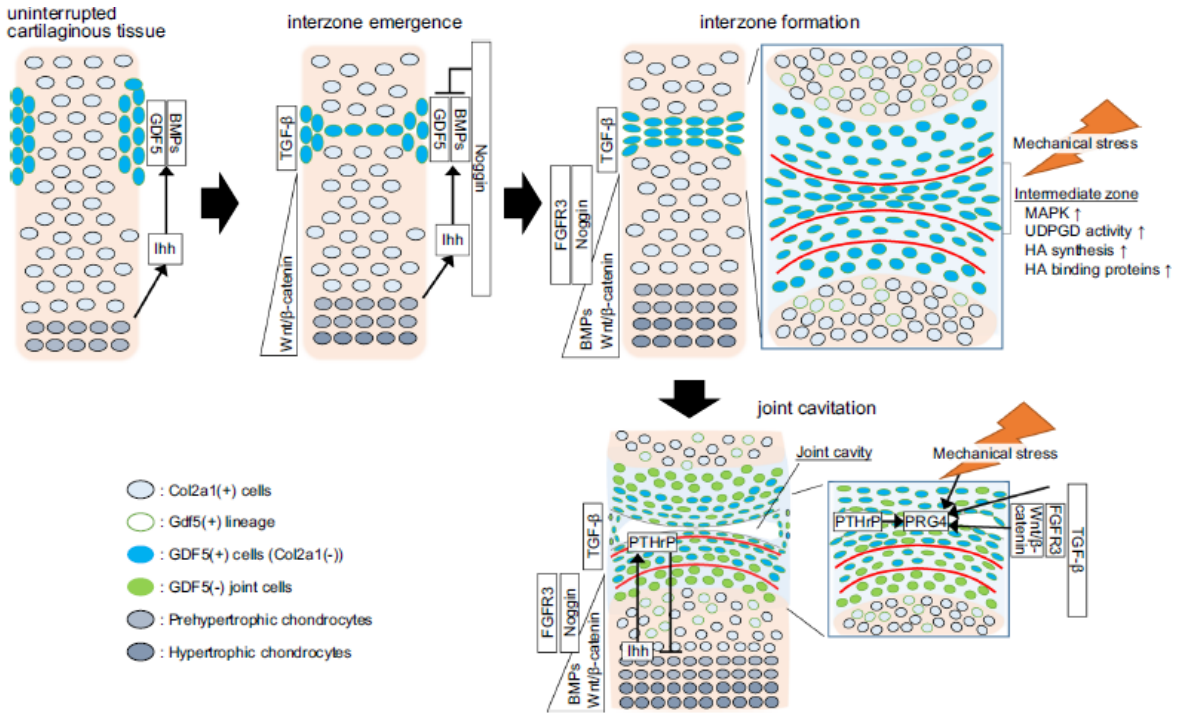


Figure 8. Joint formation and articular cartilage development in the early stage (Reprinted from Chijimatsu & saito, 2019, with permission from Elsevier).

2.4.2.1.2 Wnt Signaling

The expression of Wnt4, Wnt9a and Wnt16 was found in interzone and the flanking area before their simultaneous expression with GDF5 within interzone (159). *In vitro,* the canonical Wnt signaling pathway suppresses chondrogenesis in limb bud mesenchymal cells (21). A couple of gain-of-function studies indicated that Wnt signaling participate in inducing the joint formation upstream of GDF5 by suppressing chondrogenesis, though the loss-of-function studies revealed that joint formation is still achieved in Wnt ligand deficient mice (21). In addition, deletion of Wnt9a did not alter markers of joint formation, but an ectopic chondrogenesis resembling synovial chondromatosis was observed, which became more severe when Wnt4 was additionally deleted (13, 160). Apart from that, Wnt signaling has a role in a later developmental phase. A loss or reduced number of flattened superficial zone cells and decreased Prg4 expression was reported in *Col2a1* or *Gdf5* lineage-specific β-catenin cKO mice (15, 16) whereas aggrecan and collagen type 10 increased (16). Collectively, canonical

Wnt signaling is less essential for interzone formation in the early joint development, but in long term it orchestrates the articular cartilage integrity and function through its chondrogenesis inhibiting effect (20).

2.4.2.1.3 Indian Hedgehog—Parathyroid Hormone-Related Protein

IHH secreted by pre-hypertrophic chondrocytes at the center of cartilage anlagen is associated with the interzone formation, as the absence of IHH led not only to dwarfism but also to fused distal limb joints in mutant mice (17,88), and to an altered distribution of GDF5(+) cells (161). These results indicated that IHH is necessary in recruiting the cartilage anlagen flanking cells into interzone (20). IHH was initially detected in the presumptive ossification center where chondrocytes start to be hypertrophic, whereas its expression was not observed in the presumptive joint forming sites (21). However, Patched-1, an IHH receptor, and two main downstream transcription factors Gli1 and Gli3 were found expressed in interzone and around the cartilage anlagen (21). In Gli3 deficient mice, the phalanges were deformative and the joint shapes were irregular (91, 161). Therefore, the IHH signaling regulates joint morphogenesis via a paracrine manner within the cartilage anlagen (20). Notably, the above IHH-mediated joint morphogenesis might not be a continuous process, as the expression of Patched-1 was detected only from E12.5 to E13.5 in elbow joints of mice (85). Conversely, when IHH signaling remains continuously active, the GDF5(+) cells did not migrate into interzone anymore, which leads to a fused joint (87). Moreover, ectopic knee cartilage formation was found when IHH signal was excessive at the interzone, but in contrast to severe joint deformity in *Col2a1-Cre*;$Smo^{fl/fl}$ strain, the disruption of IHH signal in progenies of the initial formulative interzone cells did not alter the joint morphology (88). Taken together, the importance of IHH signaling in joint development is mainly revealed prior to interzone formation, rather than after it (20, 162). Furthermore, PTHrP and IHH signals appear to be independent during the early joint development, different than in endochondral ossification (20, 21). As indicated, the disruption of PTHrP did not change much the joint morphology but sabotaged the endochondral ossification (20, 21). Even without active IHH signaling, PTHrP expressing cells could be detected over lifetime in articular cartilage (20). In addition, the recombinant PTH (1–34) could inhibit the osteoarthritis development, and PTH/PTHrP signaling was involved in a process of mechanical induction of Prg4 via CREB dependent manner (25). Thus, PTHrP probably contributed to the postnatal development and homeostasis of articular cartilage (20).

2.4.2.1.4 Bone Morphogenic Protein Signaling

BMP signaling plays a key role during both chondrogenesis and osteogenesis. Mutant mice lacking the canonical BMP regulators Smad 1, 5, and 8 have shown severe chondrodysplasia in skeletal elements (163). Apart from the previously mentioned *Gdf5*, during the articular cartilage formation, *Bmp2* and *Bmp4* are expressed in the interzone (20). As soon as the interzone is specified, Bmp signaling is down regulated, as phosphorylated Smads are not observed at the expected joint sites pre cavitation as is in the neighboring epiphyseal cartilage. This was studied using BMP antagonists such as Noggin and Chordin, which could exert their inhibiting effect by directly binding to BMP2 (164), BMP4 (165), and GDF5 (166). Noggin

mRNA was found in the early cartilage template and temporarily in the interzone (20). Mutant mice overexpressing Noggin under *Col11a2* promoter lost most of their cartilaginous tissue (65). Conversely, in a loss of function study with mutant mice lacking Noggin expression, the initial mesenchymal condensation was normal but later developed hyperplasia, and joint formation failed (167). A similar phenomenon with ectopic Col2a1 expression was observed in Noggin neutralizing antibody injected chick embryos (168). Noggin contributes to the formation of articular cartilage by antagonizing the chondrogenic effect of GDF5, as in humans, missense mutation of *Gdf5* lost its sensitivity to Noggin, thereby leading to synostosis (20). After the joint cavitation, Noggin expression is limited to the epiphyseal cartilage, preventing the local BMP ligands from being diffused into the joint region. Meanwhile, another BMP antagonist gremlin 1 was found able to regulate BMP signaling during postnatal stage (20). Recently, BMP2 was combined with soluble VEGF receptor in microfracture surgery, which resulted in hyaline articular cartilage formation instead of previously reported fibrous cartilage (169). In summary BMP signaling is important both for the development and homeostasis of articular cartilage (20).

2.4.2.1.5 Transforming Growth Factor-β

Another signaling pathway critical for joint homeostasis is TGF-β signaling, which is activated by the initial interaction between the ligands and the TGF-β type II receptor (Tgfbr2) and followed by the recruitment of Tgfbr1, thus resulting in the formation of the heterodimer complex. Signaling is then transduced to intracellular level by activating Smad2/3 or Smad-independent pathways (20). Age related cartilage degeneration was found partially related to the decrease of TGF-β receptor expression in chondrocytes. Accordingly, a clinical trial using human chondrocytes transduced with virus vector containing the construct for TGF-β1 transcription factor is ongoing for osteoarthritis treatment (170). TGF-β signaling is crucial during early joint development (20) (Fig 9). For instance, the *Prx1-Cre*; *Tgfbr2*$^{fl/fl}$ mice in which *Tgfbr2* was conditionally knocked out in the early mesenchyme displayed failure of the interzone formation within phalanges, but the influx of GDF5-expressing cells was not observed (171, 172). This is similar to the phenotype shown in IHH null mice (83). TGF-β signaling has shown chondrogenesis inhibiting effect in limb bud cultures (171). As previously stated, the interzone formation needs the suppression of chondrogenesis in the cartilage primordia where BMP signaling is active. Moreover, BMP and TGF-β signaling shared Smad4, a mediator that triggers the translocation of Smad complex into the nucleus, thus suggesting that this competition for Smad4 is maybe significant during the formation of the interzone structure (20). Furthermore, the loss of Smad4 in the early mesenchyme caused damaged limb development and joint formation, while it disrupted the endochondral ossification with a mild joint alteration (20). In addition, the expression of Tgfbr2 was detected in the interzone of interphalangeal, elbow and shoulder joints from E12.5 to E16.5 (172). Apart from such transient expression, Tgfbr2 expression was maintained in joint surrounding tissues until adulthood (173). Interestingly, during embryonic stage, Tgfbr2 positive cells also expressed certain joint progenitor markers including GDF5, Noggin, and Jagged1, suggesting that Tgfbr2 functions as an upstream regulator for their expression (173). Moreover, these Tgfbr2 positive

cells partly displayed a slowly dividing stem cell like phenotype, as indicated by BrdU proliferation assay (173).

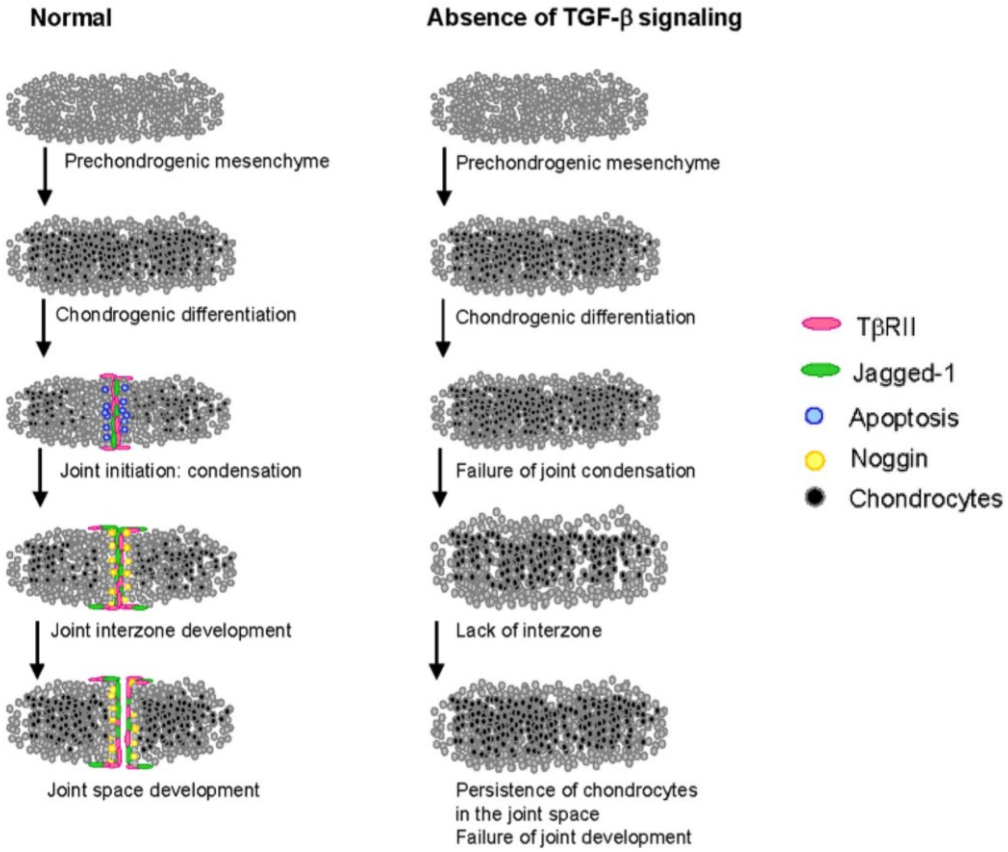


Figure 9. TGF-β is a master signaling center during the interzone formation (Modified from Spagnoli et al., 2007, with permission from Elsevier).

Apart from indicating the importance of TGF-β signaling for joint development, these studies may also support the hypothesis that growth plate and articular cartilage derived from different cell sources (20).

2.4.2.1.6 Fibroblast Growth Factor 18/Fibroblast Growth Factor Receptor 3 Signaling

Transcripts of FGF18 were detected in interzone cells, however its function was not clear, as Fgf18 null mutant limbs were not affected (20). Conditional knockout of *Fgfr3* during postnatal period induced disordered growth plate and adjacent chondroma formation via upregulated IHH signaling (174). Moreover, there is association between FGF18/FGFR3 and IHH/PTHrP and Wnt signaling (174). For instance, in mutant mice where IHH signaling was constitutively activated, ectopic cartilage hyperplasia was inhibited while the expression of Fgf18 was down regulated compared to the controls (175). The phenotypic changes induced by hedgehog activation could be attenuated by FGF18 and β-catenin stabilization (175). Moreover, FGF18 was found as direct transcriptional target within the canonical Wnt signaling (20). These results indicated that FGF18 gets involved in the joint initiation, being mutually interactive with IHH-PTHrP and canonical Wnt. During adulthood, articular chondrocytes express FGF18 and FGFR3. In infants and adult rats FGF18 was found predominantly expressed in articular cartilage compared to growth plate cartilage (176). *Fgfr3* knockout mice showed early osteoarthritis progress with increased IHH signaling (20). The current belief is that FGFR3

induces anabolic processes while FGFR1 induces catabolic ones in the articular chondrocytes (20). The representative FGFR1 ligand FGF2 was upregulated in osteoarthritis cartilage, while FGF18/FGFR3 signaling was decreased (20). Furthermore, human recombinant FGF18 has been applied for an ongoing clinical trial as osteoarthritis treatment, confirming the promising therapeutic effect (20).

2.4.2.2 Late Stage Regulators

2.4.2.2.1 Lubricin

Lubricin is encoded by *Prg4* and it is secreted by the superficial zone chondrocytes for joint lubrification, being a critical component of synovial fluid. Its expression is decreased in osteoarthritis, and accordingly intra-articular injection of exogenous Prg4 is believed a promising treatment (20). Prg4 expression was observed at the initiation of joint cavitation and it becomes intense as cavitation (20). After the joint formation, Prg4 is mainly expressed in the superficial cells of cartilage and synovium. Moreover, factors such as TGFβ, PTHrP, and mechanical loading have been identified as upstream regulators of Prg4 (20). Transient canonical Wnt activation promotes the superficial zone cells proliferation and with an improved Prg4 expression, and conversely, Wnt deletion impairs the superficial zone cells maintenance with a down regulation in Prg4 expression (15, 160). Besides, *Prg4* deletion did not alter the neonatal skeleton, but with aging it caused deposition of an acellular layer at the articular surface (20). This suggests that Prg4 is more crucial in articular cartilage homeostasis, which was further supported by a study from Kozhemyakina et al. to identify a Prg4 expressing progenitors with *Prg4GFPCreERt2* mice. The genetic tracing revealed that at one month of age, only 3 top layers of SZ chondrocytes are Prg4 positive. However, when it progresses to one year, the descendants occupied the whole depth of articular cartilage (23, 177). Regarding the mechanism of how lubricin contributes to articular cartilage maintenance Maenohara et al. found evidence that lubricin may suppress the differentiation of superficial zone chondrocytes, and that NF-κB-Mmp9-TGF-β pathway downstream of lubricin plays a role in superficial zone maintenance (178). Zhang et al. showed that superficial zone chondrocyte death does not contribute to cartilage damage, by using the DTA mediated cell ablation method. Nevertheless, increased damage was found in articular cartilage with intact chondrocytes compared to those with incomplete Prg4(+) chondrocytes ablation in medial meniscus destabilized animals. This further implies that the catabolic activity of superficial chondrocyte rather than their death is responsible for articular cartilage damage after the injury (179).

2.4.2.2.2 Notch

Canonical Notch signaling is initiated through the interactions between cell surface ligands (Jagged1, 2, Delta- like 1, 3, 4) and notch extracellular domain (NECD) of receptors (Notch1–4). Notch intracellular domain (NICD) is then released and translocates into the nucleus for the activation of downstream genes, including Hes/Hey family members, forming a complex with regulator RBPjκ and co-activator MAML (180). Non-canonical or RBPjκ-independent pathway exists in drosophila and mammals, whereby NICD binds to membrane associated

small GTPase R-Ras to activate integrin pathway (180) (Fig 10). IKKa in NF-kB pathway or LEF1 in Wnt pathway interact with NICD to activate non-canonical target genes (180). Notch inhibits chondrogenesis during the early pre-cartilage primordia period, as the deletion of RBPjκ in the early limb mesenchyme led to chondrogenesis improvement, and skeletal development was interrupted severely, in the case of NICD overexpression in chondrocytes (20). This inhibitory effect was realized by suppressing the expression of SOX9, a vital transcription factor of chondrogenic cell proliferation and differentiation (181). Though being important during endochondral ossification, Notch signaling seems not indispensable during joint creation and later development, because in mutant mice with RBPjκ deleted specifically in cartilage (*Col2a1-Cre*) or in early mesenchyme (*Prx1-Cre*), the joint and articular cartilage structure were normal (182, 183).

In addition, there were controversial outcomes regarding the role of Notch signaling in articular cartilage development and homeostasis, specifically whether Rbpjκ deletion promoted osteoarthritis progress (20). The observed bifacial roles of Notch might arise from differences in animal models and experiment schedules used, resulting in reports stating that increased Notch signaling contributed to osteoarthritis progress, while Notch expression was confirmed in superficial zone cells and was regarded as a marker of cartilage progenitor (20). Overall, Notch signaling has to be in an appropriate cascade for a normal endochondral ossification within developing skeletal elements.

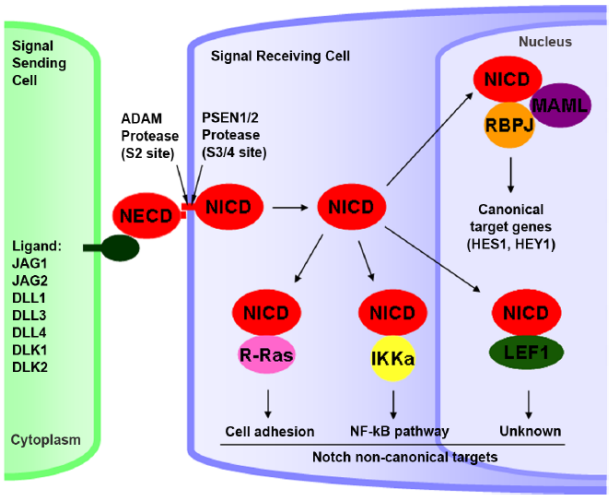


Figure 10. Canonical and non-canonical Notch signaling in mammals (Reprinted from Tao et al., 2010, with permission from Elsevier).

2.5 ARTICULAR CARTILAGE REPAIR

The treatment of articular cartilage repair has been largely demanding not only because it is technically arduous but also because the injuries and defects could lead to post-traumatic osteoarthritis (OA) (184). Secondary or post-traumatic OA could be caused by traumatic injuries, the aberrant extracellular matrix (ECM) of cartilage, or the anatomical joint deformity, whereas for primary OA it is not clearly understood in terms of the underlying joint structure abnormalities or other potential causing factors. Irrespectively of the category, the main characteristic of OA is significant articular cartilage degeneration (184). The identified risk factors include age, genetics, obesity and joint surface defects. Up to now a therapeutic

medicine for OA treatment has not been licensed. As AC is inherently composed of densely organized fibril network and has low cell-to-matrix ratio plus lack of a direct blood supply to articular cartilage, the endogenous healing is challenging. This has led to reparative strategies, aiming to recruit more cells into the injury site, either via marrow stimulation or cell transplantation techniques (184).

2.5.1 Microfracture

The methodology arises from the observation that the osteochondral defect has a higher chance of regenerative tissue formation than the defect involving only articular cartilage (185). Clinically, cell homing can be activated by the microfracture of chondral defects smaller than 2–3cm^2. During the surgery, the bone marrow cavity is accessed by drilling into the debrided chondral bone at series 3-4mm apart within the damaged articular surface. The mesenchymal stromal/stem cells (MSCs) from the bone marrow are released into the formed blood clots at the injury sites and these MSCs are expected to differentiate into chondrocytes, which help rebuilding the injured cartilage. However, the reconstructed articular surface repaired in this way mostly ended up as fibrocartilage with type I collagen expression rather than the type II collagen abundant hyaline cartilage (184).

The autologous matrix-induced chondrogenesis (AMIC) is a more advanced version of microfracture which integrates a bilayer collagen membrane into the microfracture surgery stabilizing the clots and thus guiding and enhancing the marrow-derived repair. According to a multicenter study of a randomized controlled trial comparing this technique (41 patients included) with the MF along (39 patients included), at 12 months AMIC could give rise to grater lesion filling and better quality of the regenerative tissue, though clinical symptoms between two groups were equivalent (186).

2.5.2 Cell Transplantation

Autologous chondrocyte implantation (ACI) is a common technique for articular cartilage repair, it was initially reported in 1994, and first patient treated with ACI was in 1987. Thereafter, ACI has been applied to treat articular cartilage defects sized raging 3–10 cm^2 (184). The whole procedure includes three steps: (i) Chondrocyte extraction from the healthy non-load-bearing area; (ii) chondrocyte expansion *in vitro*; (iii) expanded chondrocytes are implanted under a periosteal flap. According to the 10 years follow up evaluation of multicenter studies in USA, 69% patients improved, whereas 17% failed. For 75% of the failed cases, the mean following up time was 2.5 years (187). These postoperative data were improved gradually by the secondary generation of ACI using a porcine collagen membrane rather than the periosteal flap, and third generation of ACI using cell seeded 3D porous scaffolds which can be cut to fit the injury site and fixed with fibrin glue instead of being sutured. But to compare the clinical outcomes between microfracture and ACI techniques, long term follow-up evaluations are warranted (184). Though ACI has been technically improved, the main unsolved limitation is its dependency on cellular monolayer expansion. This procedure leads to the formation of mostly fibrocartilage rather than the expected hyaline cartilage and is

detrimental to cell phenotypes due to de-differentiation of chondrocytes, during which type II collagen expression is down regulated whereas type I collagen expression is up regulated (184). For this reason, the osteochondral autograft transfer (OAT) technique might be considered as a better option. In OAT technique the defect is filled out with hyaline cartilage, by transferring cartilage from different parts of joints with the arthroscope. The donor tissue plug consists of articular cartilage and subchondral bone harvested from a non-weight bearing position and it is transplanted and press-fitted into the defect. Multiple plugs can be applied when the defect area is bigger, in that case termed as mosaicplasty (188). However, the defect area should not be bigger than 4 $cm^{2.}$ The morbidity status at the donor site is also of concern. The postoperative follow up till 10 years has revealed good or excellent results in approximately 90% of patients with femoral condyle or tibial plateau mosaicplasty. Still outcomes vary depending on lesion size, age, and gender. Increased failure rates were noticed with lesion size greater than $3cm^2$ in women older than 40 years old (186). In case of big cartilage defects when the patient autograft is not an option for cartilage repair, fresh osteochondral allograft can be employed. During this alternative procedure chondrocyte viability is directly related to the surgery success rate. Physiological temperature storage of the fresh allografts is helpful to maintain the chondrocyte viability to the maximum (186). In a study following up 129 patients postoperatively, with the definition of failure as revision or conversion to arthroplasty, survivorship was 82% at 10 years, and decreased to 74% and 66% at 15 years and 20 years respectively. Surgery failure was associated with patients older than 30 years at the time of surgery and with two or more previous surgeries (186).

2.5.3 Stem Cells and Gene Therapy

The limitations of the current approaches led to an increased interest towards stem cell therapy for OA treatment (189). Recently, the use of cell lineage tracing animal strains enabled the identification of cartilage stem/progenitor cells (CSPCs) in the superficial zone of articular cartilage in mice (155, 177, 190), which are Prg4 expressing cells and probably the descendants of GDF5(+) cells, as they are present at articular surface and at the synovial membrane. Notably, during postnatal stage, these CSPCs at the articular surface could generate chondrocytes formulating the whole articular cartilage and are able to migrate into the cartilage defect. However, their origin and healing potential need to be investigated (191). Recently, great progress has been made in identifying skeletal stem cells (SSCs) in mice (mSSCs) and humans (hSSCs) (42, 43, 192, 193). Purified SSCs are characterized by the ability of self-renewal and trilineage differentiation into bone, cartilage and stromal cells. An interesting study showed that the injury stimulated resident SSCs could be promisingly utilized for cartilage regeneration for OA treatment. Briefly in this study, microfracture was applied and effectively resulted in the expansion of local SSCs. These microfracture-activated SSCs were prone to form fibrocartilage. However, the fate of these activated SSCs was skewed to articular like hyaline cartilage when co-delivered with BMP2 and a soluble VEFGR1 antagonizing VEGFR in hydrogel (169).

As the rapid gene editing progress gene therapy studies for articular cartilage diseases started to be realized by viral and non-viral gene transfer routes (194). The viral methods have been believed more efficient in its targeting approach to transfer the genes of interest. The commonly used viral vectors include adenoviruses, retroviruses, and lentiviruses, herpes simplex viruses for interested gene delivery. However, with the ongoing safety concerns of using viral vectors *in vivo*, non-viral methods using lipid-based systems, as well as other DNA-conjugates started to draw much attention and support with the strengths of easy handling, being safe and cost effective for *in vivo* studies (194). A representative example of using the viral gene delivering system is TissueGene C that is composed of primary chondrocytes and irradiated human chondrocytes modified with retroviral vector to over-express TGF-β1 and the cells mixture is injected into the OA knee joint (195). For the non-viral gene delivery application, certain stem cell populations and SOX5/6/9 plasmid DNA are seeded into scaffold models for pursued chondrogenesis *in vivo* (195). Overall, it seems like joint structural and functional improvement is still limited with the methods above and further optimization based on a more comprehensive understanding about biological physiology of joint cartilage is warranted.

3 RESEARCH AIMS

The main goal of this thesis was to explore the role of the local microenvironment on chondrocyte differentiation in growth plate and articular cartilage and study its implications for articular cartilage repair and regeneration.

To reach this overarching aim this thesis includes four studies with the following specific aims:

1) To systematically optimize sensitivity and specificity of situ hybridization (ISH), immunohistochemistry (IHC) and immunofluorescence (IF) on skeletal tissues while maintaining tissue morphology

2) To investigate the role of the local microenvironment on growth plate and articular chondrocyte differentiation.

3) To investigate the contribution of transplanted perichondrial cells and their progenies in the regeneration of full-thickness articular cartilge injuries after perichondrium transplantation.

4 MATERIALS AND METHODS

Method distribution in papers and manuscripts is listed below and will be discussed in general. For technique details please move to individual papers.

- Animal Models (Papers I-IV)

- Tissue Processing and Sectioning (Papers I-IV)
- Histological staining (Papers I-IV)
- Immunohistochemistry (Papers I-IV)
- Immunofluorescence (Papers I, III)
- Confocal microscopy (Papers I, III) and Whole Slide Imaging (Papers I-IV)
- *In Situ* Hybridization (Papers I-IV)
- Real-time Polymerize Chain Reactions (Papers II, III)
- Liquid Chromatography (Paper II)

4.1 ANIMAL MODELS

Animal models have been used during recent decades as the mainstay of the cartilage repair research and are constantly required by regulatory ethics for approvals in various clinical uses. Historically small animals including rats and rabbits, as well as large animals including horses, sheep, goats, and dogs have been used as research models in cartilage repair. The surgical creation of focal defects in the knee is a typical procedure for each of them. Each animal model has both pros and cons considering safety and efficacy for cartilage repair (196).

Animal models used in this thesis are rat (Papers I-III), rabbit (Paper II), and goat (Paper IV). The use of rodents is cost- and space-effective and the related genetic modification techniques are well developed. In addition, it allows the use of xenogeneic cells and tissues, and the short (2 to 4 years) lifespan with a faster development compared to large animals enables time evaluations of skeleton. Moreover, rodents have a rapid reproduction rate and litters are large (10 pups in average). Collectively, all these advantages have made rodents an efficient model for mechanism determination, variable assessment and drug screening, all of which could be further tested in large animals (196).

The limitation of using rodents to study growth plate cartilage is that unlike humans, their growth plates do not fuse. Still the long bone growth dramatically declines after 13 weeks of age when they have skeletal maturation, and the eventual grow plate ceasing is commonly reached at 26 weeks (36). Rabbits have larger joints thus providing an easier surgical manipulation of cartilage, and growth plate fusion in rabbits is similar to the one in humans. One complication of using rabbits is that they display remarkable cartilage healing capacity compared to humans, and additionally, young rabbits are susceptible to fatal mucoid enteritis induced by surgical stress, early weaning and transportation (36). Goats belong to the category of large animal models, and its usage addresses the above limitations with rodents and rabbits. Importantly, healing potential of goat cartilage is comparable to what is observed in humans, as reported by many studies, and the joint sizes as well as the articular cartilage thickness are amenable to the relevant clinical managements and the outcome evaluations (177, 196). Limitations in using goats are mainly more laborious tissue collection and processing steps, which require bone saw, longer fixation times and decalcification periods which can affect RNA integrity.

All animal studies in this thesis were approved by the regional animal ethics committee in Stockholm (Permit no. N248/15, N118/16, and 15635- 2017). Maximum effort has been made according to the three Rs of humane animal experimentation: animal replacement with alternative methods or organisms with limited sentience, reduction of animal numbers, and refinement of methodology to minimize animal suffering (197).

4.2 TISSUE PROCESSING AND SECTIONING

Tissue morphology and preservation of the target molecules is very important to be able to perform histological analysis and *in situ* targeting experiments. The general processing for skeletal tissues starts from tissue fixation where a bone saw might be used to divide tissue samples into smaller parts for better penetration of the fixation reagent. Neutral-buffered formalin which contains 4% formaldehyde has been commonly used as fixation reagent, as it creates cross linkages between proteins or between proteins and nuclear acids involving hydroxymethylene bridges (198). Shorter fixation time impairs the formalin fixation effect, which may be rescued by coagulation fixation during the tissue dehydration by alcohol. However, this admixture fixation mostly leads to variations in immunostaining. Conversely, over fixation may give rise to false negative results because of the excessive epitope masking (198). After fixation, the skeletal tissues used in this thesis underwent decalcification to make them softer for the subsequent sectioning. There are different decalcification protocols that commonly use acids with varying ionic strength or chelating agents. Strong acids such as hydrochloric or nitric acid are used for ionizing and solubilizing calcium ions, whereas chelating agents such as ethylenediaminetetraacetic acid (EDTA) link calcium ions to form an insoluble complex. Decalcification with EDTA solution is believed to be less deleterious to tissue samples due to its neutral pH. At the end of decalcification, the tissue has to be properly washed to get rid of the chelated calcium to avoid tissue damage. This represents one of the limitations of using EDTA (199). After decalcification and washing, different embedding methods can be chosen depending on the subsequent experimental purposes. Paraffin embedding is often performed with tissue that will be used for histological staining, immunohistochemistry and *in situ* hybridization assays. Generally, the tissue undergoes series of dehydration steps of gradient ethanol and vacuum, followed by paraffin penetration before the final paraffin embedding. In this thesis these steps were professionally conducted by the Histocore at Bioclinicum (J8:02) at Karolinska university hospital.

Alternatively, if the downstream analysis is *in situ* immunofluorescent targeting, OCT embedding is the primary choice because of its advantage of avoiding autofluorescence diffusion problem which has not been well solved with paraffin embedded tissue (200).

Sectioning is another critical factor that can significantly impact the experimental outcomes. The proper sectioning blade and sectioning microtome greatly influence the quality of tissue sections in various subsequent *in situ* assays. Blade types with different physical parameters should be tested and cautiously selected for certain types of tissues. Commercial sectioning blade N35 (Feather Safety Razor Co., Ltd., Osaka, Japan) is compatible with continuous sectioning of formalin-fixed and decalcified skeletal tissues and was used for studies in this

thesis. Commercial microtomes are available for sectioning of paraffin and OCT blocks. The microtomes for paraffin-embedded tissues vary depending on cooling system of the blocks, the water-based section transfer and water bath components. Microtome HM355S from ThermoFisher was used in this thesis for sectioning and mounting of paraffin-embedded tissues. Different parameters such as waterflow, sample block positioning, the angle between the sectioning blade and the block surface were all optimized in this thesis work to obtain qualitative sections.

Less variation exists among OCT sectioning modules. The anti-rolling function of the glass connected to the blade holder helps to maintain a satisfying frozen section morphology, thus being a critical factor according to the feedback from different technicians and my individual experience. Technical parameters such as cooling temperatures, block positioning and the angle against the blade were also optimized for this thesis, as for paraffin sectioning. Moreover, glass slides with coating layers are necessary to properly mount sections and to maintain them attached to the slides. After testing different slide versions, the commercial ones usually used in our lab include TruBond 380 (Electron Microscopy Sciences, Hatfield, PA, USA) and Superfrost Plus (Thermo Fisher Scientific, Waltham, MA, USA).

4.3 HISTOLOGICAL STAINING

Histological staining comprises series of technical steps utilizing specific dyes to characterize the histology of tissues in microscopic studies (201). Since the seventeen-year-old Leeuwenhoek used dyes extracted from plants such as madder, indigo and saffron to stain tissues and then put them under his rudimentary microscope for observation, improvement in the related chemistry, molecular biology and immunology has been achieved and contributed to great progress in staining techniques facilitating organ and tissue studies (201). Histological staining is commonly used to study the four phases of wound healing: homeostasis, inflammation (early and late), proliferation and remodeling phases (202). A number of indicators are used to evaluate the histopathological changes, such as depth and lengths of the healed wound, infiltration of leucocytes and macrophages, epithelial stratification, fibroblast migration, elastin formation, and the most crucial collagen fiber structural appearance, which determines the structural integrity of the healing (202). The use of conventional hematoxylin and eosin (H&E) staining to study wound healing is challenging because it is not able to characterize collagen deposition and scab formation, which may lead to misinterpretation of the results. Some other microscopic and computer-based methodologies have been tried to overcome this drawback but all of them need complicated steps and certain equipment beyond the wet histopathological laboratory. Masson's trichrome (MT) staining is a promising solution as it is able to well distinguish hemoglobin, keratin, and muscle fiber (red color), cytoplasm and adipose cells (light red or pink), cell nuclei (dark brown to black) and stain collagen fibers in blue, which could be measured and analyzed with compatible software (202).

Another histological staining method usually used in cartilage biology is Safranin O staining, in which the cationic dye Safranin O that under certain conditions exhibits metachromasia (203). The orthochromatic form in Safranin O binds stoichiometrically to glycosaminoglycan,

making this staining a useful method to quantify the GAG and proteoglycan content within the cartilage. In addition, when combined with fast-green/ hematoxylin as the counterstaining, collagens and nuclei can be visualized as well, useful for computerized image analysis. Moreover, a number of cartilage diseases are characterized by changes of distribution or amount of glycosaminoglycan in tissues, therefore the disease status could be effectively revealed and analyzed using Safranin O staining (203). Alcian blue also stains GAG but with less reproducible results. It is important to include a cartilage sample to be used as control for both Safranin O and Alcian blue staining, so that the variations due to fixation and technical performances leading to under or over estimation can be avoided (203).

4.4 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a powerful technique that uses monoclonal and polyclonal antibodies to determine the distribution of targets of interest within healthy or diseased tissues (204). It plays an important role in oncologic pathology, neuropathology, and hematopathology. The overall aim of IHC is to target antigen effectively with the least possible cell or tissue damage (204). The principles of IHC can be dated back to 1930s, but the first IHC study was reported in 1941, when Coons et al. used FITC-labeled antibody and fluorescent dye for the localization of pneumococcal antigens in infected tissues (Coons et al., 1994, as cited in Duraiyan et al., 2012) (204). Since then, technical development has led to labeling enzymes such as peroxidase and alkaline phosphatase. IHC was used in this thesis to specifically trace the eGFP(+) transplanted cells and their progenies at different postoperative time points in the recipient skeletal samples.

The basic IHC technical procedure with paraffin sections can be divided into pretreatment, primary antibody binding to targets of interest, followed by the chromogenic detection of the bound primary antibody to reveal the target distribution in tissues (36). In this thesis, FFPE rat femoral sections were rehydrated through xylene and gradient ethanol series. Proteolytic-induced epitope retrieval with proteinase K was chosen for the proper antigen retrieval while maintaining a satisfying tissue morphology. The endogenous enzymatic activity was blocked by hydrogen peroxidase, and unspecific primary antibody binding was blocked with buffer containing BSA and normal serum from the secondary antibody host species. A polyclonal rabbit anti-GFP primary antibody was used for the direct targeting, followed by a biotinylated secondary goat anti-rabbit antibody with avidin-biotin system for signal amplification. Then biotinylated HRP and free avidin were added with a certain proportion as one avidin can bind 4 biotin molecules. Incubation at specific temperature enables the formation of secondary Ab-biotin-avidin-biotin-HRP complex. The HRP activity in this complex is then subjected to develop a chromogenic brown color by DAB for the final signal revealing.

4.5 IMMUNOFLUORESCENCE

Immunofluorescence (IF) is an important immunochemical technique to localize various antigens of interest in many tissues. IF has an outstanding sensitivity, allows specific signal amplification compared to IHC and allows the use of diverse microscopic platforms thanks to

the various fluorophore-conjugated antibodies. After tissue processing and sectioning, the limiting step is antigen retrieval, which is achieved by either HIER or PIER. In this thesis PIER was selected and optimized to expose the epitopes of the target since tissue sections frequently got damaged and even detached totally during HIER.

IF can be direct (primary) or indirect (secondary), depending on the antibodies used (205). In direct IF the primary antibody is already conjugated with fluorophores, so that after the incubation with primary antibody, sections will be ready for imaging and signal distribution analysis. In the indirect IF, the primary antibody is free from fluorophore labeling, but the fluorophore-conjugated secondary antibody is used to bind the primary one. The advantage of using direct IF is obviously a shorter time needed to complete the assay. However, indirect IF is often selected due to the superior signal specificity and signal amplification. Moreover, the indirect IF allows multiple targeting in one experiment, enabling colocalization studies requiring high-resolution multiantigen imaging (205). Indirect IF was used in this thesis considering the abovementioned strengths.

The choice of the proper targeting antibodies is important during IHC. The primary antibody has to be hosted in a different species to prevent cross binding of the secondary antibody with the endogenous immunoglobin of the tissue (205). Moreover, during multiplex targeting assay, the secondary antibody conjugated fluorophores have to be combined carefully to ensure signal specificity without wave lengths of the excitation light overlapping much in the fluorescence spectrum. Both single and double indirect IF labeling were used in this thesis. In the single GFP targeting, a rabbit polyclonal anti-GFP was used followed by a donkey Alexa Fluor 488 conjugated secondary antibody. During the double targeting of GFP and osteocalcin, a chicken primary anti-GFP and a rabbit primary anti-osteocalcin, followed by a goat Alexa Fluor 647 conjugated secondary antibody and a donkey Alexa Fluor 488 conjugated secondary antibody were used. For each target, the concentration of primary antibody was optimized. Nonspecific binding of the primary antibodies was blocked by buffer containing BSA and the serum of the secondary antibody hosted species.

4.6 CONFOCAL MICROSCOPY AND WHOLE SLIDE IMAGING

Confocal laser scanning microscopy (CLSM) has been one of the most popular fluorescence imaging platforms to acquire three-dimensional information from cells or tissues since it started to be commercialized in the late 1980's. CLSM enables different applications ranging from fast scanning of cells for revealing dynamic processes to analysis of particular tissue morphology and colocalization of target proteins (206). The term confocal in microscopy refers the conjugated focal plane between the objective lens and the detector, whereby the in-focus plane is isolated from the adjacent axial planes. The defining feature of the confocal microscopy is using diffraction-limited spot of light to illuminate the sample and the conjugate focal plane positioned pin hole at the collection light path, with which optical sectioning at certain depth of the sample is generated (206).

The CLSM module used in this thesis is Zeiss LSM 700, for z-stack tile scanning of 10 μm-thick frozen rat femoral sections which had been stained with specific fluorophore labeled antibodies as stated above. “Best signal” approach was selected for fluorophore combination. Several parameters such as frame size, scanning speed, laser power, pin hole size, the tile scanning area and z-stack scanning depth were individually optimized. When scanning was completed, raw data was imported into open-source software ImageJ (NIH, Bethesda, Maryland) for brightness and contrast adjustment.

The whole slide imaging (WSI) includes the following sequential steps: image acquisition, storage, processing, and visualization. The hardware used for image acquisition consists of two components one for image capture and one for image display. Image capture is performed by a digital slide scanner comprised by a trinocular microscope, robotic elements and a camera. The robotic elements control the illumination intensity, slide loading and unloading, change of objectives, and focusing facilities (207). Different than still microscope, the digital slide scanner acquires sequential images in either tile or a lining manner and all images required will then be compressed and/or stitched into the whole virtual slide, thus creating an exact replica of the real slide with stained tissues. The image resolution depends on the selected objective magnification. Magnification of 20x is appropriate for routine surgical pathology and immunohistochemistry analysis.

All histological staining, IHC and ISH slides in this thesis were scanned by WSI using a panoramic digital slide scanner (3DHISTECH). Images were analyzed with the compatible Caseviewer software (3DHISTECH) for brightness and contrast adjustment.

4.7 *IN SITU* HYBRIDIZATION

In 1969, *in situ* hybridization (ISH) was initially utilized for DNA identification in Xenopus (208). Since then this technique has been applied continuously for DNA/RNA detection in whole mount or tissue sections. The broad applications of ISH even led to the establishment of databases, such as EMAGE where it is possible to search and analyze *in situ* gene expression data in the mouse embryo with the accompanying suite of tools (67, 209).

RNA ISH is as a powerful tool to detect mRNA species, as it enables a rapid detection of temporary expression pattern of genes of interest but avoiding tedious procedures such as producing the antibody against a certain protein target (210). However, RNA ISH requires high molecular skills to efficiently clone the complementary RNA chain of the target. In addition, to acquire a satisfying target specificity, some radioactive isotypes had been used widely to label riboprobes before the invention of non-radioactive labeling methods. Moreover, RNA ISH does not give much insights about translational events and the subsequent protein properties such as localization, stability etc. Nevertheless, these minor limitations did not prevent researchers from using it to rapidly predict developmental functions of genes (210).

A general ISH procedure includes tissue processing, embedding, sectioning, deparaffinization, permeabilization, prehybridization, hybridization with labeled probes, and detection of DIG labeled hybrids (211). As commonly suggested in protocols, permeabilization is required

because DNA/RNA targets are cross-linked with surrounding proteins during tissue fixation (212). Permeabilization is the process by which the structural membranes surrounding the target are dissolved by detergents or enzymes, so that targeting probes or antibodies can access and bind native mRNAs or antigen epitopes respectively (213). Permeabilization is the most crucial step during the ISH optimization as it may differ depending on fixation status of the samples, especially on FFPE sections. A titration test is thus recommended. Many studies have been investigating how to best optimize this crucial step, starting from the order of fixation and permeabilization steps, including: (a) permeabilization, fixation and staining, (b) fixation, permeabilization, and staining, and (c) simultaneous fixation and permeabilization. Fixation followed by permeabilization has been the most commonly used, though there are reports of success for mRNA ISH via the other two methods as well (213). Pepsin has been used for epitope exposure during ISH, together with HCl in different protocols (212), which might damage the morphology of the fragile skeletal sections. Moreover, pepsin needs to be frozen for storage and freshly prepared just before use, which could introduce variations into the already complex ISH protocol. Proteinase K is more stable and easier to use for permeabilization and was therefore chosen and its concentration was optimized during ISH assays in this thesis.

The overall aim of post hybridization is to minimize the background noise, which might be caused by unbound probes and/or the mis annealed hybrids. This very important step involves the RNase A digestion. As one of the major types of endoribonucleases for laboratory use, RNase A has an outstanding thermo stability, as it can be isolated simply by boiling crude cellular extract so that all the other enzymes are denatured. RNase A specifically cleaves unpaired C and U residues of single strand RNAs (215), meaning that a single base mismatch in RNA hybrids with DNA/RNA can be cleaved by RNase A. Therefore, a single RNA probe or several overlapping ones could be employed to detect single base substitution or locate mutation sites unambiguously (216). However, certain sequences that could be related to such RNase A cleavage were not found, but it looks like that 30% to 50% possible mismatches could be cleaved (217, 218). Numerous published ISH protocols advocated that the RNase A-digestion step after the hybridization induces better signal specificity, though with a potential risk of deleterious effect on the annealed hybrids (219). RNase A concentrations may differ in different protocols and usually range around 10-20 μg/mL (212). In ISH assays in this thesis 20 μg/mL RNase A was applied for targeting *Col10a1* and *Prg4* during post hybridization, whereas 2000 μg/mL RNase A was used for targeting *Col1a1* and *Col2a1*.

4.8 REAL-TIME POLYMERIZE CHAIN REACTIONS

The term “real time” refers to the detection of PCR products as they accumulate. Current quantitative real time PCR (qPCR) procedures are based on a set of probes and primers which determines the high specificity of this technique (220). Two approaches have been developed by Applied Biosystems for detection of qPCR products. First, the Taqman approach uses the oligonucleotide probe containing a fluorescent reporter (VIC or FAM) at 5’ end, and fluorescent quencher TAMRA or non-fluorescent quencher NFQ at 3’ end. The conventional

Taqman probes are generally linked with TAMRA resulting in 30-40 bases in length and thus less specific. The other more specific kind of Taqman probes is minor groove binder (MGB) probes linked with NFQ. Its higher specificity results from the increased melting temperature of the MGB probe, which enables a shorter length of the probe (36). During the extension phase, as the 5'-3' exonuclease activity of Thermophilus aquaticus (Taq) DNA polymerase, the probes are degraded so that reporter dye is released off the quencher group and then its excited fluorescence can be captured by a laser integrated detector (220) of the facility. Alternatively, SYBR green approach uses a dye which binds to the groove of double stranded DNA and thus forming the dye-DNA complex absorbing blue and emitting green light (36). In both cases, the reporter signal increases with PCR amplification cycling. The PCR cycle number at which the fluorescence value equals to 10 times of standard deviation of the base line fluorescence emission is used for expression quantification. This cycle number is termed the cycle threshold (Ct) and it is inversely proportional to the starting amount of target gene. Accordingly, multiplex qPCR is possible when using different fluorescent reporter linked probes against a set of genes of interests (220). Gene expression can be calculated either with the standard curve-based quantification or the relative expression to the endogenous reference gene (e.g. GAPDH, β-actin, 18S ribosomal RNA) which is expressed in a similar level within all cells. In qPCR analysis of this thesis, gene expression was calculated using the formula $2^{-\Delta\Delta Ct}$.

4.9 LIQUID CHROMATOGRAPHY

Chromatography is a crucial biophysical technique that is used to separate, purify and identify mixture components for qualitative and quantitative analysis (221). Proteins have their specific properties such as molecular sizes, shapes, charging status, hydrophobic groups at the surface, and the binding affinity to the stationary phase. Different molecules in mixtures can be separated based on those properties. While flowing with the aid of the mobile phase, some of the molecules of the mixture will be rained longer in the stationary phase thus moving slowly in the chromatography system, while some others rapidly flow through the stationary phase and move faster (221).

The solid phase in chromatography can be solid or a liquid phase coating a solid one. The mobile phase flowing through the stationary phase can be liquid or gaseous. When the mobile phase is liquid the system is termed liquid chromatography (LC), whereas if it is gaseous is named gas chromatography (GC) (221, 222).

4.9.1 Column chromatography

Column chromatography is a commonly used method to purify characteristic proteins from mixtures. In this system the column (column material inside with fiberglass support) is used as the stationary phase in which biomolecules get separated. Washing buffer as the mobile phase is used to push the separated components through the device, so they flow at the end of the system in a volume and time dependent manner based on their biophysical properties (221, 222).

4.9.2 Ion-exchange chromatography

The mechanism of ion-exchange chromatography is based on the electrostatic interactions between charged molecule groups and stationary matrix, which is loaded with oppositely charged ions. The affinity between the groups of molecules and the matrix is realized by ionic ties, and molecules can be separated by changing buffer pH, the concentration of ionic salts or the ionic strength of the buffer solution (221, 222). The positively charged matrices are termed anion-exchange matrices which adsorbs the negatively charged groups, while the positively charged groups are adsorbed by negatively charged matrices which are termed as cation-exchange matrices (221).

4.9.3 Gel-permeation (molecular sieve)/Size exclusion chromatography

The rationale behind this chromatography is to use a matrix with micropores to separate macromolecules based on their molecular sizes. The column (stationary phase) is filled with inert materials with micropores. The mobile phase containing molecule groups passes through the column at a stable flow velocity. Molecules with a bigger size than the pores cannot pass through and thus bypass by flowing through the column rapidly, whereas the smaller molecules get trapped into the micropores and thus are retained for a longer time by the column (221, 222). Sample fractions can be collected in a time or volume-dependent manner. Furthermore, molecular size calibration kits are available to exactly determine the size ranges for certain fractioned chromatographic peaks that might contain the proteins of interest. Sephadex G type, poly- acrylamide, agarose and dextran are commonly used column materials (221, 222).

4.9.4 High-pressure/performance liquid chromatography (HPLC) and Fast protein liquid chromatography (FPLC)

Using HPLC, it is possible to perform structural and functional analysis and separation of molecule groups within a short period of time. This is achieved by the application of hundreds of atmospheric pressures and a high flow rate. These special conditions yield excellent results in separating and identifying biomolecules such as carbohydrates, amino acids, lipids, proteins, nucleic acids, steroids, etc. The essential components of this system include high-pressure pump, solvent depot, commercially manufactured columns, detector and recorder. Self-automatic control is realized with the aid of computerized system (221, 222). However, these high pressure-based settings always use organic solvents and generally have the low sample loading limitation. FPLC was developed in Sweden by Pharmacia in 1982, and was originally termed as fast performance liquid chromatography to be distinguished from HPLC. FPLC has the full range of chromatography modes with versatile categories of columns which are compatible with greater sample loading amount than the conventional HPLC, but also it uses a wide range of aqueous, biocompatible buffer systems. There are many reports using FPLC since its introduction, with the two most popular used being ion exchange and gel permission (size exclusion) FPLC (221, 222).

Some other chromatography types not listed above include affinity chromatography, paper chromatography, thin-layer chromatography, gas chromatography, dye-ligand

chromatography, hydrophobic interaction chromatography, and pseudo-affinity chromatography (221). Size exclusion chromatography (SEC) (Äkta Pure FPLC, GE Healthcare technology) with a Superdex 200 Increase 10/300 GL column (GE Healthcare technology) was used in this thesis with the aim of estimating the molecular size of the synovial factor. A calibration kit (Gel filtration calibration kit, GE healthcare technology) was applied in a parallel running according to manufacturer's instructions to investigate fraction sizes.

5 RESULTS AND DISCUSSION

5.1 *IN SITU* TECHNIQUES WITH SKELETAL TISSUES

Optimized protocols of *in situ* hybridization, immunohistochemistry, and immunofluorescence for skeletal tissue (Paper I)

In situ techniques to study gene and protein expression are powerful tools to characterize the distribution of differentiation markers on tissue sections. Techniques such as *in situ* hybridization (ISH), immunohistochemistry (IHC) and immunofluorescence (IF) have been utilized to study mRNA and proteins. However, these techniques tend to produce false positive or false negative results unless carefully optimized. In addition, poor adhesion of skeletal tissue sections to glass slides is an additional challenge when using these methods. In this study we optimized ISH, IHC and IF respectively with the aim of to obtain protocols with high reproducibility, sensitivity and specificity to investigate chondrocyte differentiation markers in skeletal sections, while with preserving their morphology.

For ISH of rat femoral FFPE sections, we firstly tested different epitope unmasking methods, specifically heat induced epitope retrieval (HIER), and proteolytic-induced epitope retrieval (PIER). Consistent to previous reports, HIER often resulted in impaired tissue morphology and sometimes even caused complete detachment of tissue section. We optimized PIER by titration of proteinase K (0 μg/mL, 10 μg/mL, and 100 μg/mL) to characterize the expression of superficial chondrocytes marker *Prg4* and hypertrophic chondrocytes marker *Col10a1*. For both, 10 μg/mL proteinase K resulted in the expected expression pattern with strong signal intensity, while 0 μg/mL or 100μg/mL proteinase K resulted in false negative results (Fig 11a). To remove the background staining in *Col1a1* and *Col2a1* ISH, we found that intensified RNase A digestion, but not increased stringency of post hybridization washes successfully removed the *Col1a1*-like background in *Col2a1* ISH and *Col2a1*-like background in *Col1a1* ISH (Fig 11b). Most likely, these background stainings were the result of mis-annealed *Col2a1* probe to *Col1a1* mRNA and vice versa, as they have 70% or more of sequence similarities. These findings demonstrate the importance of selecting an optimized epitope retrieval methodology that effectively exposes antigen epitopes while maintaining tissue morphology. Moreover, the increased RNase A concentration beyond the traditional range effectively removes the background caused by partially annealed hybrids.

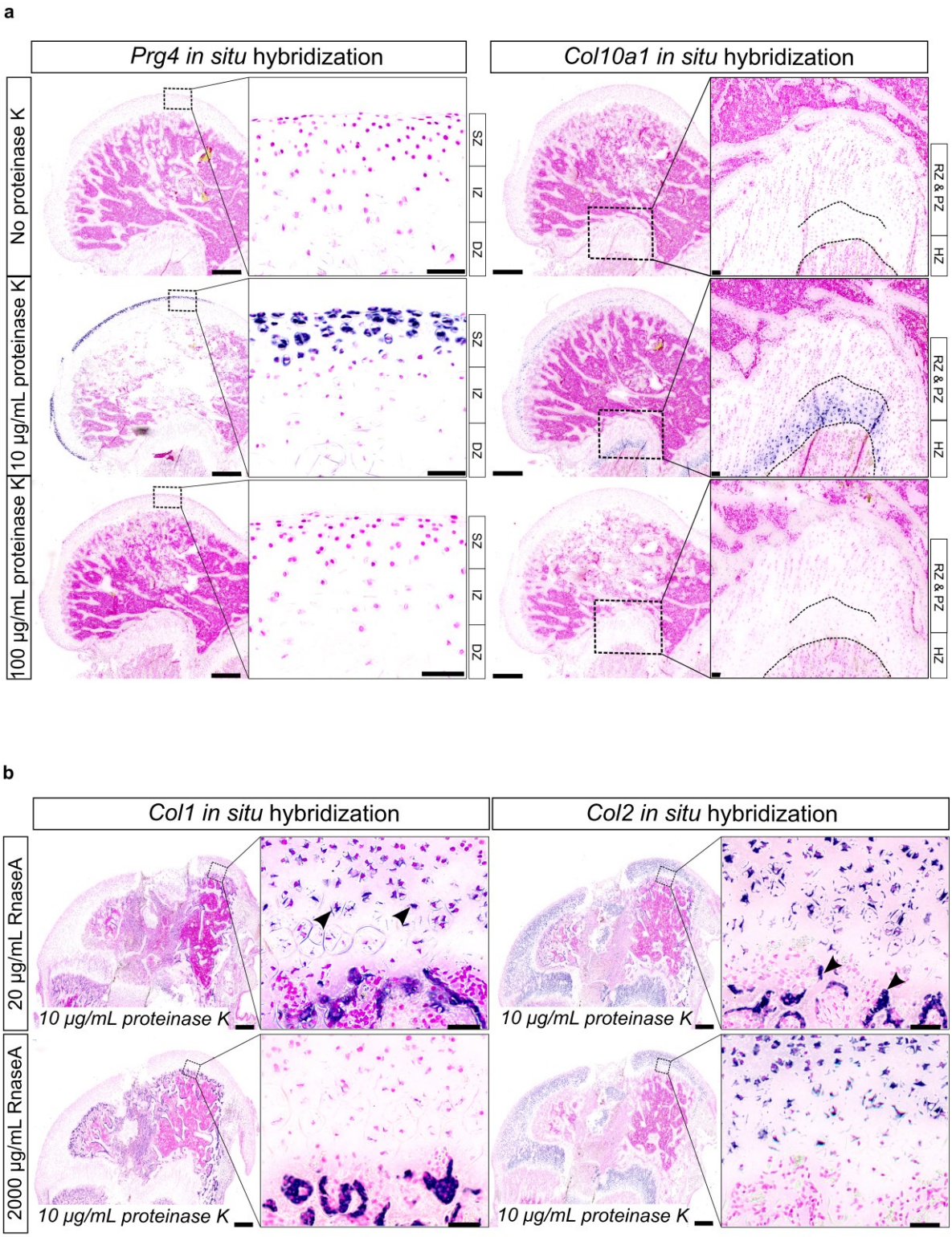


Figure 11. Titration of proteinase K and RNase A concentrations for ISH. Titration of proteinase concentration for ISH detection of *Prg4* and *Col10a1* on formalin fixed, decalcified, and paraffin embedded rat distal femur sections displayed in low and high-power magnification (a). RNase A conditions was performed with 20 or 2000 µg/mL to determine whether a highly concentrated rather than a typical RNase A concentration could prevent cross-reactivity of *Col1a1* and *Col2a1* riboprobes during ISH. Representative images of *Col1a1* and *Col2a1* ISH with standard (20 µg/mL, 30 min, 37 ◦C) and high (2000 µg/mL) RNAse concentration. Black arrowheads point to probe-specific background staining of chondrocytes and osteoblasts with the *Col1a1* and the *Col2a1* probe, respectively (b). Scale bar: 500 µm in low and 50 µm in high- power images. **SZ:** Superficial zone. **IZ:** Intermediate zone**. DZ:** Deep zone. **RZ**: Resting zone. **PZ**: Proliferative zone. **HZ**: Hypertrophic zone.

For GFP IHC of rat femoral FFPE sections, HIER had been tested first and it most often damaged tissue sections. We then performed proteinase K titration (0 µg/mL, 10 µg/mL, and 100 µg/mL) for the optimal PIER condition, to maintain a good tissue morphology and to avoid false negative results. Similar to IHC, 10 µg/mL constantly gave rise to sensitive and specific GFP tracing results, while both 0 µg/mL and 100 µg/mL led to false negative results. Proteinase K concentrations of 50 µg/mL and even higher impaired tissue histology by depleting hypertrophic chondrocytes in deep zone of articular cartilage and hypertrophic zone at the growth plate, even causing tissue section detachment off the slide (Fig 12). However, when targeting the intranuclear BrdU by IHC, the same PIER condition mostly gave negative results, which were rescued by adding a pretreatment with HCl step (DNA hydrolysis). These data indicated that PIER is a working alternative for antigen retrieval in situations where HIER impairs the morphology of FFPE skeletal tissues. Furthermore, PIER has to be individually optimized for IHC with FFPE skeletal sections. Moreover, targeting by IHC has to include a DNA hydrolysis step in combination with the optimized PIER for intra nuclear antigens such as BrdU, for a good target sensitivity while maintaining the skeletal tissue section intact. There are conflicting reports whether HCl-induced DNA hydrolysis is required for BrdU IHC. However, we found that at least when using PIER, HCl pretreatment is required for successful BrdU IHC. A possible explanation to the conflicting results is that we used PIER, whereas previous studies used HIER. Other possible explanations include differences in the tissue, species, and fixation protocols.

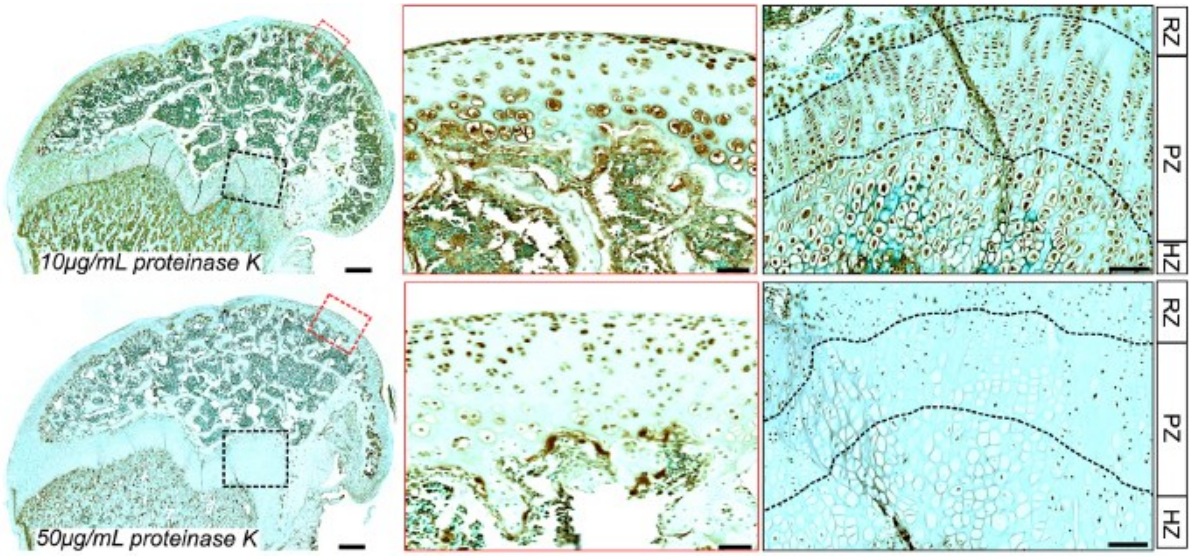


Figure 12. Titration of proteinase K concentration for immunohistochemistry. Immunohistochemistry of GFP (brown coloration) was performed on sections of formalin fixed, decalcified and paraffin embedded distal femur from eGFP-positive rats (strain: LEW-Tg(CAG-EGFP)YsRrrc). Different concentrations of proteinase K for epitope retrieval were evaluated. Slides were counterstained with methyl green and shown in low (left columns) and high power. Note the loss of hypertrophic chondrocytes in the hypertrophic zone with proteinase K concentrations higher than 10 µg/mL. The open dashed lines roughly define the individual growth plate zones. Scale bar: 500 µm in low and 100 µm in high-power images. **RZ**: Resting zone. **PZ**: Proliferative zone. **HZ**: Hypertrophic zone.

IF on OCT-embedded frozen rat skeletal tissues was also optimized in this study. Generally, the morphology of frozen skeletal sample sections is somewhat inferior compared to their FFPE counterparts, as freezing artifacts can be formed when tissues are embedded in OCT, disrupting tissue morphology, which is instead always well preserved during paraffin-embedding, thanks to steps of dehydration, vacuum and paraffin penetration. In addition, the subsequent sectioning at a freezing temperature instead of at room temperature can make a difference in tissue morphology. Considering the concern in maintaining tissue morphology, PIER was therefore not used during antibody optimization for GFP IF analysis of perichondrium recipient samples, though the indirect two-step IF results in a better signal specificity with a signal amplification effect, according to previous reports. However, in this study, indirect two-step IF did not improve GFP targeting compared to direct IF, with the same primary antibody. Two-step IF combining primary anti-GFP antibody (Ab290, Abcam, Cambridge, Storbritannien, UK) and the secondary donkey anti-rabbit IgG (A-21206, Thermo Fisher Scientific, Waltham, Massachusetts, USA) gave rise to the optimal GFP targeting (Fig 13). Consistent to the previous reports, these results indicate the importance of antibody selection and combination during IF.

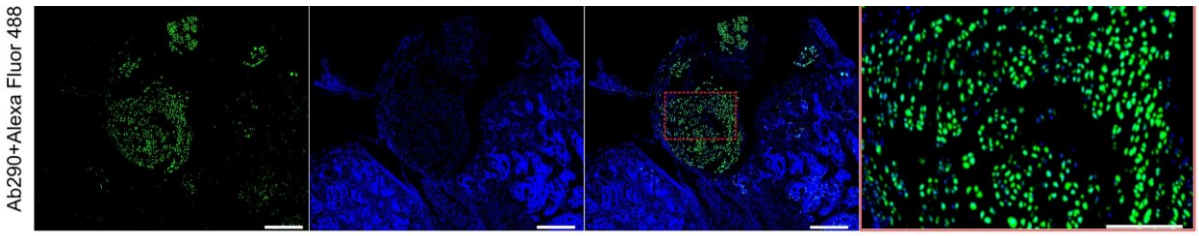


Figure 13. The optimal antibodies combination for GFP immunofluorescence. GFP immunofluorescence was performed with the primary anti-GFP antibody Ab290 and the secondary donkey anti-rabbit IgG A-21206 on distal femur sections from wild-type animals containing eGFP-positive perichondrium transplants. Immunofluorescence images displaying eGFP (green), DAPI (blue) and merge channels in low power and selected areas (red dashed squares) of merge channel displayed in high power. Scale bar: 500 µm in low power and 100 µm in high power images.

Despite this, the above optimized methodology for PIER did not work for periosteum allograft recipient femoral samples. With these samples we used 10 µg/mL proteinase K, as in previous ISH and IHC assays, but for a shorter time (3 min instead of 15 min incubation) to avoid tissue section detachment. The above antibody combination strategy and optimized PIER was successfully used for the double IF of GFP and bone marker osteocalcin.

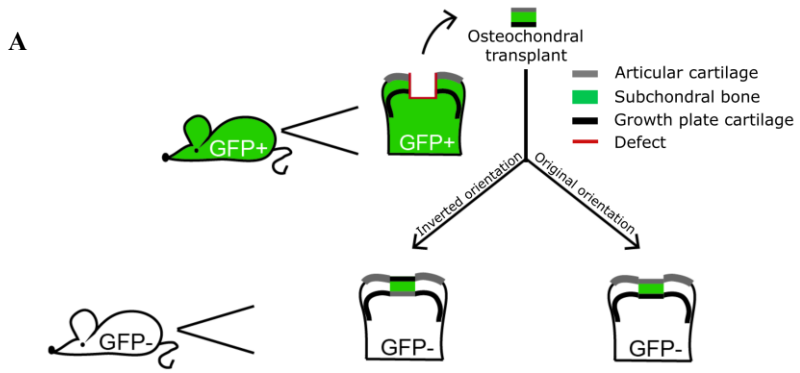
In conclusion, our results for this part show that PIER improves sensitivity and specificity of IF on formalin fixed and decalcified skeletal tissue sections, against the common thinking that antigen retrieval step can be avoided during IF of frozen tissues.

5.2 SYNOVIAL MICROENVIRONMENT EFFECT ON ARTICULAR CARTILAGE DIFFERENTIATION

5.2.1 Synovial cells secrete a temperature-stable protein that inhibits hypertrophic differentiation and induces articular cartilage differentiation of chondrocytes (Paper II)

During endochondral ossification, chondrocytes undergo hypertrophic differentiation in transient growth plate (GP) cartilage, and the newly formed cartilage is replaced by bone. In contrast, articular cartilage (AC) is a permanent cartilage in which hypertrophic differentiation and endochondral bone formation only occurs in the deep layers, closest to the subchondral bone. The microenvironments of the superficial zone chondrocytes of AC and growth plate cartilage is very different as articular chondrocytes are exposed to loading and shear stress during joint movement, as well as in direct contact with the synovial fluid. To investigate the role of the local microenvironment on articular and growth plate chondrocyte differentiation, we surgically altered the localizations of GP and AC at the distal femur in rats, by osteochondral biopsy transplantation surgery. We placed growth plate cartilage ectopically at the joint surface and articular cartilage inside the epiphysis close to the position of the growth plate. In this way, the local microenvironments of both were switched (Fig 14A).

At postoperative day 3, 7, 28 the recipient samples were collected for histological analysis by Masson's trichrome staining, for transplant localization by GFP IHC and for differentiation characterization by *in situ* hybridization. Microscopically, the integration of the graft increased over time, to become indistinguishable at day 28 post-surgery. Meanwhile, the thickness of the transplant was initially greater and eventually became similar to the adjacent native AC, without showing any proliferative columnar structure, as was seen on postoperative day 3 (Fig 14B). With time, the C*ol10a1* expression pattern of the transplanted GP shifted and became consistent with the adjacent native AC, while its original hypertrophic cells at the articular surface were altered, with smaller Prg4(+) cells at day 28 post-surgery (Fig 15). In addition, for the AC ectopically transplanted into epiphysis, some cells migrated out from the superficial zone and appeared as *Col1* positive but *Col2a1* and *Col10a1* negative. These results indicated that the GP ectopically transplanted at the articular surface gradually remodeled into an articular-like cartilage, and the ectopic AC transplanted into the epiphysis lost its AC phenotype with time. These findings suggested an important role of the synovial joint local microenvironment on maintaining AC characteristic, by inhibiting hypertrophic differentiation of chondrocytes while promoting AC differentiation.



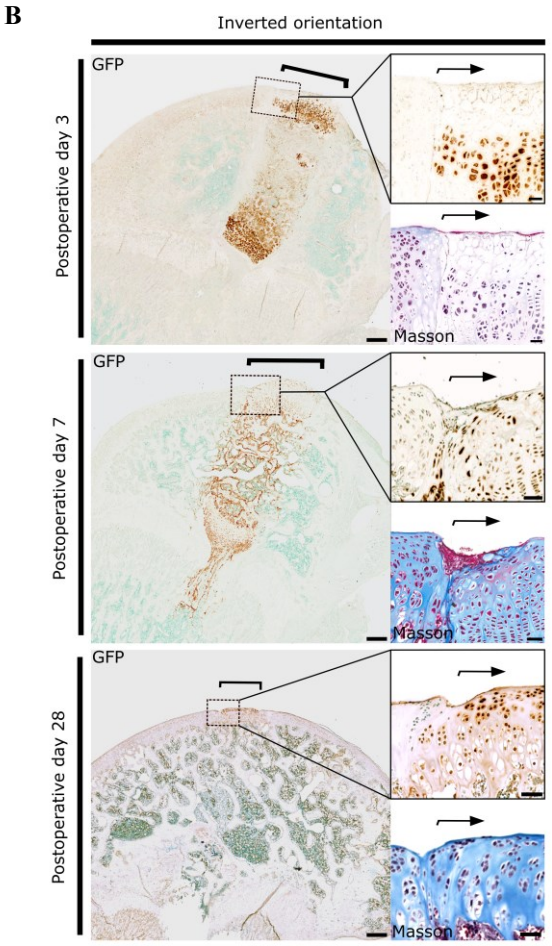


Figure 14. Experimental design, histological analysis and GFP transplanted cells tracing. Osteochondral allografts consisting of articular cartilage, epiphyseal bone and growth plate cartilage from distal femoral intercondylar articular surfaces of inbred eGFP-expressing rats were transplanted to matching sites in inbred wild-type rats in original (control) or inverted orientation. Donor and recipient animals were 4 weeks of age on the surgery day (*A*). Representative GFP immunohistochemistry staining in low and high power (upper insert) images, and representative Masson's trichrome staining (lower insert) images in high power. Arrows and brackets delineate osteochondral allografts. Scale bar: 500 µm and 50 µm in low and high-power images respectively (*B*).

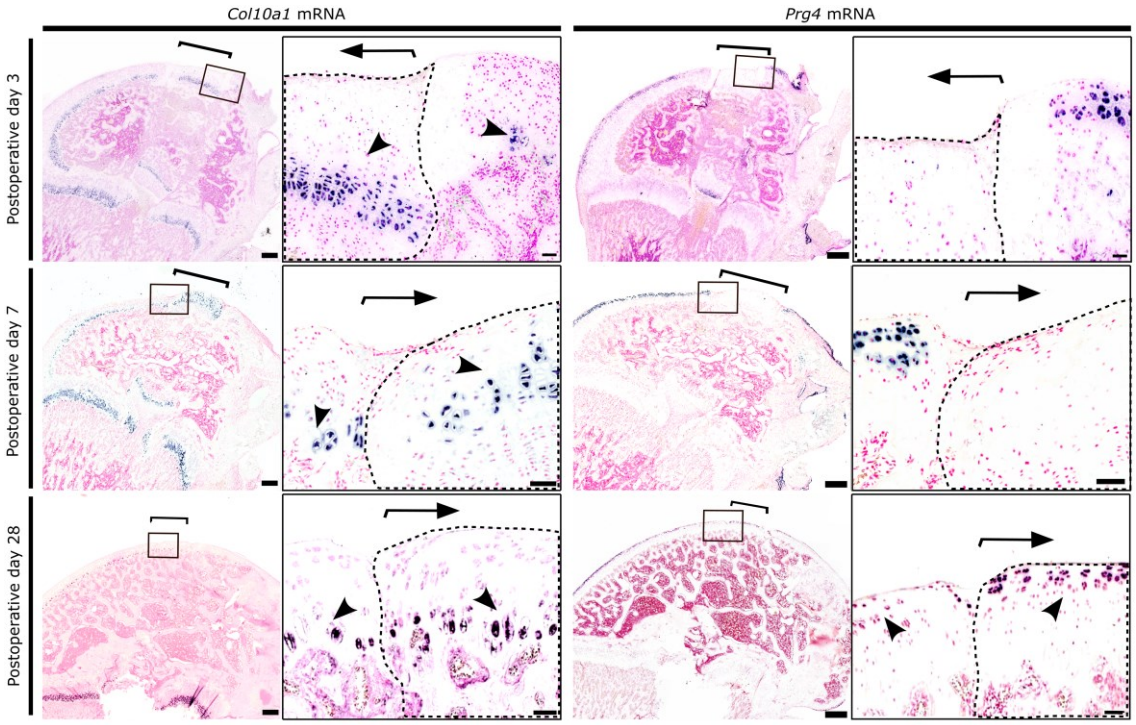


Figure 15. Targeting *Col10a1* and *Prg4* mRNAs in inverted transplants. Representative *Col10a1* and *Prg4* expression by non-radioactive digoxigenin *in situ* hybridization (stained with purple NBT/BCIP substrate and counterstained with nuclear fast red) on recipient femoral tissue sections at different postoperative time points. Arrowheads and dashed lines delineate osteochondral allografts. Scale bar: 500 µm and 50 µm in low- and high-power images respectively.

To test this hypothesis, chondrocyte pellets were cultured *in vitro* in regular (control) or synoviocyte-conditioned media and found that chondrocytes in pellet cultures exposed to synoviocyte-conditioned media were smaller, expressed less dramatically *Col10a1* (Fig 16), *ALP*, and *IHH* but higher levels of *Prg4* (Fig 16) than controls. This effect was specific to synoviocyte-conditioned media and did not occur when pellets were cultured with media conditioned by myoblasts, chondrocytes or osteoblasts. The putative synovial factor is highly thermostable and its bioactivity was found in two fractions of 35-80kDa and > 500kDa, derived from fractionation of synoviocyte conditioned media. In addition, if synoviocyte conditioned media was treated with proteinase K, the factor bioactivity decreased. Taken together, these findings suggest that synoviocytes secrete a thermostable, large (> 35kDa) protein factor that acts on the articular cartilage to inhibit hypertrophic differentiation and promote *Prg4* expression.

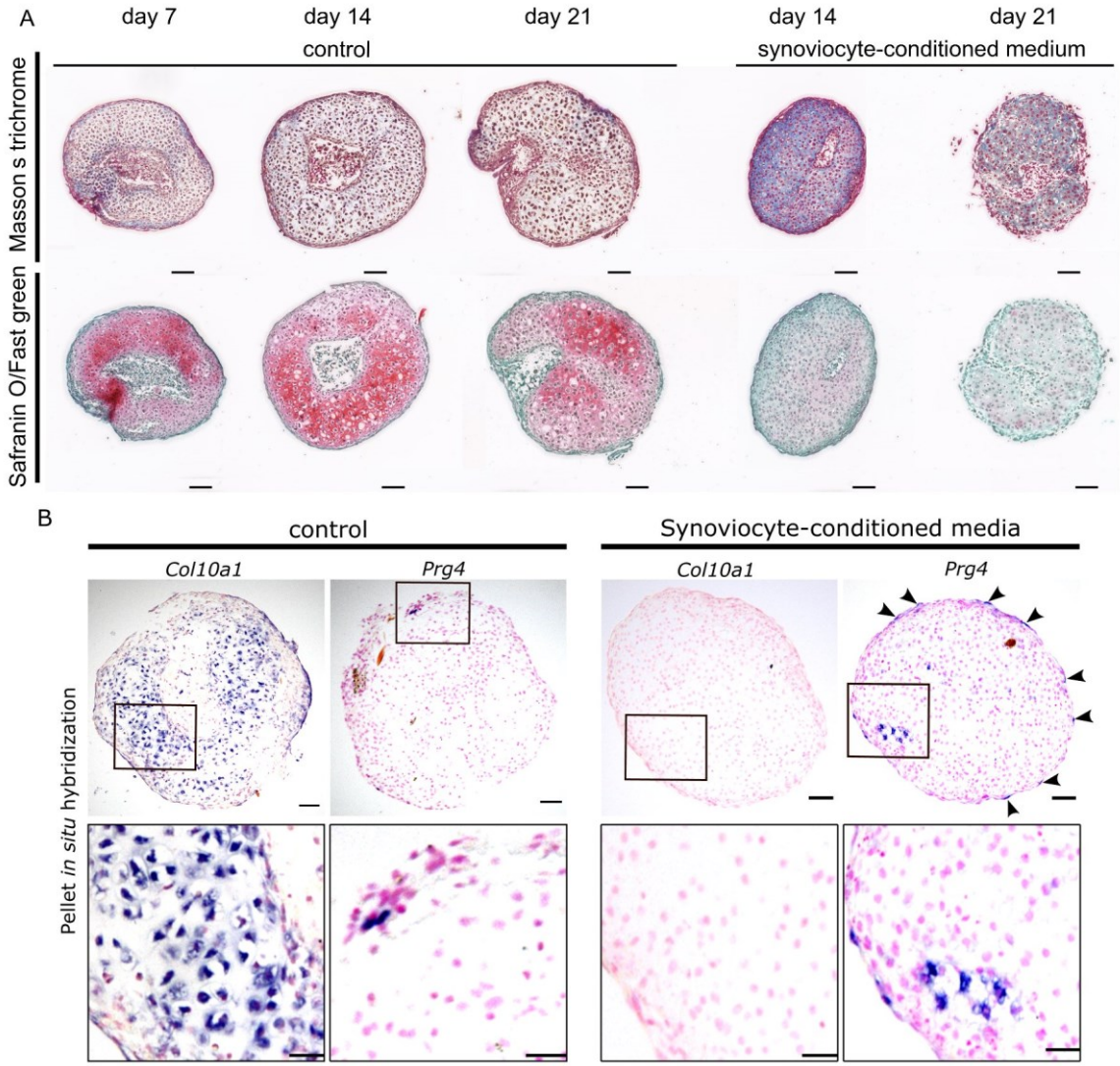


Figure 16. Synovium-conditioned medium induces articular cartilage-like phenotype *in vitro*. Histological examination of collagen content by Masson's Trichrome staining and GAG content by Safranin O/Fast green staining in representative pellet sections over time. Bars represent 100μm (*A*). Gene expression of *Col10a1* and *Prg4* analyzed in representative chondrocyte pellet sections at day 14 of culture by *in situ* hybridization. Arrowheads point to *Prg4* signal at the edge. Bars represent 100μm and 50μm in low- and high- power images (*B*).

The identification of this putative factor may represent an effective way to clinically manipulate the local synovial microenvironment to suppress the commonly reported chondrocyte hypertrophy and phenotype loss in cartilage grafts after transplantation. In this way alteration of the synovial microenvironment could lead to articular cartilage maintenance and regeneration without an invasive procedure. Currently there has not been a licensed clinical treatment for degenerative cartilage diseases such as osteoarthritis (OA). Identification of such articular chondrogenic factor(s) will shed light on understanding the pathogenesis of degenerative AC disease and AC tissue engineering.

5.2.2 Microenvironmental Influences on Articular Chondrocyte Differentiation: An *In Vivo* Study in Juvenile Goat (Paper III)

Many systems involved in the regulation of longitudinal bone growth have been identified, whereas regulation of articular cartilage formation is still less well characterized. The previous study within this thesis has identified a novel regulatory mechanism by which the local microenvironment inhibits endochondral bone formation and promotes articular cartilage differentiation and maintenance. To explore this finding further we used a large animal (goat) model, which has a thick articular cartilage including a growth plate-like structure at the bottom, next to the subchondral bone, i.e the articular-epiphyseal cartilage complex (AECC). We hypothesized that the cellular fates of the different chondrocyte layers might not be definitely determined during the embryonic stage, instead might be changed if the surrounding biochemical and biomechanical stimuli were modified. To test this hypothesis, 3-week-old goats were used for transplantation surgery during which AECC were isolated from the lateral condyle and then transplanted into the medial condyle in inverted orientation, while the same way to transplant the mentioned cartilage layers from the medial condyle to the lateral condyle of the joint. Likewise, the same transplantation operation was performed between the distal and proximal sites at the trochlear groove (Fig 17). In this way, the local cellular microenvironment of articular and growth layers of the AECC were switched.

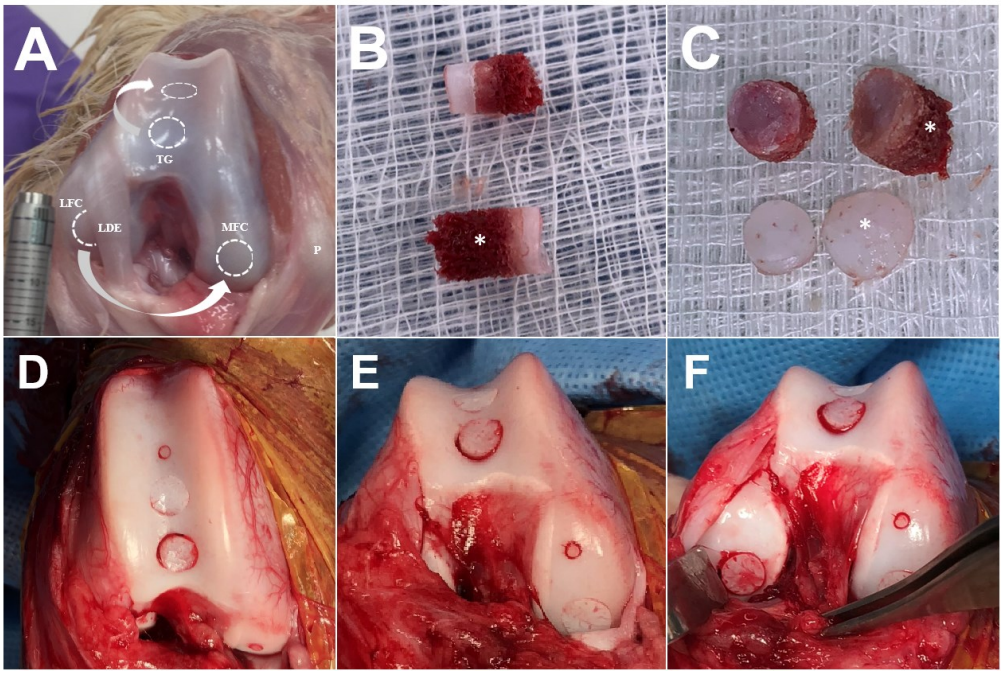


Figure 17. Schematic of donor (lateral condyle and distal trochlea) and recipient sites (medial condyle and proximal trochlea), arrows illustrate the direction of donor plug to recipient site, A. Osteochondral autografts measuring 4.75 mm x ~7 mm harvested from donor (lateral condyle or distal trochlea) and recipient sites (medial condyle or proximal trochlea) in the distal femur, B. Subchondral bone sharply dissected from the articular epiphyseal cartilage complex leaving a thin layer of cartilage to avoid transplanting osseous tissue, C. Asterisks denote the donor plug, which is approximately 1 mm larger in diameter compared to the recipient plug to facilitate press-fitting. Intraoperative articular-epiphyseal cartilage complex autograft transfers at the femoral trochlea, D, medial condyle, E, and lateral condyle, F.

At week 1, 2, 3, 6, 12, and 24 post-surgery, osteochondral samples from the joints were collected for microscopic observation, for histological analysis by Masson's trichrome staining, and for differentiation characterization by Col2a1-immunohistochemistry and *in situ* hybridization of *Col2a1*, *Col10a1* and *Prg4.* We showed that articular cartilage maintained its phenotype expressing type II collagen (Fig 18) and *Prg4* at all time points post-surgery, though it was inserted into the epiphysis and surrounded by bone.

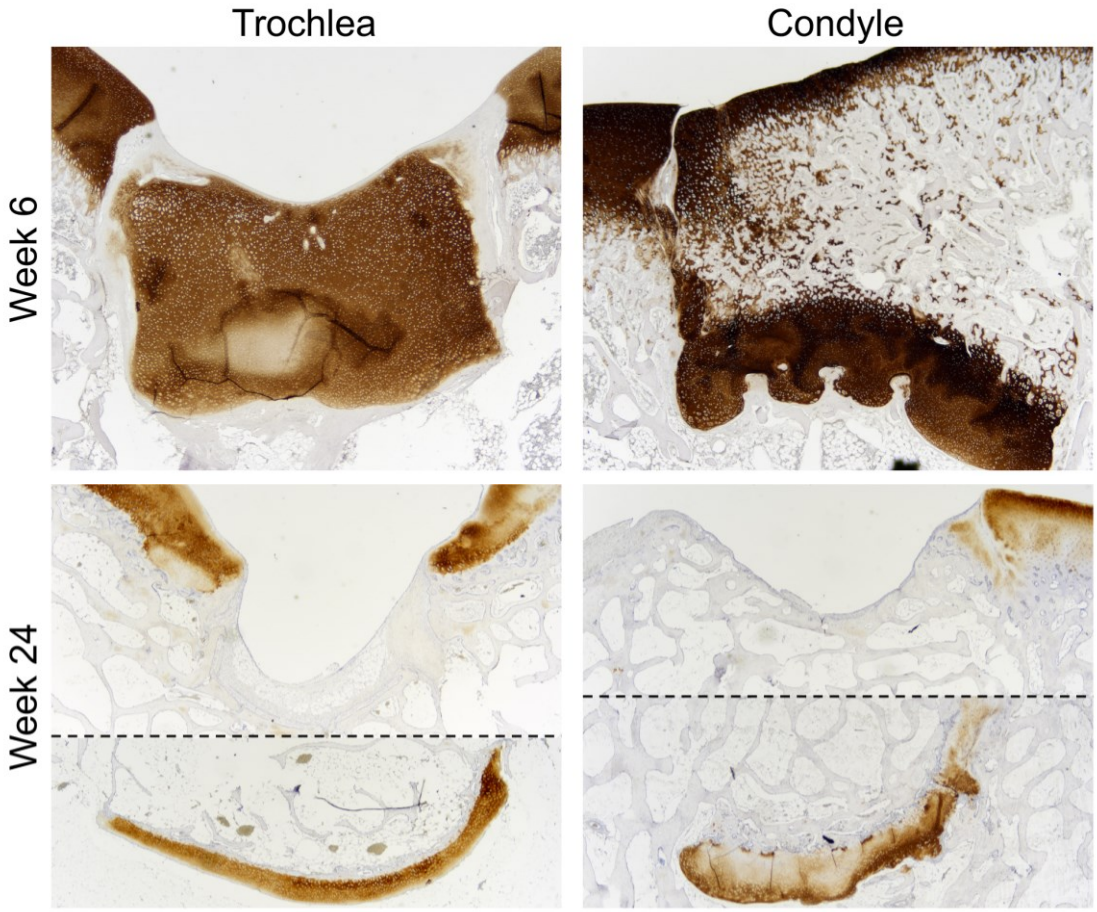


Figure 18. Type II collagen immunohistochemistry of grafts in the trochlea and condyles at 6 and 24 weeks postoperatively. Brown color staining indicate the presence of type II collagen protein. Dotted line is a break in the image.

The transplanted epiphyseal growth cartilage ectopically placed at the articular surface initially adopted an articular-like morphology, with densely packed small cells, some of which expressed *Prg4* (Fig 19 and 20). However, with time, an ossification center formed in the center of the transplanted cartilage at postoperative week 12, leaving an articular-like cartilage at the

surface which had thinned and also mostly lost type II collagen expression at week 24 (Fig 18).

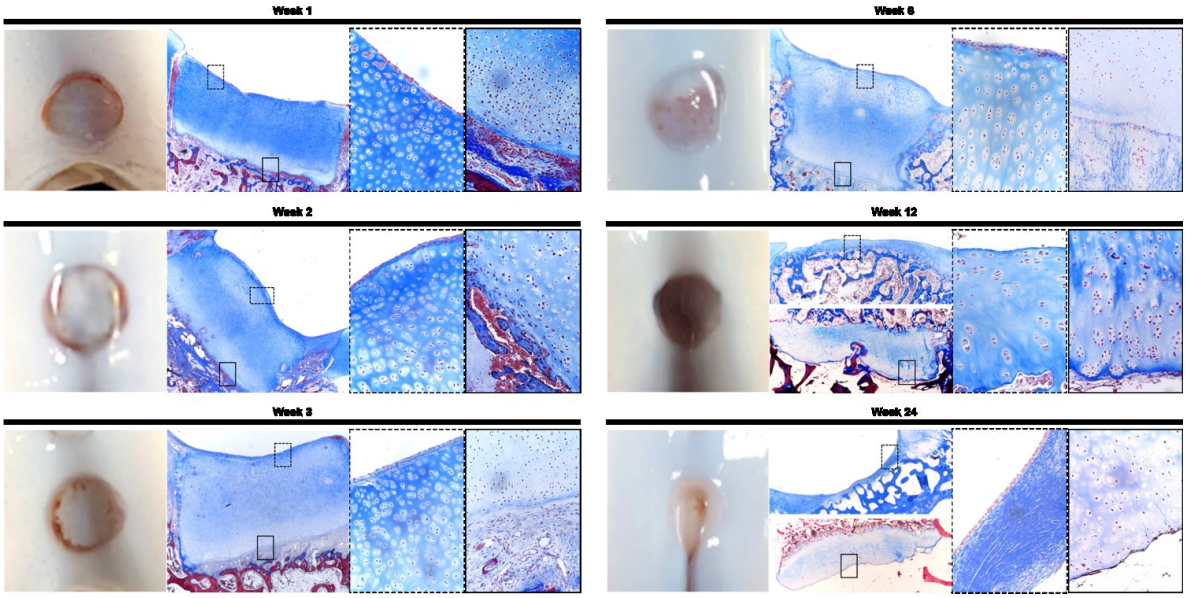


Figure 19. Masson trichrome stain of grafts in the trochlea at 1, 2, 3, 6, 12, and 24 weeks postoperatively juxtaposed with their macroscopic appearance. Connective tissues stain blue, nuclei stain black, and cytoplasm stains red. Low magnification (2x) is shown in the second column and high magnification (20x) is shown in the third and fourth columns. Dotted insets focus on the articulating surface of the grafts while solid insets focus on the epiphyseal side of the grafts.

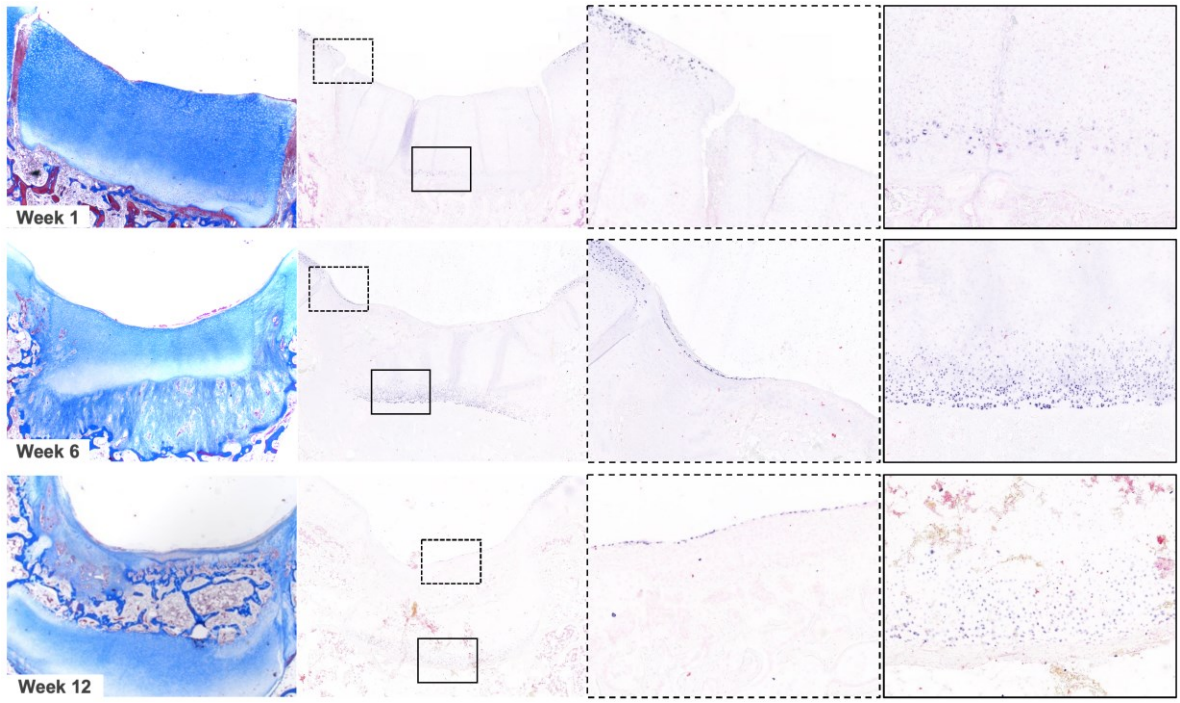


Figure 20. Lubricin (Prg4) in situ hybridization of grafts in the trochlea at 1, 6, and 12 weeks postoperatively. Purple staining demonstrates Prg4 gene expression. Tissues were counterstained with nuclear fast red.

These results suggested that some of the functions and fates of AECC chondrocytes are at least partly predetermined prenatally and might not able to be postnatally changed even if the biochemical and biomechanical microenvironment is changed. The experimental goat data indicated that the local synovial microenvironment is able to skew but could not circumvent the terminal differentiation program within the epiphyseal growth chondrocytes. Interestingly, the observed ossification started at the center of the transplant, with a morphology reminiscent of secondary ossification center. This was not found in control grafts in original orientation. Collectively these findings suggest that the protective factor(s) from the synovial microenvironment can stabilize the phenotype of AC superficial layer.

Results in this study support the conclusion of previous studies that the immobilization could delay the endochondral ossification process while extra mechanical loading was related to promoted maturation and mechanical stiffness. Grafts located at the low weight bearing trochlear groove had better integration with native articular cartilage, improved *Col2a1* expression, and experienced the central ossification later, with a time difference of 6 months when compared to grafts at high weightbearing condyle sites.

Additional studies are needed to identify the specific biochemical and/or biomechanical signals and their responsibilities for the mild cellular remodeling.

5.3 THE EFFECT AND ROLE OF PERICHONDRIUM TRANSPLANTS IN THE REPAIR OF ARTICULAR CARTILAGE INJURY

Rat perichondrium transplanted to articular cartilage defects forms articular-like, hyaline cartilage (Paper IV)

Perichondrial transplantation has been clinically applied for articular cartilage repair and resurfacing, with sometimes excellent long-term outcome (223). However, the contribution of the transplanted cells and their progenies and the quality of the reconstructed articular surfaces have not been fully investigated yet.

To this end, we harvested perichondrium and periosteum grafts from ubiquitously eGFP expressing donor rats and transplanted them into the whole-thickness defected distal femoral articular cartilage of the wild-type litter mates. Injuries were left without a transplant as control. At postoperative day 3, 14, 56, and 112 surgery distal femurs were collected for histology, transplant tracing and cellular differentiation analysis. We found that transplanted cells could be traced by GFP immunofluorescence and that almost all cells located in the defects were transplant-derived (Fig 21). Perichondrium transplants filled the defect, differentiated into hyaline cartilage that was maintained for at least sixteen weeks after the surgery. Periosteum transplants also initially filled the defect and briefly differentiated into hyaline cartilage, but it remained more prone to form fibrocartilage formation. Specifically, at postsurgery day 3, both perichondrium and periosteum transplants expressed SOX9 (Fig 22A) and by day 14 both types of transplants expressed *Col2a1* (Fig 22B) and were positive to proteoglycan, which is a hallmark of chondrogenic differentiation. SOX9 expression remained high in perichondrium samples at all time points. In contrast SOX 9 was lost at post-surgery day 14 in periosteum

transplants (Fig 22A). Consistently, *Col2a1* expression was high in both perichondrium and periosteum at day 14 and was maintained till day 112 post-surgery in perichondrium, but mostly not detected in periosteum transplants on postoperative day 56 and 112. The perichondrium samples were negative to *Col1a1* expression at all time points, while periosteum samples started to express *Col1a1* at day 14 until postoperative day 112 (Fig 22B). Interestingly, *Prg4* expression was detected in perichondrium-derived superficial cells at day 56 and 112.

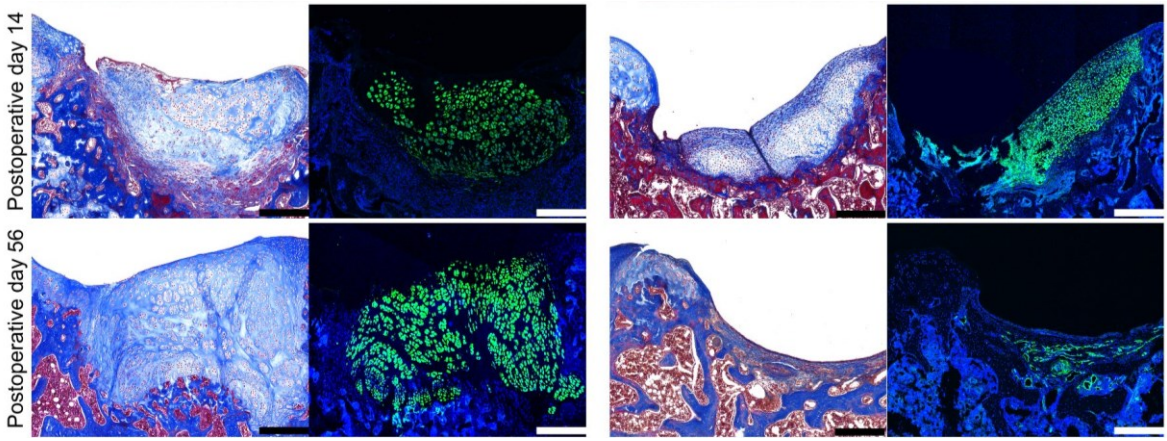
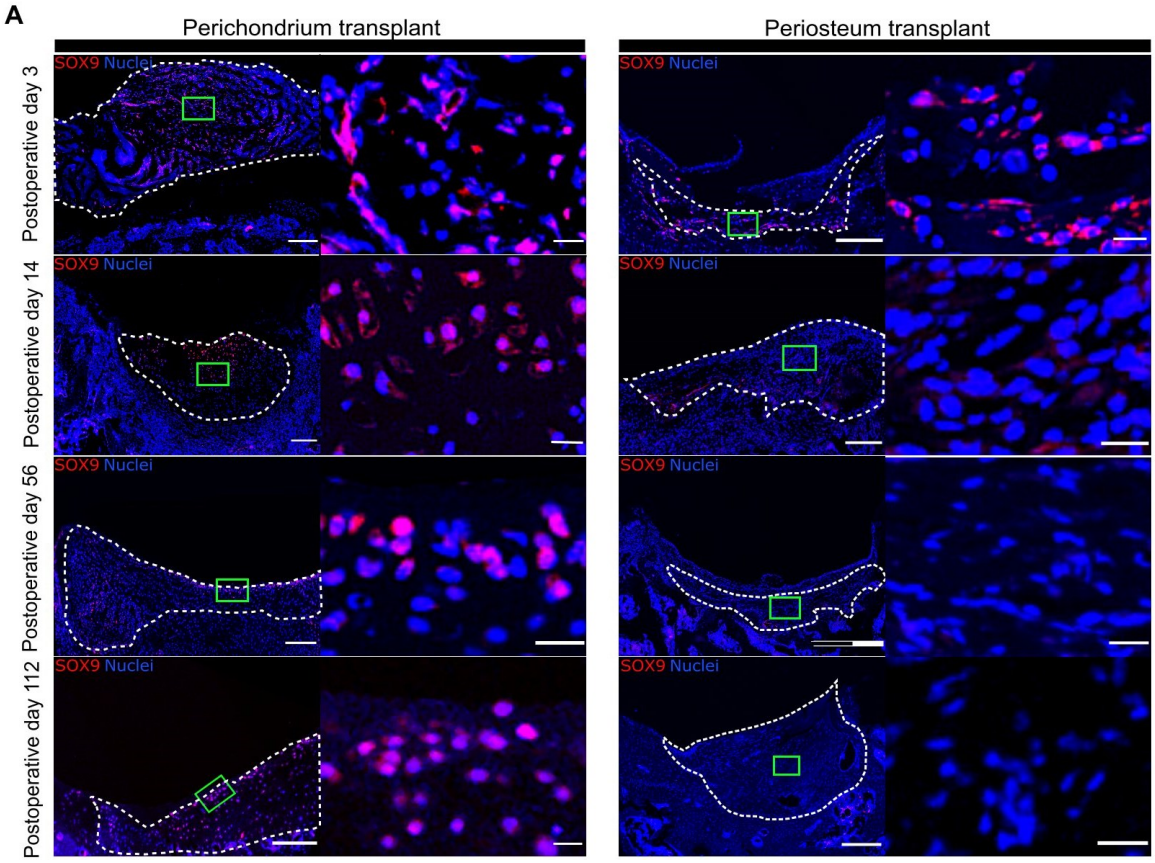


Figure 21. Tracing of transplant derived cells. Transplant derived cells were visualized using GFP immunofluorescence on frozen sections of distal epiphyses at post-surgery days 14, 56. Representative immunofluorescence images were aligned with corresponding Masson's trichrome stained tissue sections. Scale bar represents 500 μm.



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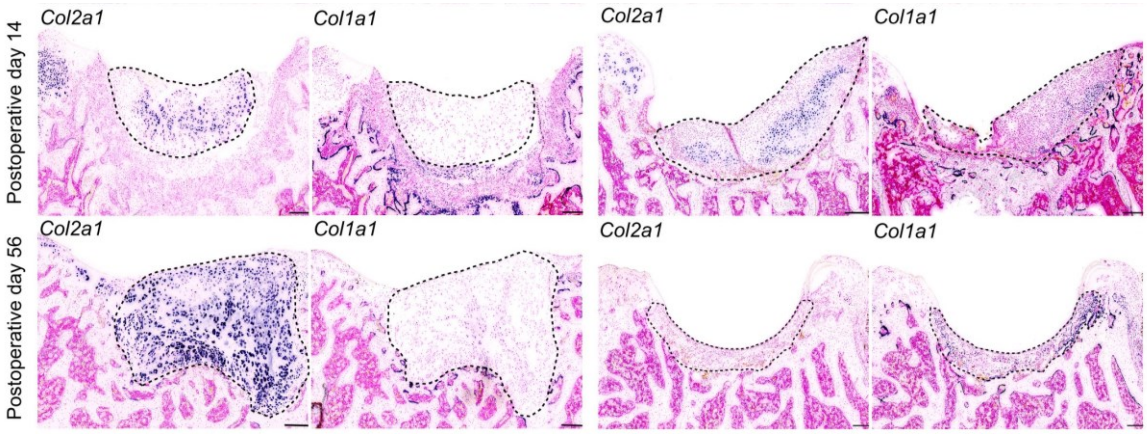

Figure 22. SOX9 immunofluorescence and collagen type I and II *in situ* hybridization of perichondrium and periosteum grafts. SOX9 protein expression was localized by immunofluorescence on frozen sections of distal epiphyses at post-surgery days 3, 14, 56 and 112. Representative immunofluorescence images of perichondrium and periosteum transplant groups with grafts indicated by white dashed lines. Scale bar represents 200 µm and 20 µm respectively in low and the green square focused high-power images (A). *Col2a1* and *Col1a1* mRNA were visualized (purple coloration) with non-radioactive digoxigenin labeled riboprobes in consecutive paraffin sections of distal femoral epiphyses. Representative *Col2a1 Col1a1 in situ* hybridization images of perichondrium (left) and periosteum (right) transplants (grafts indicated by dashed lines). Scale bar represents 200 µm (B).

In addition, *Col10a1*-positive hypertrophic chondrocytes could be detected in the central and at the bottom of perichondrium transplants, but not close to the joint surface, consistent with the hypothesis that hypertrophy might be suppressed by the synovial microenvironment. Interestingly, both perichondrium- and periosteum-derived cartilage appeared to undergo active remodeling to bone at the cartilage-bone interface. Moreover, double IF of GFP and bone marker osteocalcin suggested that both perichondrium- (Fig 23) and periosteum-derived cells differentiated and contributed to the osteoblastic cell pool of the subchondral bone.

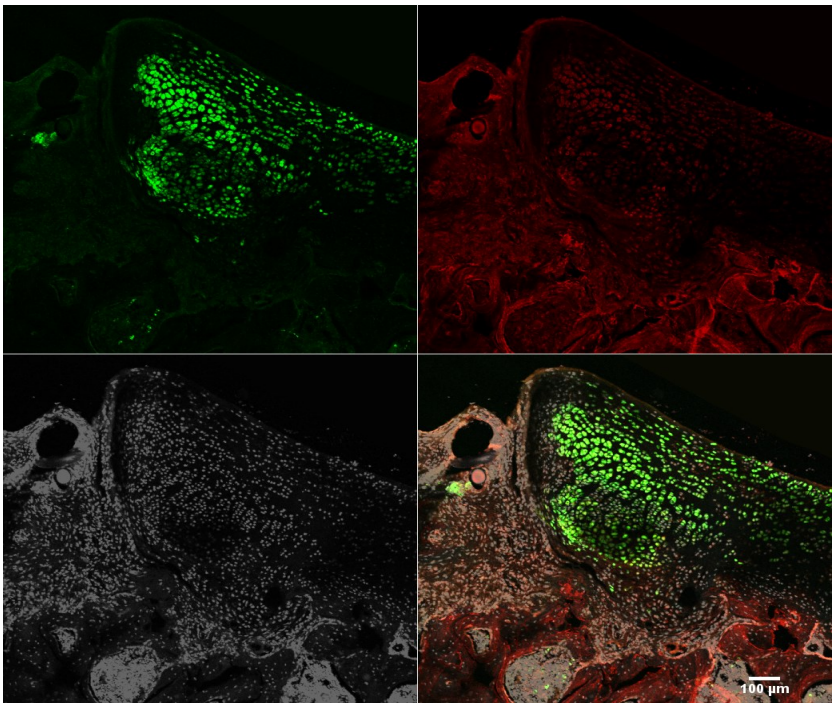

Figure 23. Double immunofluorescence of GFP and osteocalcin on the distal femur from a perichondrium recipient at day 56 post-surgery. GFP and osteocalcin were co-localized using immunofluorescence on frozen sections, and immunofluorescence images are displayed with individual channels for GFP (green) Osteocalcin (red), DAPI (grey) and merged image. Scale bar represents 100 µm.

In summary, the cells derived from transplanted rib perichondrium proliferated and expanded out of the articular cartilage defect and with time differentiated and attained a phenotype that is very similar to that of the native articular cartilage. Periosteum transplant did not show such chondrogenic potential, instead it produced a thin fibrocartilage layer providing more cells into the subchondral bone rather than articular cartilage itself. These findings suggested that perichondrium is a suitable tissue for articular cartilage repair and the applications of resurfacing injured joints and cartilage tissue engineering. Further studies exploring the above possibilities are warranted.

6 CONCLUSIONS

1. *In situ* hybridization, immunohistochemistry and immunofluorescence protocols need systematic optimization to achieve high sensitivty and specificity, epecially when applied to skeletal tissues. Optimized proteolytic-induced epitope retrieval could effectively unmask epitopes while maintaining tissue morpholgy with both FFPE and frozen skeletal tissue sections for *in situ* hybridization, immunohistochemistry and immunofluorescence. For *in situ* hybridization, cell- and probe-specific background from partly annealed hybrids occured when sequence similarity were 70% or higher. This cell- and probe-specific background could be prevented by intensified RNase A digestion. BrdU immunohistochemistry with proteolytic-induced epitope retrieval required DNA hydrolysis with HCl for optimal sensitivity and specificity. The optimizations reported in this study can be a useful reference for protein or mRNA expression studies in formalin-fixed and decalcifed skeletal tissues.

2. By switching the articular and growth plate microenvironments in rats we found evidence that synoviocytes produce a factor or several factors that inhibits hypertrophic differentiation prevent endochondral ossification at the joint surface and also promote chondrocyte differentiation towards the articular cartilage phenotype. This novel mechanism and the future identification of this putative factor may have important clinical implications for the understanding of articular cartilage development and maintenance as well as for the pathophysiology of degenerative articular cartilage disease and articular cartilage engineering.

3. We hypothesized that chondrocytes' fate towards the permanent articular cartilage or the transient growth plate cartilage which will be substituted by bone via endochondral ossification is mendable postnatally. Therefore, the location and orientation of the articular-epiphyseal cartilage complex (AECC) was surgically changed. The switch of local microenvironment did not affect the phenotype of articular cartilage surrounded by bone. On the other hand, the epiphyseal growth cartilage transplanted to the articular surface underwent morphological remodeling with the appearance of densely packed small cells at the superficial layer but then gradually lost *Col2a1* expression, and

eventually all but a thin fibrocartilage layer developed into bone. The observed transient remodeling and the central ossification of inverted epiphyseal growth cartilage grafts suggest that protective factor(s) within the synovial microenvironment promote the articular chondrocytes differentiation and/or inhibit its hypertrophic differentiaton. Besides, higher biomechanical load was associated with decreased grafts integration, graft migration, and earlier graft ossification.

4. Rib perichondrium and tibial periosteal allografts transplanted to full thickness articular cartilage defects largely contributed to the regenerated articular surfaces as they expanded, filled the injuries and integrated with the underlying bone. Perichondrium transplants formed a hyaline, articular-like cartilage with abundant proteoglycan and *Col2a1* expression, *Col1a1* negative, and at later time-points even exhibited superficial *Prg4*-positive chondrocytes. Periosteum transplants initially expressed SOX9 and *Col2a1*. However, they expressed *Col1a1* at all timepoints, were thinning and lost proteoglycan and *Col2a1* expression with time. This study demonstrate that rat perichondrium has great chondrogenic potential, and together with previous findings in humans suggest that it is a proper tissue source for repair and resurfacing of injured joints.

7 POINTS OF PERSPECTIVES

In almost all vertebrates, the formation of secondary ossification center at around the time of birth pushes the articular cartilage away from the growth plate cartilage, and since then the two cartilaginous elements only overlapped at groove of Ranvier, which may act as a source of SSCs. However, cartilage is difficult to regenerate if injured, as angiogenic factors are lacking. During the recent decades, a great deal of efforts has been made to overcome challenges towards developing preclinical and clinical practices such as microfracture, cell transplantation, stem cell identification and activation, leading to exciting findings. However, not many researches explored the protective local synovial microenvironment, though some reports indicated that synovium derived Prg4 (+) cartilage stem/progenitor cells contribute to articular cartilage injury healing.

First of all, protocols were optimized to produce optimal target sensitivity and specificity in these studies. We confirmed the hypertrophy inhibiting and articular cartilage promoting effect of the synovial microenvironment, by tracing cellular differentiation and remodeling of transplanted growth plate at the articular surface. Moreover, we found evidence that the synoviocytes secreted large-sized protein (> 35kDa) that drives the remodeling. However, this cellular fate skewing was extremely mild in our study in goats, which resulted in only transient cellular morphological changes and graft central hypertrophy. The inverted epiphyseal growth layer gradually lost type II collagen expression, formed thin fibrocartilage and ended up in central bone formation. These results suggested the existence of protective biochemical and/or biomechanical factors in the synovial local microenvironment. The identification of these

factors will help understanding of cartilage biology and will improve clinical methods of articular cartilage repair.

We also investigated the healing contribution of transplanted perichondrial cells at the full thickness defected articular cartilage. We found that perichondrium transplants could resurface the injured articular cartilage better by producing qualitative surface layer, with abundant proteoglycan content and *Col2a1* expression and Prg4 secreting superficial cells, compared to the periosteum transplants which produce *Col1a1* (+) fibrocartilage surface and induce a higher number of cells to migrate into epiphyseal bone. These findings indicated the great chondrogenic potential of perichondrium allograft, therefore its use as tissue source for cartilage engineering and regeneration should be further studied.

In the near future, firstly we will continue to refine our FPLC purification pipeline to identify the putative synovial factor(s) responsible for the effect we observed. We will use single RNA sequencing to compare the expression profiles of cells cut by laser capture microdissection (LCM) from the transplanted growth plate, native growth plate, transplanted articular cartilage and native articular cartilage *in vivo*, to study the underlying mechanisms. Based on the results, we expect to use corresponding genetic knockout animal models to functionally test the biological role of the factor(s), as well as genetic tracing of specific cell populations at different developmental stages. In addition, we may perform intra-articular collagenase injection or meniscus destabilization surgery to create OA animal models to test the therapeutic potential of the factor(s). The function of the synovial factor(s) in large mammals such as goats may also need to be studied. To summarize, all the above furthering studies will help to unveil novel regulatory possibilities for articular cartilage homeostasis at the normal or the reconstructed articular surfaces and therefore holds tremendous clinical significance for articular cartilage regeneration and the degenerative joint diseases treatment.

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