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REGULATION OF GROWTH PLATE AND ARTICULAR CHONDROCYTE DIFFERENTIATION: IMPLICATIONS FOR LONGITUDINAL BONE GROWTH AND ARTICULAR CARTILAGE FORMATION

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Karolinska Institutet

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Cover photo: Osteochondral allograft transplantation surgery – postoperative week 1. A cylindrical graft consisting of articular cartilage, epiphyseal bone, and growth plate cartilage from distal femur of an enhanced green fluorescent protein (EGFP)-expressing Lewis rat was transplanted in inverted orientation to a matching site in a Lewis rat without the EGFP transgene. Donor and recipient animals were inbred and 4 weeks of age. Graft cells are stained brown by immunohistochemistry for detection of EGFP and the whole tissue is counterstained with methyl green.

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"Success is stumbling from failure to failure with no loss of enthusiasm"
– Winston Churchill

To my beloved Family

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ABSTRACT

Overall height and body proportions in humans are determined primarily by bone growth. Linear bone growth occurs at the growth plate, a thin layer of cartilage at the ends of long bones between the epiphysis and metaphysis. In the growth plate, resting/stem-like chondrocytes divide and give rise to proliferative chondrocytes, which, in turn, enlarge to become hypertrophic chondrocytes that ultimately undergo apoptotic cell death and are replaced by bone. Articular cartilage is an embryologically related but permanent tissue that lines the ends of long bones providing a lubricated surface for articulation and distributing loads to minimize stress on underlying subchondral bone. In both growth plate and articular cartilage, precise cell signaling mechanisms ensure normal bone growth and joint maintenance, respectively, by regulating cell differentiation, proliferation, and hypertrophy as well as matrix synthesis and turnover. A better understanding of these mechanisms has broad clinical implications for preventing, diagnosing, and treating skeletal diseases.

The aim of this thesis was to study the molecular mechanisms regulating growth plate and articular chondrocyte differentiation. In this regard, similarities and differences between these structurally similar yet functionally distinct skeletal tissues were also investigated.

We first explored gene expression related to the BMP signaling system in different layers of rat growth plate cartilage using manual microdissection, microarray, and real-time PCR (Paper 1). Our findings suggest a functional BMP signaling gradient across the growth plate where BMP antagonists are highly expressed in the resting and proliferative zones and BMP agonists are highly expressed in the hypertrophic zone. Gradients in BMP action may thus provide a key mechanism responsible for the spatial regulation of chondrogenesis in growth plate cartilage and thereby contribute to longitudinal bone growth.

Another important mechanism is the Ihh/PTHrP feedback system, which prevents premature hypertrophic differentiation in embryonic epiphyseal cartilage. However, less is known about its organization in the growth plate after birth when the area undergoes substantial remodeling. We therefore explored Ihh/PTHrP-related gene expression in postnatal rat growth plate and surveyed Ihh activity in the *Gli1-lacZ* mouse growth plate (Paper 2). We found that the embryonic Ihh/PTHrP feedback system is maintained postnatally except that the source of PTHrP has shifted to a more proximal location in the resting zone. This finding provides insight into the potential role of Ihh/PTHrP signaling in growth plate senescence and fusion.

Similar to the growth plate, articular cartilage is structurally organized into chondrocyte layers; however, its cellular differentiation program is not as well characterized. Thus, we explored the similarities and differences between articular and growth plate cartilage by comparing gene expression profiles of individual rat epiphyseal cartilage layers using bioinformatic approaches (Paper 3). Our findings revealed unexpected transcriptional similarities between the deeper zones of articular cartilage and the resting zone of growth plate cartilage as well as between articular cartilage superficial zone and growth plate cartilage hypertrophic zone, suggesting that in articular cartilage, superficial chondrocytes differentiate from chondrocytes in the deeper layers following a program that has some similarities to the hypertrophic differentiation program in growth plate cartilage.

Based on these findings, we hypothesized that microenvironment regulates chondrocyte differentiation into either articular or growth plate cartilage. We tested this hypothesis by transplanting growth plate cartilage to the articular surface in an EGFP rat model that enabled cell tracing (Paper 4). We found that hypertrophic differentiation appeared to be inhibited in growth plate cartilage transplanted to the articular surface. The transplanted cartilage also underwent structural remodeling into articular-like cartilage, which suggests that the synovial microenvironment inhibits hypertrophic differentiation and promotes articular cartilage formation.

POPULAR SCIENCE SUMMARY

Ever wondered how just one cell, a fertilized egg, is able to become the tens of trillions of cells that make up your entire body? Or how the long bones in your body know when to start and stop growing and thereby determine your final body proportions and standing height as an adult? Or how two bones in a joint are able to rub against each other without eroding away like the rocks you find on the beach? These questions are worth answering not only for the sake of our curiosity but also because disturbances in the normal processes of skeletal growth, development, and maintenance are responsible for a whole host of human skeletal diseases, ranging from skeletal dysplasia causing disproportionate short stature to osteoarthritis causing inflamed and painful joints.

In order for cells to become whole organisms, you for example, they must divide and differentiate. Differentiation means that cells adopt unique functions based on where they are in your body. This occurs even though every cell in your body contains the same genetic code and originates from the same fertilized egg. For example, your bone and cartilage cells form the rigid skeleton that is enabling you to be sitting, standing, or lying down as you are reading this thesis, whereas your brain cells send and receive neurological signals, such as to and from your eyes, which are allowing you to be reading this thesis and either agreeing or disagreeing with what is being presented. This thesis specifically focuses on understanding what controls cartilage cell division and differentiation in growth plate and articular cartilage.

Wait, not so fast. Growth plate and articular cartilage are thin tissues located at the ends of long bones. Growth plate cartilage is the site of longitudinal bone growth and thus also where final body proportions and standing height are determined, whereas articular cartilage covers and protects the ends of long bones where they make contact with each other in joints. Hence, normal skeletal development and maintenance depend largely upon the functions of these two relatively small tissues. It is also worth noting that growth plate cartilage disappears after it fulfills its job at the end of pubertal development, while articular cartilage persists throughout life serving its purpose granted the absence of disease.

So here is the million-dollar question. How do your cartilage cells know when to divide and differentiate? There are many levels of complexity in the world of a cartilage cell and scientists still do not perfectly understand how cartilage cells make decisions. Similar to understanding how a car works by removing the brake, adding an extra engine, or examining individual parts separately, scientists are trying to understand what regulates cartilage cell division and differentiation by knocking out or in genes in mice and observing for abnormal skeletal growth or studying the cells in petri dishes out of their normal environment. This thesis contributes to these efforts by exploring two well-known molecular signaling pathways, called BMP and Ihh/PTHrP, as well as trying to discover novel regulatory mechanisms.

Finally, you might be asking what is a molecular signaling pathway? Cells in your body communicate with one another to exchange information just like people do. For instance, you send a letter to a friend (endocrine signaling), you talk with a friend over drinks or dinner (paracrine signaling), or you talk to yourself in the mirror (autocrine signaling). Endocrine, paracrine, or autocrine signals can trigger a cascade of activities in a cell such as DNA transcription to RNA, RNA translation to protein, or inhibition of any of these processes to help the cell decide when to divide and differentiate. In the medical field, understanding and targeting key molecular signaling pathways can potentially help prevent, diagnose, and treat human diseases.

LIST OF PUBLICATIONS

I. Nilsson O, Parker EA, Hedge A, **Chau M**, Barnes KM, Baron J. Gradients in Bone Morphogenetic Protein-Related Gene Expression Across the Growth Plate. *Journal of Endocrinology*. Apr 2007; 193(1): 75-84.

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RELATED PUBLICATIONS

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LIST OF ABBREVIATIONS

ACI	Autologous cartilage implantation
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
CACP	Camptodactyly-arthropathy-coxa vara-pericarditis syndrome
CMP	Cartilage matrix protein
COMP	Cartilage oligomeric matrix protein
DIG	Digoxigenin
DXA	Dual-energy X-ray absorptiometry
EGFP	Enhanced green fluorescent protein
EXT	Exostosin
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan
GDF	Growth differentiation factor
Gli	Glioma-associated oncogene family zinc finger
Hox	Homeobox
HZ	Hypertrophic zone of growth plate cartilage
IDZ	Intermediate/deep zone of articular cartilage
IGF-I	Insulin-like growth factor I
Ihh	Indian hedgehog
LacZ	Lac operon Z
LCM	Laser capture microdissection
LRP	Low-density lipoprotein receptor-related protein
MMP	Matrix metalloproteinase
NPR2	Natriuretic peptide receptor-2
OA	Osteoarthritis
OATS	Osteochondral autograft/allograft transplantation surgery
Osx	Osterix
Prg4	Proteoglycan 4
Ptch	Patched
PTH	Parathyroid hormone
PTHR1	Parathyroid hormone 1 receptor
PTHrP	Parathyroid hormone-related protein
PTPN11	Protein-tyrosine phosphatase, non-receptor type, 11
PZ	Proliferative zone of growth plate cartilage
qPCR	Quantitative polymerase chain reaction
Runx2	Runt-related transcription factor 2
RZ	Resting zone of growth plate cartilage
SHOX	Short stature homeobox
Smo	Smoothened
Sox	SRY-related high mobility group box
SZ	Superficial zone of articular cartilage
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
Wnt	Wingless-type MMTV integration site family
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 FORWARD

This thesis focuses on understanding the cellular and molecular mechanisms regulating growth plate and articular chondrocyte differentiation. The general aim was to study selected molecular signaling pathways regarding their roles in endochondral ossification and articular cartilage formation. To achieve this, two methods were developed: (1) manual microdissection of rodent epiphyseal cartilage to isolate individual cartilage layers and (2) microsurgical manipulation of growth plate and articular cartilage in small animals. Based on subsequent findings from high-throughput genetic analyses and histological observation, the hypothesis that an unknown growth factor in the synovial joint microenvironment supports chondrocyte differentiation into articular cartilage and/or prevents hypertrophic differentiation was explored.

The motivation for this thesis is based on the idea that understanding the normal patterns of human growth and development can lead to new ways of preventing, diagnosing, and treating human diseases. Thus, elucidating the cellular and molecular mechanisms regulating skeletal growth will potentially shed light on how skeletal diseases occur. This knowledge can, in turn, be used to develop or refine medical and surgical treatments, such as pharmacologic agents targeting specific molecular signaling pathways and tissue engineering to repair, replace, or augment dysfunctional skeletal tissues. For instance, engineered growth plate cartilage can be used to treat damaged growth plates, whereas engineered articular cartilage can be used to treat joint linings inflicted by degenerative, inflammatory, or autoimmune diseases.

2 INTRODUCTION

Skeletal development is one of the most fundamental processes of human growth and development. In caring for the pediatric patient, skeletal growth is routinely monitored with growth charts for height, and, if medically indicated, with hand x-rays for bone age, DXA scans for bone mineral density, X-rays and computer tomography scans for fractures and tumors, and blood and urine tests for signs of metabolic bone disease. Continuous monitoring of growth is important since deviations can signify the presence of primary skeletal diseases (e.g. achondroplasia and osteogenesis imperfecta), chronic illnesses (e.g. inflammatory bowel disease and celiac disease), genetic diseases (e.g. Down syndrome and Turner syndrome), endocrine disorders (e.g. growth hormone deficiency and hypothyroidism), and malnutrition (e.g. vitamin D deficiency and vitamin A toxicity).

Maintaining the structural and functional integrity of skeletal tissues (i.e. bone and cartilage) is essential for health and wellbeing during childhood as well as throughout the rest of life. The skeleton plays a multifaceted role throughout life by providing a rigid framework for the body, protecting vital organs, leveraging biomechanical movement, storing minerals, and serving as the site of hematopoiesis. Proper maintenance of skeletal tissues allows the individual to perform daily physical activities in the absence of pain and without increased risk of injury. Conversely, disturbances in skeletal integrity (e.g. osteoarthritis, rheumatoid arthritis, osteoporosis, skeletal dysplasia, rickets, osteoporosis, and malignancy) can lead to chronic pain, fracture, deformity, incapacitation, and even death.

2.1 THE SKELETON

The human skeleton is a marvellous composition of over 206 bones conventionally categorized into two subdivisions: the axial and appendicular skeletons. Based on shape, bones are further classified as long, short, flat, sesamoid [Gr. *sesamoeides*, sesame seed-like], or irregular. Bone formation occurs by two distinct processes: endochondral ossification by which bone originates from mesenchymal cell-derived cartilage condensations and intramembranous ossification by which bone arises directly from mesenchymal cell condensations. In general, long bones form by endochondral ossification, whereas flat bones form by intramembranous ossification. A summary of the major bones composing the human skeleton is presented in **Table 1** (Standring, 2008).

During early fetal life, the skeleton is predominantly cartilaginous but most of it is replaced by bone later in development. After birth, cartilage persists only in certain areas of the body, including articular surfaces, larynx, trachea, bronchi, nose, ears, ribs, intervertebral disks, pubic symphysis, joint capsules, wrists, ligamentous insertions, and epiphyseal growth plates in long bones. Cartilage is a tough and flexible tissue inhabited by only one but highly specialized cell type – the chondrocyte – sparsely embedded in an extensive extracellular matrix built of collagens, proteoglycans, and noncollagenous, also referred to as multiadhesive glycoproteins or nonproteoglycan-linked proteins. Based on appearance and matrix composition, cartilage is classified as hyaline, yellow elastic, or white fibro as presented in **Table 2** (Standring, 2008). Of interest, growth plate and articular cartilage are classified as hyaline cartilage.

Table 1. Summary of the human skeleton: 206 bones plus ossicles of the middle ears, hyoid of the throat, and sesamoids of the hands and feet.

Axial skeleton (Number of bones: 80)	**Appendicular skeleton** (Number of bones: 126)	Other
Skull: cranial: ethmoid^{I,e} (1), frontal^{F,i} (1), occipital$^{F,e\ and\ i}$ (1), parietal^{F,i} (2), sphenoid^{I,e} (1), and temporal$^{I,\ e\ and\ i}$ (2); facial: inferior nasal concha^{I,e} (2), lacrimal^{F,i} (2), mandible^{I,i} (1), maxillae^{I,i} (2), nasal^{F,i} (2), palatine^{I,i} (2), vomer^{F,i} (1), and zygomatic^{I,i} (2) **Vertebral column^{I,e}:** cervical (7), thoracic (12)**,** lumbar (5)**,** sacral (4-5), and coccygeal (3-4) **Rib cage^{F,e}:** rib (24) and sternum (1)	**Pectoral girdles:** clavicle^{L,i} (2) and scapula^{F,i} (2) **Upper limb:** arm^{L,e}: humerus (2), radius (2), and ulna (2); wrist carpals: scaphoid^{S,e} (2), lunate^{S,e} (2), triquetrum^{S,e} (2), pisiform^{SS,e} (2), trapezium^{S,e} (2), trapezoid^{S,e} (2), capitate^{S,e} (2), and hamate^{S,e} (2); hand^{L,e}: metacarpals (10), and phalanges (28) **Pelvic girdle^{F,i}:** hip (2) **Lower limb:** leg: femur^{L,e} (2), tibia^{L,e} (2), fibula^{L,e} (2), and patella^{SS,e} (2); ankle tarsals^{S,e}: calcaneus (2), talus (2), navicular (2), medial cuneiform (2), intermediate cuneiform (2), lateral cuneiform (2), and cuboid (2); foot^{L,e}: metatarsals (10), and phalanges (28)	**Middle ear ossicles^{I,e}:** malleus, incus, and stapes **Throat^{I,e}:** hyoid **Sesamoids^{SS,e}:** hand: two consistently at distal end of first metacarpal embedded within adductor pollicis and flexor pollicis brevis tendons**;** foot: two consistently at distal end of first metatarsal embedded within flexor hallucis brevis tendon

Classification of bones: L, long; S, short; F, flat; SS, sesamoid; I, irregular. Two distinct processes of bone formation: e, endochondral ossification; i, intramembranous ossification.

Table 2. Classification of cartilage based on appearance and matrix composition.

Cartilage type	**Structure**	**Function**	**Location**
Hyaline [Gr. *hyalos*, glassy]	Firm tissue containing type II collagen, proteoglycans (predominantly aggrecan), and multiadhesive glycoproteins synthesized by chondrocytes. Surrounded by perichondrium except at articular surfaces.	Serve as shock absorbers and minimal friction gliding surfaces for articulating bones, templates for endochondral bone formation, and structures for the respiratory system.	Articular surfaces, epiphyseal growth plates, ribs, nose, larynx (thyroid, cricoid, and base of arytenoid), trachea, bronchi
Yellow elastic	Flexible elastic tissue containing extensive elastic fiber networks synthesized by chondrocytes in addition to components of hyaline cartilage matrix. Surrounded by perichondrium.	Provide flexible support particularly at sites with vibrational functions.	External ear (pinna), external acoustic meatus, Eustachian tube, larynx (epiglottis, corniculate, cuneiform, and apex of arytenoid)
White fibro	Tough and dense fibrous tissue containing type I collagen and proteoglycans (predominantly versican) synthesized by fibroblasts in addition to components of hyaline cartilage matrix. No surrounding perichondrium.	Resist deformation under stress and strain.	Intervertebral discs (annulus fibrosus), joint capsules (menisci), pubic symphysis, articular discs (sternoclavicular and temporomandibular joints), wrist (triangular fibrocartilage complex), insertion of tendons and ligaments

The unique biomechanical properties of hyaline cartilage are attributed to its composition. Collagen is a structural matrix protein shaped into elongated fibrils. Type II, IX, X, and XI collagen are found in significant amounts only in cartilage and, thus, are referred to as cartilage-specific collagens, whereas type III, V, VI, XII, and XIV collagen, which are also found in cartilage, are more universally distributed (Eyre, 2002; Hughes et al., 2005; Thomas et al., 1994). Proteoglycans (e.g. aggrecan, decorin, biglycan, fibromodulin, versican, etc.) are heavily glycosylated proteins with a structure consisting of a core protein with covalently attached glycosaminoglycans (GAGs) (Knudson and Knudson, 2001; Ross and Pawlina, 2006; Roughley and Lee, 1994). GAGs are long unbranched polysaccharides made of a repeating disaccharide unit and, depending on the disaccharide unit, are classified as heparin/heparan sulfate, chondroitin/dermatan sulfate, keratin sulfate, or hyaluronan. The predominant proteoglycan in hyaline cartilage is aggrecan, which is composed of a core peptide conjugated with approximately 100 chondroitin sulfate and 60 keratan sulfate chains. At the c-terminal, aggrecan attaches to hyaluronan to form large proteoglycan complexes consisting of over 300 aggrecan molecules. Due to the presence of sulfated GAGs, aggrecan has a large negative charge and thus strong affinity for water, giving hyaline cartilage its remarkable osmotic properties and resilience to compression. Proteoglycan complexes are bound to collagen fibrils by electrostatic interactions and noncollagenous proteins. Noncollagenous proteins (e.g., thrombospondin/COMP, matrilin/CMP, link protein, anchorin CII/annexin V, fibronectin, tenascin, etc.) also modulate interactions between chondrocytes and matrix molecules (Neame et al., 1999).

Individual bones of the skeleton are held together at joints. Based on structure and degree of movement, joints are classified into three types. Fibrous/synarthrosis joints, such as the sutures between cranial bones of the skull, are fused by dense connective tissue and allow no appreciable movement. Cartilaginous/amphiarthrosis joints, such as the pubic symphysis and intervertebral disks, are connected by fibrocartilage and allow only limited movement. Synovial/diarthrosis joints are freely movable and characterized by a cavitary space internally lined by synovial membrane that may or may not contain ligamentous or meniscal structures. Based on shape and specific movement, synovial joints are further classified into plane (gliding in one plane), hinge (bending in one plane), condyloid (bending in two planes), saddle (bending in two planes), pivot (rotation), and ball and socket (all movements except gliding). Synovial joints are found mainly within the appendicular skeleton where they form connections between long bones and provide enriching local environments for articular cartilage.

2.1.1 Limb and Synovial Joint Formation

Limb (i.e. long bone) and synovial joint formation occur in parallel (Andersen, 1961; Gardner and O'Rahilly, 1968). These processes begin during embryonic life with the migration and subsequent condensation of mesenchymal cells from the lateral plate mesoderm to the outgrowing limb buds. An essential genetic switch for patterning of skeletal elements is the expression of *Hox* genes, which encode a highly conserved family of transcription factors (Goodman, 2002). Mesenchymal condensations are initially uninterrupted and at a later time differentiate into chondrocytes that express type II collagen (Craig et al., 1987; Fell, 1925; Thorogood and Hinchliffe, 1975). This cellular transformation is marked by genetic changes that activate the chondrogenic

phenotype, in particular the expression of *L-Sox5*, *Sox6*, and *Sox9*, which encode transcription factors (Bi et al., 1999; Lefebvre et al., 2001).

Cartilage templates are thereby set at the sites of future long bones and continue to elongate by cell proliferation and matrix deposition. The proximal regions give rise to the humerus and femur, whereas the more distal regions form the radius/ulna or tibia/fibula and digits. At the future sites of synovial joints, chondrocytes flatten, down-regulated type II collagen expression, and form three-layered structures known as interzones that comprise two chondrogenic cell layers separated by a thin flattened cell layer (Craig et al., 1987). The locations of joint formation are marked by the expression of *Wnt9a* (formerly *Wnt14*) (Hartmann and Tabin, 2001) and thereafter *Gdf5* (Storm and Kingsley, 1996; Storm et al., 1994), both of which encode signaling proteins. Additionally, there is a significantly reduced expression of *Sox9* and type II collagen. Articular cartilage formation and chondrocyte differentiation are further discussed in section 2.3.

At the start of the fetal period, chondrocytes in the middle of cartilage templates stop proliferating, hypertrophy, and release growth factors, such as VEGF, that attract blood vessels and osteoblasts that, in turn, develop primary ossification centers. Expression of the transcription factor *Runx2* is essential for permitting chondrocyte maturation and vascular invasion (Komori et al., 1997), and the transcription factor *Osx* acts downstream to permit subsequent bone formation (Nakashima et al., 2002). Concurrently, cells in the interzone differentiate into fibrous capsules, synovial membranes, menisci, and cruciate ligaments and undergo central delimitation to give rise to joint cavities (Khan et al., 2007; Pacifici et al., 2005). Shortly after birth, secondary ossification centers form in the middle of the epiphyses at one or both ends of long bones, thereby compartmentalizing articular cartilage to the joint surface and growth plate cartilage between the epiphysis and metaphysis (**Fig. 1**).

2.1.2 Longitudinal Bone Growth

Longitudinal bone growth occurs at the growth plate by endochondral ossification (**Fig. 1**), a process by which new cartilage is formed and continuously remodelled into new bone tissue. During long bone development, the thickness of the growth plate remains relatively constant, as the amount of cartilage produced matches the amount of cartilage replaced by bone (Kember and Sissons, 1976). Chondrocyte proliferation and hypertrophic differentiation as well as production of new cartilage matrix collectively extend the epiphyses away from the diaphysis to lengthen the bone. In order to retain proper proportions and unique shapes during the elongation process, long bones undergo preferential remodeling of metaphyseal surfaces by bone resorption and deposition (Whalen et al., 1971). The cellular and molecular mechanisms by which the growth plate forms new cartilage that subsequently becomes remodelled into bone are discussed in section 2.2.

Longitudinal bone growth ceases at the end of puberty when growth plates are completely replaced by bone, fusing the epiphysis and metaphysis. The mechanisms leading to this terminal event are characterized by functional decline and structural involution of the growth plate and are collectively termed growth plate senescence (Nilsson and Baron, 2004). There is a dramatic decline in growth rate that is in large due to less cell division in the proliferative zone and also a decrease in the size of the hypertrophic chondrocytes. These functional changes are accompanied by structural

changes, including a gradual reduction in growth plate height due to a decrease in the number of proliferative and hypertrophic chondrocytes and thus cell density of the columns (Sissons and Kember, 1977). With increasing age there is also a quantitative and qualitative depletion of progenitor cells in the resting zone (Schrier et al., 2006).

On the molecular level, the endocrine hormone estrogen is a key regulator of growth plate senescence. This has been demonstrated by two unique cases of individuals with homozygous nonsense mutations in the gene encoding estrogen receptor α that resulted in estrogen resistance (Quaynor et al., 2013; Smith et al., 1994), and multiple cases of males and females with autosomal recessive mutations in *CYP19* causing aromatase enzyme deficiency and, in turn, a complete lack of estrogen (Morishima et al., 1995). These patients exhibited drastically delayed bone age, incomplete growth plate fusion, and tall stature due to continued longitudinal bone growth into adulthood. Conversely, premature estrogen exposure, as in precocious puberty, leads to bone age advancement, early growth cessation and hastened epiphyseal fusion. Studies in rabbits demonstrated that estrogen does not hasten epiphyseal fusion directly, but instead accelerates the program of growth plate senescence leading to earlier cessation of chondrocyte proliferation and growth, thereby indirectly causing earlier fusion (Weise et al., 2001). In particular, the finding that estrogen irreversibly accelerates the depletion of progenitor cells in the resting zone may explain the mechanism by which estrogen accelerates growth plate senescence and hastens epiphyseal fusion (Nilsson et al., 2014).

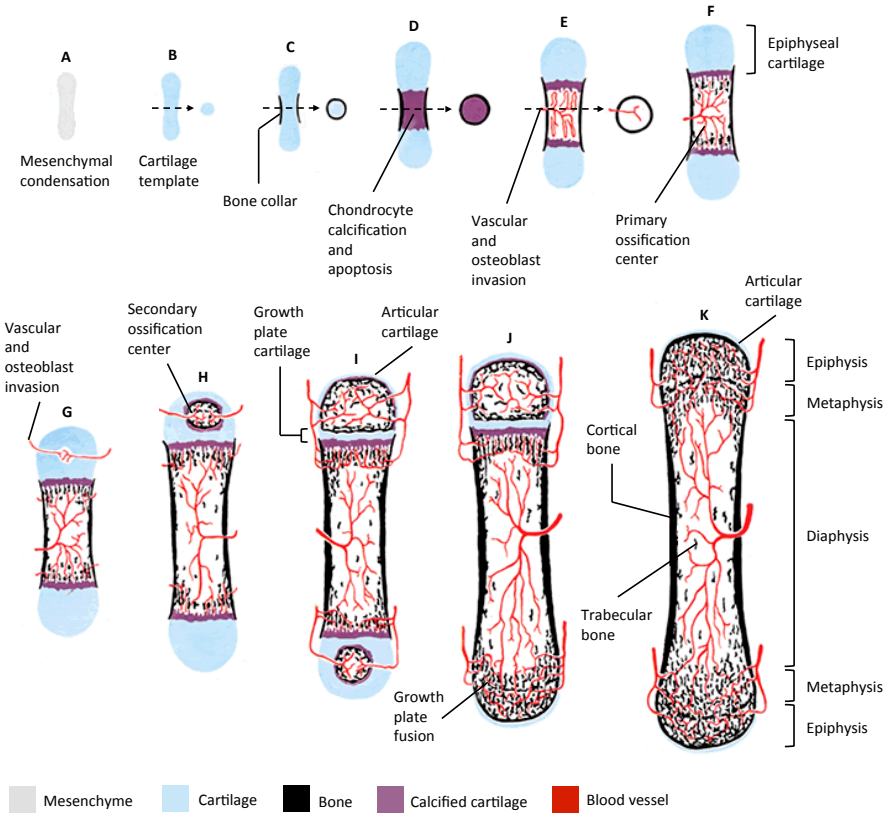

Figure 1. Schematic diagram of long bone development. **A**, mesenchymal cells condense. **B**, cells differentiate into chondrocytes that proliferate; cells at the periphery differentiate into a thin layer of perichondrial cells. **C**, chondrocytes at the center stop proliferating and become hypertrophic; perichondrial cells adjacent to hypertrophic chondrocytes become osteoblasts and form a bone collar **D**,

hypertrophic chondrocytes direct calcification, attract blood vessels and osteoblasts, and undergo apoptosis. **E**, blood vessels and osteoblasts invade the diaphysis. **F**, primary ossification center forms. **G**, blood vessels and osteoblasts invade the epiphyses. **H**, secondary ossification centers form; the developing growth plate forms orderly columns of proliferating chondrocytes. **I**, secondary ossification centers expand and compartmentalize growth plate and articular cartilage. **J**, growth plates undergo senescence and fuse. **K**, longitudinal bone growth ceases. Adapted and modified from (Ross and Pawlina, 2006).

2.1.3 Preservation of Articular Surfaces

Unlike the growth plate, articular cartilage does not normally undergo endochondral ossification and, barring disease and injury, is preserved throughout life. Articular cartilage is only 2 to 5 millimeters thick but possesses exceptional resilience against sheer and compressive forces, the ability to distribute loads that effectively minimizes stress on subchondral bone, and durability that in many people grants normal joint function for 80 years or more (Buckwalter et al., 2005). Unfortunately, the repair of articular cartilage once damaged is not trivial. Articular cartilage by nature of its composition and metabolism has a poor regenerative capacity and, with age, articular chondrocytes become less efficient at producing matrix macromolecules and undergoing cell division (Buckwalter et al., 2005; Ulrich-Vinther et al., 2003).

Articular cartilage lacks blood vessels, lymph vessels, and nerves. Resident chondrocytes derive all of their nutrients and oxygen by simple diffusion from the synovial fluid, which is a plasma ultrafiltrate in the joint cavity containing water, nutrients such as electrolytes, small molecules, and glucose, and synovial cell secretions such as proteoglycans, proteases, and cytokines (Huber et al., 2000). To reach articular chondrocytes, nutrients and other small molecules pass through a double diffusion system consisting of first the synovial membrane and then the cartilage matrix. Diffusion through the cartilage matrix relies heavily upon intermittent joint loading, which flushes out interstitial fluid and allows new fluid containing nutrients to flow in (O'Hara et al., 1990). Intermittent joint loading also affects articular chondrocyte metabolism in the synthesis and breakdown of matrix macromolecules. This internal remodeling process involves a complex interplay of cytokines with catabolic and anabolic effects. TNF-α and IL-1 induce the expression of MMPs, COX-2, and nitric oxide synthetase that cause matrix degradation, whereas IGF-I and TGF-β stimulate matrix synthesis and cell proliferation (Ulrich-Vinther et al., 2003). Joint loading stimulates internal remodeling by altering interstitial hydrostatic pressure, ion concentration, pH, and pericellular proteoglycan concentration as well as deforming chondrocytes (Kim et al., 1994). Conversely, extended joint immobilization leads to reduced proteoglycan synthesis and cartilage loss (Palmoski et al., 1979).

The cellular organization of articular cartilage is discussed in section 2.3. In general, articular cartilage is a hypocellular tissue and resident chondrocytes make up only 3 to 5% of the overall wet weight (Ross and Pawlina, 2006). Taking together an ungainly means of receiving nutrients and cell-to-cell signaling, a low metabolic rate, and a low cell density with declining proliferative capacity with age, articular cartilage has limited potential for self-regeneration. Therefore, any damage to articular cartilage be it repetitive minor insults or a traumatic injury risks leading to progressive tissue deterioration.

2.2 GROWTH PLATE CARTILAGE

2.2.1 Structure and Function

The growth plate is a thin disk of hyaline cartilage located between the epiphysis and metaphysis at one or both ends of long bones (**Fig. 2**). It is avascular but located in sufficient proximity to systemic vasculature at the two chondro-osseous junctions and perichondrium (Williams et al., 2007). Based on chondrocyte size, shape, organization, and function, the growth plate is subdivided into three distinct layers or zones: resting, proliferative, and hypertrophic. The resting zone lies directly beneath the secondary ossification center and contains "stem-like" cells that give rise to daughter proliferative chondrocytes (Abad et al., 2002). Proliferative chondrocytes arrange into columns resembling "stacks of coins" parallel to the long axis of the bone and undergo rapid cell division that contributes to longitudinal bone growth (Abad et al., 2002; Kember and Walker, 1971). At the bottom of the proliferative zone, chondrocytes stop proliferating and undergo hypertrophy, a process characterized by gains in cell height, intracellular volume, and organelle size up to 4-, 10-, and 3-fold, respectively, and that also contributes to longitudinal bone growth (Cooper et al., 2013; Hunziker et al., 1987). Hypertrophic chondrocytes modify their genetic program to express type X collagen, alkaline phosphatase, MMPs, and VEGF that direct matrix calcification and invasion of blood vessels and osteoblasts from the underlying metaphysis (Gerber et al., 1999; Schmid and Linsenmayer, 1985; Vu et al., 1998; Xu et al., 1994). Finally, hypertrophic chondrocytes undergo apoptotic cell death leaving behind a scaffold for endochondral bone formation (Kronenberg 2003). This stepwise program of growth plate chondrocyte proliferation and differentiation attributes to longitudinal bone growth.

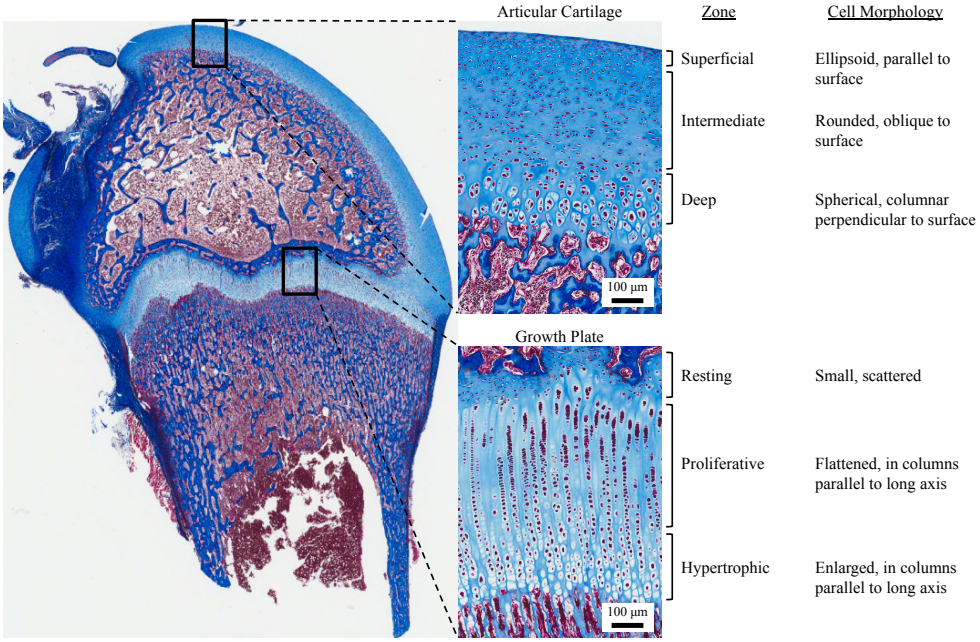

Figure 2. Morphology of growth plate and articular cartilage. Masson's Trichrome stain of a 1-month-old New Zealand white rabbit distal femur. The height of articular cartilage progressively declines until 3 months, which is when puberty begins in this species, and the growth plate progressively thins and fuses by 8 months (Hunziker et al., 2007). Colors: black = nuclei; blue = collagen and bone; red = cytoplasm.

2.2.2 Regulation of Chondrocyte Proliferation and Differentiation

To ensure normal longitudinal bone growth, systemic (endocrine) and local (autocrine and paracrine) signaling pathways must regulate the proliferation and differentiation of growth plate chondrocytes (Nilsson et al., 2005; van der Eerden et al., 2003). Among the key local signaling molecules in the growth plate are bone morphogenetic proteins (BMPs), Indian hedgehog / parathyroid hormone-related protein (Ihh/PTHrP), wingless-type MMTV integration site family (Wnts), and fibroblast growth factors (FGFs).

2.2.2.1 Bone Morphogenetic Proteins

BMPs were initially discovered as the component in demineralized cell-free bone matrix capable of inducing ectopic bone formation in soft tissue (Urist and Strates, 1971; Urist, 1965). Successful isolation, purification, and cloning of the first BMPs occurred over several decades (Luyten et al., 1989; Sampath and Reddi, 1981; Wozney et al., 1988). BMPs are now known to be part of the TGF-β superfamily of signaling polypeptides, which includes TGF-β, growth differentiation factors (GDFs), activins, and inhibins. To date, more than 20 BMPs have been identified and characterized, some of which overlap with the GDF subfamily (Bragdon et al., 2011; Reddi, 1997).

BMPs signal through cell surface serine/threonine kinase receptors, either type I (e.g. BMPR-IA, -IB, and ActR-I) or type II (e.g. BMPRII, ActR-II, and ActR-IIB), to trigger intracellular pathways involving Smad proteins. Two of the three transducer Smads (Smad-1, -5, or -8) complex with Smad-4 and translocate into the nucleus to bind specific DNA sequences to influence gene transcription (Derynck and Zhang, 2003). BMP signaling is regulated by various mechanisms, including extracellular binding of BMP ligands by the antagonistic proteins Noggin (Zimmerman et al., 1996), Chordin (Blader et al., 1997), and Gremlin (Hsu et al., 1998) as well as intracellular obstruction of type I BMP receptors by inhibitor Smad-6 (Imamura et al., 1997).

BMPs play diverse roles in the morphogenesis and homeostasis of many organs (Reddi, 2005). In the growth plate, BMPs and their receptors are expressed by chondrocytes and perichondrial cells and have been shown to regulate cartilage formation and maturation (Kobayashi et al., 2005; Tsumaki et al., 2002). For instance, infection of embryonic chick limbs with retroviruses encoding BMP-2, -4, and GDF-5 increased chondrogenesis and final sizes of skeletal elements (Duprez et al., 1996; Francis-West et al., 1999). Similarly, *in vitro* administration of BMP-2 to rat fetal metatarsal bones or mouse embryonic stem cell lines increased chondrocyte proliferation and hypertrophy, whereas addition of Noggin elicited the opposite effect of preventing hypertrophic differentiation, thus indicating endogenous production of BMPs (De Luca F et al., 2001; zur Nieden et al., 2005). Furthermore, mice deficient in both BMPR-IA and -IB receptors in cartilage lacked most skeletal elements that form by endochondral ossification and those that formed were rudimentary, demonstrating the importance of BMP signaling in early chondrogenesis (Yoon et al., 2005). Conversely, mice overexpressing the BMPR-IA receptor had shortened proliferative columns and accelerated hypertrophic differentiation in the growth plate, suggesting BMP signaling also stimulates chondrocyte maturation (Kobayashi et al., 2005). Moreover, loss of BMP antagonism in Noggin (Brunet et al., 1998) and Gremlin (Khokha et al., 2003) knock-out mice led to multiple skeletal abnormalities,

including enlarged growth plates and defective patterning and outgrowth of limbs. Conversely, cartilage-specific overexpression of antagonist Smad6 in mice caused significantly delayed chondrocyte hypertrophy, thin trabecular bones, and dwarfism (Horiki et al., 2004, p. 6).

Taken together, BMPs have essential roles at many stages of endochondral bone formation and accelerates longitudinal bone growth at the growth plate by promoting chondrocyte proliferation and differentiation. BMPs are potent morphogens and are currently used in tissue-engineering applications to repair bone and cartilage. Since 2001, the U.S. Food and Drug Administration has approved the use of recombinant human BMP-2 and -7 for treating spinal fusion and nonunion long bone fractures (Ong et al., 2010).

2.2.2.2 Indian Hedgehog / Parathyroid-related Protein

The role of PTHrP on the skeleton was first observed by investigators identifying PTHrP as the tumor-secreted protein responsible for hypercalcemia of malignancy (Strewler, 2000; Suva et al., 1987). PTHrP is structurally similar to parathyroid hormone (PTH) at the amino-terminal from amino acid 1 to 34 (Martin et al., 1991) and both signal through the PTH/PTHrP G-protein-coupled receptor (PTHR1) (Jüppner et al., 1991). In relation, Ihh belongs to the hedgehog family of signaling proteins including Desert and Sonic hedgehog, the latter of which regulates patterning in many systems including the limb (Riddle et al., 1993). Ihh binds to and impairs the function of the cell surface receptor Patched, which normally represses the activity of the transmembrane protein Smoothened (McMahon, 2000). Reduced inhibition of Smoothened allows Gli transcription factors to translocate to the nucleus and upregulate target gene expression, including *PTHrP, Patched*, *Hip*, and *Gli1* itself (di Magliano and Hebrok, 2003; Koziel et al., 2005).

In humans, activating mutations of PTHR1 cause Jansen's metaphyseal chondrodysplasia characterized by short bowed limbs with normal growth plates but disorganized metaphyseal regions (Schipani et al., 1995), whereas inactivating mutations of PTHR1 cause Blomstrand lethal chondrodysplasia characterized by short limbs, increased bone density, and advanced skeletal maturation (Loshkajian et al., 1997). The physiological roles of PTHrP have been further studied in genetically modified mice. Mice lacking the genes for *PTHrP* (Karaplis et al., 1994), *PTHR1* (Lanske et al., 1996), or *PTH* (Miao et al., 2002) either die at birth due to skeletal underdevelopment or exhibit accelerated chondrocyte differentiation and decreased endochondral bone formation, demonstrating the importance of PTHrP signaling as a negative regulator of hypertrophic differentiation in the growth plate.

Early studies of *Ihh* expression in chick embryo localized it to the midgut, lungs, and cartilage templates of long bones (Vortkamp et al., 1996). This work contributed to the discovery of interaction between Ihh and PTHrP in embryonic epiphyseal cartilage and the formulation of the classic Ihh/PTHrP feedback loop that regulates the rate of hypertrophic differentiation (Vortkamp et al., 1996). Ihh and PTHrP have also been shown to be expressed by human growth plate chondrocytes (Kindblom et al., 2002). The Ihh/PTHrP feedback loop in prenatal epiphyseal cartilage determines the location where proliferative chondrocytes stop proliferating and start to undergo hypertrophic differentiation, and thus the length of proliferative columns.

In prenatal epiphyseal cartilage, PTHrP is expressed by periarticular chondrocytes and primarily act to maintain proliferative chondrocytes in the mitotic

state. As proliferative chondrocytes grow distant from the source of PTHrP they undergo hypertrophy. Ihh is produced by prehypertrophic and hypertrophic chondrocytes and signals by perichondrium dependent and independent pathways to periarticular chondrocytes to express PTHrP, proliferative chondrocytes to increase the rate of cell division, and perichondrial cells to form bone collar (Chung et al., 2001; Karp et al., 2000; Kronenberg, 2003; St-Jacques et al., 1999).

2.2.2.3 Wingless-Type MMTV Integration Site Family

Wnts comprise a family of highly conserved secreted signaling glycoproteins that regulates cell differentiation, proliferation, and fate determination during embryonic development and adult homeostasis. There are a total of 19 Wnt proteins that signal through three distinct pathways: canonical Wnt/β-catenin, noncanonical planar cell polarity, and noncanonical Wnt/calcium.

Canonical Wnt/β-catenin signaling ensues when a Wnt protein binds cell surface receptor Frizzled and co-receptor LRP5/6, that in turn recruit the protein Dishevelled and disrupt the Axin degradation complex (Axin, APC, CK1, and GSK3) (MacDonald et al., 2009). These actions allow the transcriptional co-activator β-catenin to accumulate in the cytoplasm, translocate to the nucleus, and couple with the transcription factors TCF or LEF to induce target gene expression (Logan and Nusse, 2004; Nelson and Nusse, 2004). In the absence of Wnt signaling, cytoplasmic β-catenin levels are suppressed by the actions of the Axin complex causing ubiquitination and subsequent proteasomal degradation of β-catenin. Wnt signaling is regulated by secreted protein families that competitively bind Wnts, such as sFRPs and WIFs (Bovolenta et al., 2008), or antagonize the LRP5/6 co-receptor, such as Dkk and Wise/SOST (Semënov et al., 2005, 2001).

The importance of Wnt signaling in skeletal development was realized when a loss-of-function mutation in LRP5 was found to cause osteoporosis-pseudoglioma, a syndrome marked by low peak bone mass (Gong et al., 2001). Later, a kindred with an autosomal dominant syndrome characterized by high bone density was found to have a gain-of-function mutation in LRP5 impairing the antagonistic action of Dkk and thus increasing Wnt signaling (Boyden et al., 2002). Wnt signaling in synovial joint formation, chondrogenesis, and osteogenesis has since been widely explored, and, in general, has been shown to inhibit chondrocyte differentiation from mesenchymal progenitor cells and promote bone formation (Day et al., 2005; Guo et al., 2004).

In the postnatal growth plate, six members of the Wnt family are expressed, including Wnt-2b, -4, and -10b of the canonical β-catenin pathway and Wnt-5a, -5b, and -11 of the noncanonical calcium pathway. Their mRNA expressions are unanimously low in the resting zone, elevated in proliferative and prehypertrophic zones, and decreased in the hypertrophic zone (Andrade et al., 2007). Furthermore, chondrocyte-specific inactivation of β-catenin in mice causes dwarfism due to decreased proliferation and delayed hypertrophic differentiation (Akiyama et al., 2004). Conversely, β-catenin retroviral misexpression in chick limbs increases growth plate chondrogenesis due to increased proliferation and hypertrophic differentiation (Hartmann and Tabin, 2000).

2.2.2.4 Fibroblast Growth Factors

FGFs form a family consisting of at least 23 polypeptide growth factors. They bind heparan sulfate proteoglycans and activate 4 distinct cell surface receptor tyrosine

kinases to regulate cell proliferation, differentiation, and migration during embryogenesis as well as tissue repair in response to injury in adults (Ornitz and Itoh, 2001). Tissue specific alternative splicing of FGF receptor mRNAs increases the complexity of FGF signaling by creating epithelial (b isoform) and mesenchymal (c isoform) receptor variants.

The importance of FGF signaling in skeletal development was first realized when an activating mutation in the *FGFR3* gene (i.e. G1138A or G1138C resulting in Gly380Arg), was found to be the cause of achondroplasia, the most common form of short-limbed dwarfism in humans (Rousseau et al., 1994; Shiang et al., 1994). Studies have since investigated the role of FGF signaling in the developing limb bud (Martin, 1998), early mesenchymal condensation (Delezoide et al., 1998), and endochondral ossification (Ornitz and Marie, 2002) and found that FGF signaling negatively regulates growth by inhibition of chondrocyte proliferation and hypertrophic differentiation.

In the growth plate, FGFs and their receptors negatively regulate longitudinal bone growth (De Luca and Baron, 1999). Perichondrial cells produce FGF-1, -2, -6, -7, -9, -18, -21, and -22, whereas growth plate chondrocytes only express FGF-2, -7, -18, and -22 at very levels, suggesting that FGFs from the perichondrium are the main regulators of chondrogenesis (Lazarus et al., 2007). In the growth plate and surrounding tissues, FGFR1 is expressed by prehypertrophic and hypertrophic chondrocytes, FGFR2 is expressed by the perichondrium and the c isoform by resting chondrocytes, FGFR3 expression has been more controversial being suggested in all zones, and FGFR4 is expressed by resting and proliferative chondrocytes (De Luca and Baron, 1999; Lazarus et al., 2007; Peters et al., 1993, 1992; Yu and Ornitz, 2007).

As in humans, mice with an activating mutation of *FGFR3* develop achondroplasia characterized by small size and growth plate distortion with an expanded resting zone and narrowed proliferative and hypertrophic zones (Chen et al., 1999; Naski et al., 1996; Wang et al., 1999). Mice with *FGFR3* null mutations exhibit the opposite phenotype with long proliferative columns and enhanced endochondral bone formation (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998). Furthermore, FGF-2 was the first ligand to be isolated from the growth plate (Gonzalez et al., 1996) and overexpression in mice causes shortened body length, expanded resting and proliferative zones, and reduced hypertrophic zone (Coffin et al., 1995). *In vitro* culture of rat fetal metatarsals in growth medium supplemented with FGF-2 decreases chondrocyte proliferation, hypertrophy, and matrix synthesis (Mancilla et al., 1998).

2.3 ARTICULAR CARTILAGE

2.3.1 Structure and Function

Articular cartilage is a thin layer of hyaline cartilage lining the contact surfaces of long bones within synovial joints (**Fig. 2**). Similar to the growth plate, articular cartilage is subdivided into the superficial/tangential, intermediate/transitional, and deep/radial zones based on cell size, shape, organization, and function as well as matrix macromolecule composition (Buckwalter et al., 2005; Huber et al., 2000; Hunziker et al., 1997; Onyekwelu et al., 2009; Pearle et al., 2005; Ulrich-Vinther et al., 2003). The superficial zone is exposed to the synovial fluid in the joint cavity and contains the highest cell density characterized by small chondrocytes flattened parallel to the joint

surface. The matrix of the superficial zone is composed of densely packed type II collagen fibrils oriented parallel to the joint surface and a low proteoglycan content with preference for lubricants, such as proteoglycan 4 (Prg4), previously named lubricin. The intermediate zone is thickest and contains round chondrocytes arranged oblique to the joint surface and supported by arching bundles of type II collagen fibrils. In the deep zone, chondrocytes are largest and organized into cell columns perpendicular to the joint surface and interspersed between radial bundles of type II collagen fibrils. In general, from the superficial to deep zone, cell density decreases, cell volume increases, the ratio of proteoglycan to collagen increases, and water content decreases (Kim et al., 2003). Altogether, articular cartilage protects the integrity of the ends of long bones by minimizing friction at the superficial zone and distributing loads at the intermediate and deep zones.

2.3.2 Regulation of Chondrocyte Proliferation and Differentiation

The articular chondrocyte differentiation program remains poorly characterized compared to that of the growth plate. This may be due to the fact that articular cartilage once formed is a less dynamic tissue or that diseases related to articular chondrocyte differentiation are less obvious compared to those related to growth plate dysfunction such as skeletal dysplasia. One relevant disease perhaps is the rare camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome caused by autosomal recessive loss-of-function mutations in *Prg4*, resulting in early-onset joint dysfunction associated with disappearance of chondrocytes from the superficial layer of articular cartilage and hyperplasia of intimal cells of the synovium (Marcelino et al., 1999; Rhee et al., 2005). However, CACP does not reveal much about articular chondrocyte differentiation. The traditional view seems to be that articular cartilage represents the remnants of early epiphyseal cartilage that do not undergo endochondral ossification (Archer et al., 2003). Current research is continuing to refine our understanding in this area.

Cell kinetic studies have suggested that most of the epiphyseal cartilage that is present at birth, except for a distinct layer at the joint surface, is replaced by bone, and articular cartilage is thereafter formed by appositional growth (Archer et al., 1994; Hayes et al., 2001; Hunziker et al., 2007). Other studies have corroboratively reported the isolation of progenitor cells from the superficial zone of articular cartilage based on expression of the mesenchymal stem cell markers *Notch-1*, *Stro-1*, and *VCAM-1* and Hoechst dye 33342 staining, which typically stains hematopoietic stem cells (Dowthwaite et al., 2004; Grogan et al., 2009; Hattori et al., 2007; Karlsson et al., 2008). Indeed, the existence of a progenitor cell population in the superficial zone could support the hypothesis of appositional growth. Interestingly, however, in articular cartilage of patients with osteoarthritis, only the intermediate zone increases expression of *Notch-1*, *Stro-1*, and *VCAM-1*, implying an activation of growth potential in this cartilage layer that is stimulated by disease (Grogan et al., 2009).

An early cell fate mapping study using vital dye showed that articular chondrocytes do not derive from the original cartilage template but rather from the interzone, which, in turn, may have formed by differentiation of local chondrocytes or migration of peri-joint mesenchymal cells to the joint site (Pacifici et al., 2006, 2000). Other genetic cell lineage tracing studies exploiting the ROSA26-*LacZ*-reporter mouse demonstrated that either interzone cells expressing *Gdf5-Cre* (Koyama et al., 2008) or early chondrocytes lacking *Matn1-Cre* expression (Hyde et al., 2007) by embryonic

day 13.5 contribute to the formation of synovial joint and articular cartilage. A follow-up *Col2a1* lineage tracing study showed that the interzone is formed from dedifferentiated *Col2a1*-positive chondrocytes and it was postulated that these cells may incorporate into articular cartilage but that articular cartilage is mostly derived from a subpopulation of cells in the original cartilage condensation (Hyde et al., 2008).

Studies on articular cartilage homeostasis may also shed light on articular cartilage formation and differentiation. For instance, a *Gdf5-Cre* system deleting a floxed *BMPR1A*, resulted in mutant mice with premature joint degeneration resembling osteoarthritis (Rountree et al., 2004). This suggests that BMP signaling is required for articular cartilage maintenance. Furthermore, overexpression of *Notch-1* in the ATDC5 chondrogenic cell line inhibited chondrogenesis (Watanabe et al., 2003) and expression of Notch markers were shown to decline with the differentiation of human articular chondrocytes in pellet mass cultures (Karlsson et al., 2007). These findings suggest that Notch signaling maintains chondrocytes in an immature state. Finally, whole-genome gene expression microarray of pediatric growth plate and articular cartilage revealed decreased Wnt signaling in articular cartilage compared to the growth plate due to increased expression of Wnt antagonists *FRP* and *Dkk-1* (Leijten et al., 2012). This suggests that inhibitors of Wnt signaling downregulates hypertrophic differentiation.

2.4 CARTILAGE DISEASES

2.4.1 Arthritis

Arthritis is joint inflammation that causes persistent pain, swelling, and stiffness. There are many forms of arthritis associated with various conditions, such as joint degeneration (e.g. osteoarthritis), autoimmune diseases (e.g. rheumatoid arthritis, ankylosing spondyloarthritis, Reiter syndrome, enteritis-associated arthritis, psoriatic arthritis, and systemic lupus erythematosus), metabolic disorders (e.g. gout and pseudo-gout), and microbial infections (e.g. bacterial, tuberculosis, Lyme disease, and viral). Arthritis usually occurs in adults but also in children as juvenile idiopathic arthritis.

2.4.1.1 Osteoarthritis

Osteoarthritis (OA), or degenerative joint disease, is the most common form of arthritis worldwide. It is characterized by progressive degeneration of articular cartilage, eburnation and sclerosis of subchondral bone, osteophyte formation, and joint-space narrowing. It occurs most frequently in the proximal and distal interphalangeal joints of the fingers, knees, hips, and cervical and lumbar vertebrae but also wrists, shoulders, ankles, and feet (Arden and Nevitt, 2006). Symptoms include deep, achy pain that is exacerbated by activity and improved by rest, brief morning stiffness, crepitus, and limited range of movement (Arden and Nevitt, 2006; Bijlsma et al., 2011; Creamer and Hochberg, 1997). Risk factors include advancing age beyond 40, female sex particularly after menopause, obesity, joint trauma, and small, repetitive insults to articular cartilage (Arden and Nevitt, 2006; Bijlsma et al., 2011; Creamer and Hochberg, 1997). Interestingly, the medical literature does not support a causal relationship between low- or moderate-distance running and OA (Willick and Hansen, 2010). OA is not a simple consequence of aging or wear and tear but rather involves a complex interplay of metabolic, biochemical, and biomechanical factors. In addition, genetic predisposition with polygenic inheritance is an important etiological factor, as

evidenced by twin and non-twin sibling, population, and genome-wide association studies (arcOGEN Consortium et al., 2012; Holderbaum et al., 1999).

2.4.1.2 Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis is defined as arthritis occurring in children before 16 years of age and that persists for a minimum of 6 weeks (Prakken et al., 2011). The etiology of juvenile idiopathic arthritis is unknown. It is categorized into seven discrete subsets intended to guide clinical evaluation, treatment, and prognosis: 1) systemic arthritis, 2) rheumatoid factor-positive polyarthritis, 3) rheumatoid factor-negative polyarthritis, 4) pauciarthritis or oligoarthritis, 5) enthesitis-related arthritis, 6) psoriatic arthritis, and 7) undifferentiated arthritis. Many children with juvenile idiopathic arthritis, however, cannot simply be categorized into an individual subset and some children change categories over time (Nordal et al., 2011), which indicates the heterogeneity of the disease. The long-term prognosis of juvenile idiopathic arthritis is variable with some affected children developing severe functional disability.

2.4.2 Growth Plate Disorders

Since growth plates are the sites of longitudinal bone growth, environmental and genetic factors affecting the mechanisms regulating chondrogenesis can cause variations in normal skeletal growth as well as abnormal bone growth. Growth plates are also the weakest structures in the pediatric skeleton, especially during growth spurts when chondrocytes undergo rapid proliferation. Thus, growth plates are vulnerable to injuries from acute trauma and overuse. Common injuries include fracture, Osgood-Schlatter disease (apophysitis of the tibial tubercle), Sever's disease (calcaneal apophysitis), and slipped capital femoral epiphysis. Furthermore, since growth plates lie adjacent to metaphyseal bone, which is highly vascularized, they are prone to hematogenous spread of cancer metastases, microbial infections, and pharmaceutical agents. Growth plates are also susceptible to extreme cold and radiation.

2.4.2.1 Short Stature and Skeletal Dysplasia

Growth failure may be caused by malnutrition, physical or psychological stress, illness, or growth plate dysfunction. Short stature is defined as a standing height of more than 2 standard deviations below average for age and sex. Short stature is thus not necessarily due to growth failure and when determining whether a child has pathological growth failure, the growth pattern may be more informative than the current height. Short stature can be proportional, which means that all growth plates in the body have been affected similarly and therefore suggests a systemic or endocrine cause. Alternatively, some growth plates (e.g. long bones) can be affected more than others (e.g. the spine) resulting in disproportionate short stature, which suggests an underlying growth plate dysfunction or skeletal dysplasia. After excluding growth failure due to nutritional, systemic, endocrine, and genetic abnormalities, many children remain without a mechanistic diagnosis, so called idiopathic short stature. Recent progress in genetic methodology, including next-generation sequencing, has been able to put patients with idiopathic growth failure into subgroups, including mutations and deletions in the short stature homeobox (SHOX) gene (Rao et al., 1997), heterozygous mutations in the natriuretic peptide receptor-2 (NPR2) gene (Olney et al., 2006; Vasques et al., 2013), and protein-tyrosine phosphatase, non-receptor type 11

(PTPN11) gene (Wang et al., 2013). Further studies in these subgroups are likely to identify genetic causes of growth failure in a significant proportion of patients. It is plausible that some of these will be due to hypomorphic alleles of skeletal dysplasia genes.

Skeletal dysplasia [Gr. dys + plasis, bad formation] is the medical term for "little people," dwarfism, or disproportionate short stature. Skeletal dysplasia occurs in approximately 1 in 4000 births and is a heterogeneous disorder comprising more than 350 forms caused by all patterns of genetic inheritance as well as exposure to toxic or infectious agents (Krakow and Rimoin, 2010). Severe forms are typically discovered early in life by fetal ultrasound or at birth as the child's head grows out of proportion to the body and limbs. The more common forms of skeletal dysplasia include: 1) achondroplasia, the most common nonlethal form occurring in about 1 in 15,000 births, caused by an activating mutation in FGFR3 that increases normal inhibition of chondrocyte proliferation and hypertrophic differentiation in the growth plate (Richette et al., 2008; Vajo et al., 2000); 2) osteogenesis imperfecta ("brittle bone disease" or blue sclera syndrome) most commonly caused by mutations in COL1A1 or COL1A2 encoding the skeletal structural protein type 1 collagen (Prockop and Kivirikko, 1984); 3) lethal thanatophoric dysplasia caused by a more severe mutation in FGFR3 compared to achondroplasia (Naski et al., 1996); and 4) lethal achondrogenesis caused by mutations in TRIP11 (type 1A) (Smits et al., 2010), SLC23A2 (type 1B) (Superti-Furga et al., 1996), and COL2A1 (type 2) (Vissing et al., 1989).

2.4.2.2 Growth Plate Fracture

Injuries that cause benign sprains and strains in adults are more likely to cause damage to bone in children because the tensile strength of pediatric bone is less than that of the surrounding ligaments and tendons (Carson et al., 2006). Growth plate fractures constitute 15-30% of all childhood bone fractures, usually involve the upper extremities especially long bones of the fingers, occur twice as often in boys, and occur most often during adolescent growth spurts when growth plates are weakest and children engage in more high-risk activities (Mann and Rajmaira, 1990; Peterson et al., 1994). The Salter-Harris classification is the most widely used system for describing growth plate fractures among healthcare professionals (Salter and Harris, 1963; Tandberg and Sherbring, 1999) (**Fig. 3**). Slipped capital femoral epiphysis is an example of a Salter-Harris type I fracture. Complications of growth plate fracture result from bone bridge/bar formation across the fracture line and include complete or partial growth arrest causing limb-length discrepancy, permanently decreased range of motion, and angular varus or valgus deformity (Czitrom et al., 1981; Khoshhal and Kiefer, 2005). Growth plate fractures that cause discontinuities of the articular surface (i.e. Salter-Harris type III and IV) can lead to early osteoarthritis.

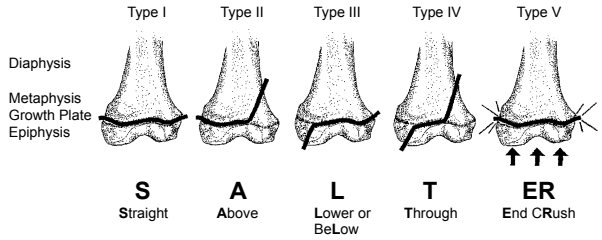

Figure 3. Mnemonic for the Salter-Harris classification of growth plate fractures. Adapted and modified from (Tandberg and Sherbring, 1999).

2.4.2.3 Chemotherapy and Radiation Damage

Many childhood cancers (e.g. leukemia, central nervous system tumors, neuroblastoma, Wilms tumor, lymphoma, rhabdomyosarcoma, retinoblastoma, and bone tumors) are treated with chemotherapy and radiation. Chemotherapeutic agents are classified based on their structure and mechanism of action as alkylating agents (directly damages DNA), antimetabolites (interferes with DNA and RNA synthesis), anti-tumor antibiotics (interferes with enzymes involved in DNA replication), topoisomerase inhibitors, mitotic inhibitors, corticosteroids (kills cancer cells or slows growth, e.g. dexamethasone), and miscellaneous (e.g. proteasome inhibitors). These drugs target cells that divide rapidly, such as cancer cells, and either inhibit growth or induce death. In children, normal cells in certain tissues, such as chondrocytes in growth plates, also proliferate at relatively fast rates and are thus susceptible to collateral damage. Similarly, radiation does not discriminate between cancer and normal cells. Radiation damage to growth plates can result in abnormal bone growth due to death of chondrocytes, disarray of proliferative columns, and decreased ability of surviving chondrocytes to synthesize functional matrix proteins (Chemaitilly et al., 2007; Hovi et al., 1999).

2.4.3 Cartilage-Forming Tumors

Cartilage-forming tumors comprise a fraction of primary matrix-producing bone tumors and include benign osteochondroma, chondroma, chondroblastoma, and chondromyxoid fibroma and malignant chondrosarcoma. All are characterized by neoplastic formation of hyaline or myxoid cartilage. Most develop in the first several decades of life and tend to originate from long bones of the extremities; however, specific types target certain age groups and anatomic sites, which can provide important diagnostic information. Clinically, most benign lesions are asymptomatic and detected as incidental radiologic findings or sudden pathologic fractures, but they can also be painful or noticed as slow growing masses.

2.4.3.1 Osteochondroma

Osteochondroma, or exostosis, is the most common benign bone tumor that occurs from childhood to early adulthood. It presents as a pedunculated bone spur capped by dysregulated cartilage arising from the metaphyseal surface of endochondral bones, especially distal femur and proximal humerus (Biermann, 2002). Approximately 80% are solitary and the rest are seen in hereditary multiple osteochondromas, which is caused by an autosomal dominant loss-of-function mutation in the exostosin genes (*EXT1* or *EXT2*) (Pannier and Legeai-Mallet, 2008). *EXT* genes encode proteins that are involved in the biosynthesis of heparan sulfate proteoglycans, which are important for cell signalling through Ihh/PTHrP and other pathways (Koziel et al., 2004; McCormick et al., 1998). Disruption in these pathways results in defective endochondral ossification and abnormal growth. Since osteochondromas are essentially ectopic growth plates, they are affected by growth factors that regulate growth plate chondrogenesis and cease to grow after skeletal maturity. Clinical complications include skeletal deformity, limb-length discrepancy, short stature, risk of fracture, and risk of malignant transformation to chondrosarcoma if the cartilage cap covering the bone spur is ≥2 cm thick in an adult, size increases after skeletal maturity, or new symptoms develop (Pannier and Legeai-Mallet, 2008).

2.4.3.2 Chondrosarcoma

Chondrosarcoma is the second most common malignant bone tumor after osteosarcoma and usually affects the pelvis, shoulders, and ribs and rarely the distal extremities of individuals in their 40s or older (Björnsson et al., 1998). It is subclassified according to location in the bone, whether it develops in the bone marrow cavity (central chondrosarcoma) or on the bone surface (peripheral or periosteal chondrosarcoma). It is also subclassified histologically as conventional (hyaline and/or myxoid), clear cell, dedifferentiated, or mesenchymal (Murphey et al., 2003). The clear cell and mesenchymal variants have a predisposition for younger patients in their teens or 20s and the clear cell variant is unique in that it originates from the epiphyses of long bones. About 90% of chondrosarcomas are central conventional and about 15% of conventional chondrosarcomas arise from a pre-existing osteochondroma or enchondroma. The most serious clinical complication is metastasis, which tends to disseminate to the lungs and other parts of the skeleton (Rizzo et al., 2001).

2.5 STANDARDS OF CARE FOR CARTILAGE REPAIR

The main goal of cartilage repair is to preserve a high level of physical functioning that supports the best quality of life. This can be achieved clinically by promoting normal skeletal growth and development, preventing disease progression, and minimizing joint pain and dysfunction. The common sense strategy is to use the least invasive intervention necessary. Specific treatment depends on the type and severity of cartilage disorder and is tailored to the patient. Guidelines for the standards of care are generated by professional organizations, including the American College of Rheumatology, the European League Against Rheumatism, the Osteoarthritis Research Society International, and the American Academy of Orthopaedic Surgeons.

2.5.1 Non-surgical Treatments

Non-surgical interventions are the mainstay of care for most skeletal disorders because of their lower risk of iatrogenic morbidity. Conservative treatments such as rest, weight loss, physical therapy, and exercise programs are first-line for patients with moderate osteoarthritis (Felson et al., 1992; Fransen et al., 2001). Pharmacologic treatments are second-line. The major pharmacologic agents for managing osteoarthritis are oral analgesics (e.g. acetaminophen and NSAIDs), topical NSAIDs and capsaicin, and intraarticular glucocorticoid injections. Alternative pharmacologic agents are oral opioid analgesics (e.g. tramadol and codeine), intraarticular injections of hyaluronic acid and growth factors (Vaquerizo et al., 2013), and prophylactic colchicine. For patients with juvenile arthritis who fail to respond to NSAIDs, alternatives include oral glucocorticoids and disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate, IL-1 and IL-6 inhibitors, and TNF-α inhibitors (Beukelman et al., 2011).

2.5.2 Surgical Treatments

Surgical treatments are reserved for conditions that, despite more conservative interventions, continue to disrupt daily living, harm skeletal growth and integrity, and/or develop malignant potential. The specific treatment is determined by the

underlying skeletal disorder and severity of the lesion.

Growth plate fractures are treated either with cast immobilization or internal fixation using pins and screws to hold the bone together and ensure proper alignment of the growth plate and joint surface (Kay and Matthys, 2001; Podeszwa and Mubarak, 2012). Additional surgical techniques exist to remove complicating bone bridges and replace with fat (Langenskiöld, 1975), cartilage (Lennox et al., 1983), autologous chondrocytes (Lee et al., 1998), or other biomaterials to prevent recurrence (Khoshhal and Kiefer, 2005).

Most osteochondromas can be observed without treatment and indications for surgical excision include local irritation or deformity and concern for malignant transformation to chondrosarcoma. For nonmetastatic chondrosarcoma surgical excision offers the only chance of cure because they are resistant to chemotherapy and radiation due to a slow growth rate and low fraction of dividing cells (Lee et al., 1999).

Options for treating osteoarthritis resistant to conservative and pharmacologic treatments include arthroscopic joint irrigation/lavage and debridement, joint realignment, joint distraction, joint fusion, and total joint replacement (Zhang et al., 2010, 2008). For standalone articular cartilage lesions, several cartilage repair procedures have been used in recent years.

Microfracture, or marrow stimulating, is an arthroscopic procedure intended to induce new cartilage growth by creating a suitable environment (Steadman et al., 2001). An awl is used to make multiple holes, or microfractures, about 3 to 4 mm apart in the subchondral bone under the lesion to create a blood clot containing mesenchymal stem cells and growth factors. The result is fibrocartilage repair with varying production of type I, II, and III collagen that provides significant short-term functional improvement but yet unknown long-term efficacy (Mithoefer et al., 2009).

Autologous chondrocyte implantation (ACI) was performed in the first patients in 1987 (Brittberg et al., 1994). It is a two-step procedure. First, healthy articular cartilage is arthroscopically collected from a non-weight bearing surface followed by chondrocyte isolation and expansion in the laboratory for 2-6 weeks. Second, an arthrotomy is performed to implant the cultured chondrocytes by injecting them under a flap of periosteum sutured over the articular defect and sealed with fibrin glue. Long-term follow-up has shown that over 80% of patients improve with relatively few complications (Brittberg, 2008). Histological studies have shown that ACI repairs primarily fill with fibrocartilage but also hyaline-like cartilage (Horas et al., 2003).

Osteochondral autograft transplantation surgery (OATS) involves the transfer of cartilage from one part of a joint to another by arthroscopy (Chow et al., 2004). A cylindrical graft consisting of cartilage and subchondral bone is taken from a non-weight bearing surface and press-fitted into a vacancy 1 mm smaller in diameter at the defective site. If the lesion is large multiple grafts can be used in a procedure called mosaicplasty (Hangody et al., 2001). Relatively recent comparative studies have shown that OATS is functionally and structurally superior to microfracture and ACI (Gudas et al., 2013; Horas et al., 2003; Krych et al., 2012).

Novel approaches for cartilage repair are on the research front. Currently, autologous stem cells can be harvested from bone marrow and differentiated into chondrocytes. However, when used for cartilage tissue engineering, the cells tend to undergo hypertrophic differentiation and matrix calcification rather than adopt an articular-like chondrocyte phenotype (Wu et al., 2011). Therefore, in the field of tissue engineering, current research is focusing on developing new methods of combining

growth factors, stems cells, and biomaterials to stimulate the body to form healthy articular cartilage (Johnstone et al., 2013; Keeney et al., 2011). High-throughput studies are being conducted to search for new growth factors that support articular chondrocyte differentiation (Johnson et al., 2012). Various non-cartilaginous tissues are being identified to contain cells that have the potency to adopt the articular chondrocyte phenotype, including bone marrow, adipose, and synovium (Leijten et al., 2013). Biomaterials are being designed to function as temporary extracellular matrices to support cartilage development while discouraging scar formation, including collagen membranes, fibrin glue, hyaluronic acid (Correia et al., 2011), and hydrogels (Moreira Teixeira et al., 2012; Sharma et al., 2013). Most of these approaches are still in the experimental phase and, although standards of care for cartilage repair exist today, the ideal of having restored cartilage perform as well as native cartilage has not yet been achieved.

3 THESIS AIMS

The main goal of this thesis is to study the molecular mechanisms regulating growth plate and articular chondrocyte differentiation. In this regard, similarities and differences between the two tissues were also investigated.

The specific aims were as follows:

1) To study the BMP signaling pathway in growth plate cartilage.

2) To study the Ihh/PTHrP signaling pathway in growth plate cartilage.

3) To compare the spatial gene expression profiles of growth plate and articular cartilage.

4) To test the hypothesis (based on study 3) that microenvironment regulates chondrocyte differentiation into either growth plate or articular cartilage.

4 METHODOLOGY

The following methods were applied in this thesis and will be described in general. For details on procedures, please consult the individual papers.

- Animal models (Papers I-IV)
- Microdissection (Papers I-III)
- RNA extraction (Papers I-III)
- Microarray (Papers I-III)
- Real-time polymerase chain reaction (Papers I-III)
- β-galactosidase staining (Paper II)
- Microsurgical manipulation (Paper IV)
- *In situ* hybridization (Paper IV)
- Immunohistochemistry (Paper IV)

4.1 ANIMAL MODELS

The ideal model for conducting scientific research benefitting human health is human, but in most cases this is infeasible or violates the bioethics principle of non-maleficence. Many studies therefore use alternative animal models, and in the field of cartilage research, these commonly include rodents (e.g. rat and mouse), rabbit, chicken, pig, dog, goat, cow, and horse (Chu et al., 2010). Each animal model has pros and cons in terms of cost effectiveness, size, lifespan, reproduction rate, offspring number, genetic manipulability, and similarity to human anatomy and physiology.

This thesis used rat (Papers I-IV), mouse (Paper II), and rabbit (Paper IV preliminary results). The benefits of rodent models include cost effectiveness, short 2- to 4- year lifespan permitting timely studies of cartilage growth and development, fast reproduction rate, large litters averaging 10 pups, feasibility of generating transgenic animals, and availability of athymic (nude) and inbred strains. A complication of using rodents to study growth plate cartilage, however, is that unlike human growth plates, rodent growth plates do not fuse. Nevertheless, longitudinal bone growth in rats drastically declines after skeletal maturity at 13 weeks of age and virtually ceases after 26 weeks of age (Hughes and Tanner, 1970; Kember, 1973). The rabbit model offers the advantages of larger synovial joints for ease of surgical manipulation and growth plate fusion similar to that in human. A complication of using rabbit to study articular cartilage, however, is that rabbit articular cartilage possesses remarkable healing capacity not seen in human (Shapiro et al., 1993). Furthermore, young rabbits are susceptible to fatal mucoid enteritis induced by early weaning, transportation, and surgical stress. In general, small animal models, such as rodents and rabbit, are useful for pilot and proof-of-concept studies.

All animal studies in this thesis were performed with permission from the Animal Ethics Committee of Northern Stockholm (permit number N290/08) and the Animal Care and Use Committee at the National Institutes of Health (Animal Study Proposal numbers: 03-011, 07-017, 11-052, and 13-087). All efforts were made to abide by the three Rs of humane animal experimentation: replacement of animals with alternative methods or organisms with limited sentience, reduction in number of animals, and refinement of procedures to minimize animal suffering (Goldberg et al., 1996).

4.2 MICRODISSECTION

Manual microdissection was used to study spatial gene expression of rat proximal tibial growth plate and articular cartilage. This technique involves using an inverted microscope, razor blades, and hypodermic needles to separate and collect individual layers of epiphyseal cartilage based on histological hallmarks into superficial, intermediate, and deep zones of articular cartilage and resting, proliferative, and hypertrophic zones of growth plate cartilage (**Fig. 4**). Tissue sections were stained with eosin to visualize histology and a layer of cartilage between adjacent growth plate zones was discarded to minimize cross-contamination.

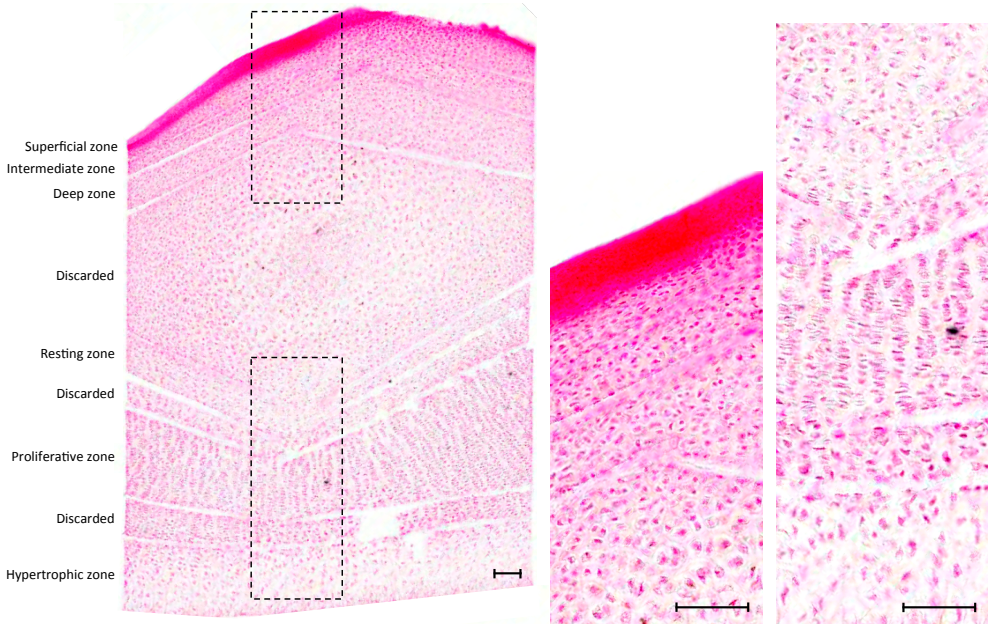

Figure 4. Photomicrograph of a longitudinal section of rat proximal tibia stained with eosin for manual microdissection. Superficial, intermediate, and deep zones of articular cartilage and resting, proliferative, and hypertrophic zones of growth plate cartilage were isolated with a razor blade. To minimize cross-contamination, a layer of cartilage between adjacent growth plate zones was discarded. High magnification is shown on right with regions delineated by dashed lines on left. Scale bars: 100 μm.

It is important to note that gene expression is analyzed on the level of DNA transcription to RNA. The extraction of high quality RNA is therefore crucial for accurate assessment of mRNA levels by microarray and qPCR. Since RNA is prone to enzymatic degradation by exogenous (i.e. from microbes and other cells) and endogenous (i.e. from host cell) RNase enzymes, strict RNase precautions were followed. Rat proximal tibial epiphyses were rapidly excised, frozen in O.C.T.

compound, sectioned (60 μm thick) onto positively charged glass slides, and stored in a -80°C freezer until manual microdissection, which was performed keeping the tissues sections immersed in xylene.

Manual microdissection provides an affordable method to isolate high quality RNA from individual layers of cartilage for gene expression studies using qPCR, NanoString, microarray, or RNA sequencing. To isolate smaller cell populations or individual cells, laser capture microdissection (LCM) provides a method for even more accuracy and precision. LCM combines an inverted microscope, infrared (IR) and/or ultraviolet (UV) laser, and digital camera to collect near pure cell populations (Emmert-Buck et al., 1996). One of several LCM systems is Arcturus, which uses an IR laser to melt/adhere a thermoplastic film onto a specified area in a tissue section and a UV laser to cut a desired shape, thereby enabling lifting of targeted cells. The main disadvantages of LCM include cost and potential heat damage to targeted cells.

4.3 RNA EXTRACTION

Total RNA isolation from cells of interest was performed using a modified version of the "single-step acid guanidinium thiocyanate-phenol-chloroform extraction" method (Chomczynski and Sacchi, 2006, 1987). A phenol-chloroform extraction is a liquid-liquid extraction that separates mixtures of molecules based on differential solubilities into two immiscible liquids. It involves adding an equal volume of phenol-chloroform to an aqueous solution of cells either homogenized or lysed by proteinase K. The combination of phenol and chloroform denatures proteins and chloroform ensures phase separation. Guanidinium thiocyanate and β-mercaptoethanol are added to deactivate nucleases (i.e. RNases and DNases) and isoamyl alcohol is added to prevent foaming. After centrifugation, the mixture is generally separated into an upper aqueous phase containing RNA and DNA, an interface containing DNA, proteins, and carbohydrates, and a lower organic phase containing DNA, proteins, and lipids. Partitioning of RNA and DNA between the acqueous and organic phases is determined by the pH of phenol and phenol to chloroform ratio (Brawerman et al., 1972; Kedzierski and Porter, 1991). For instance, a neutral to slightly alkaline pH (pH 7-8) and a 1:1 phenol to chloroform ratio maximally partitions RNA and DNA to the aqueous phase. The aqueous phase is collected and product is precipitated by sodium acetate and isopropanol (decreases solubility of nucleic acids by neutralizing negatively charged phosphates) followed by lithium chloride (does not efficiently precipitate DNA or proteins). Ethanol washes between and after precipitation steps help remove residual salts.

4.4 MICROARRAY

Microarray is a high-throughput technique used to measure the expression levels of large numbers of genes simultaneously. It involves using a 2-dimensional array composed of tens of thousands of spots or squares (each approximately 8 μm wide), called features, arranged like a checkerboard on a single glass or silicon substrate/platform/chip. Each feature contains millions of identical copies of a unique single strand of DNA (25 bp long), called a probe, representing one of several possible transcripts of a gene. The Affymetrix arrays used in this thesis, Rat Genome 230 2.0 GeneChip Arrays (Papers I-III) and GeneChip Rat Gene 1.0 ST Arrays (Paper III),

were designed to cover over 30,000 (28,000 genes) and 17,000 (16,000 genes) transcripts, respectively, accounting for multiple transcript isoforms of a given gene such as alternative splice variants. A basic schematic of a microarray workflow is shown below (**Fig. 5**). Fluorescent signals from each feature is detected and quantified by a microarray scanner and then background corrected, normalized, and summarized into relative gene expression values using microarray analysis software (e.g. Affymetrix Microarray Suite Version 5.0, BrB-array tools, Bioconductor, or Partek Genomic Suite 6.6). Log-transformed gene expression values are subsequently used to compare different treatment groups (e.g. tissues, zones, time-points, or treatment groups) in order to address the current question.

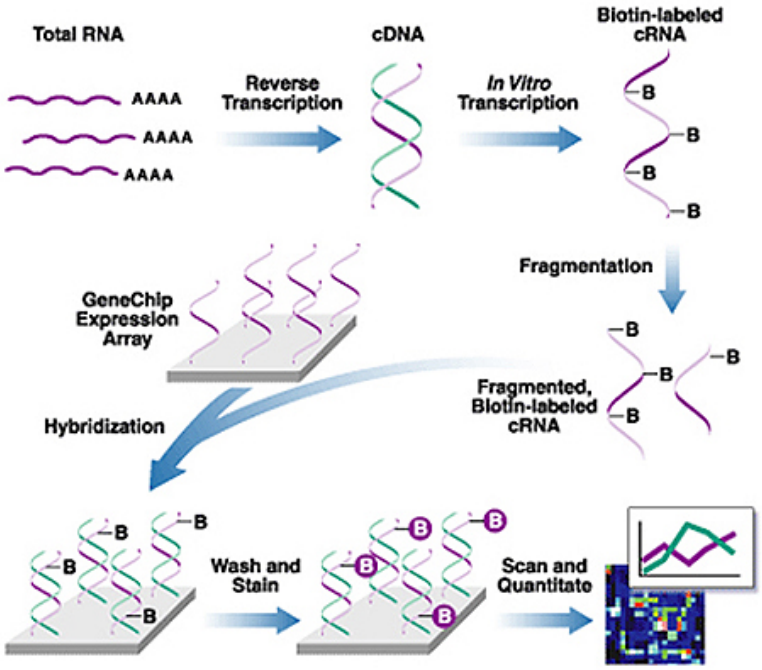

Figure 5. Basic schematic of a microarray workflow. Total RNA isolated from the cells of interest is converted to cDNA by reverse transcriptase enzyme. Next, *in vitro* transcription of cDNA to cRNA incorporates nucleotides conjugated to biotin. Biotinylated cRNA is then fragmented and hybridized to DNA probes on a 2-D array, washed and stained, and quantitated using a microarray scanner. Adapted from affymetrix.com.

4.5 REAL-TIME POLYMERASE CHAIN REACTION

Real-time polymerase chain reaction (PCR), also referred to as quantitative polymerase chain reaction (qPCR), enables quantification of targeted genes at the same time they are being amplified (i.e. in real-time). Therefore, data is collected throughout the PCR process rather than at the end of the reaction, such as in traditional PCR. In order to quantify messenger RNA (mRNA) using qPCR, the mRNA has to be reverse transcribed into complementary DNA (cDNA). The resulting relative expression values are thus not only dependent on the concentration of the targeted mRNA, but also on the efficiencies of the qPCR and reverse transcription reactions.

Two systems have been developed by Applied Biosystems to detect qPCR products. First, the Taqman system uses an oligonucleotide probe consisting of a fluorescent reporter dye (e.g. VIC or FAM) on the 5' end and a quencher dye (e.g.

fluorescent TAMRA or a non-fluorescent quencher, NFQ) on the 3'end. There are two types of Taqman probes: 1) conventional probes with TAMRA, which tend to be long (30-40+ bases) and thus less specific and 2) minor groove binder (MGB) probes with NFQ, which are more specific due to the MGB increasing the melting temperature of the probe and thus allowing for shorter probes (15-20 bases). While the probe is intact, the quencher dye reduces the fluorescence of the reporter dye. When the target DNA sequence is present, however, the probe anneals downstream to one of the primers and is cleaved by the 5' nuclease activity of Taq DNA polymerase as it elongates a new strand of DNA. Cleavage of the probe separates the reporter dye from the quencher dye and increases the reporter dye signal to detectable levels. Second, the SYBR Green system uses a dye that specifically binds double-stranded DNA resulting in a DNA-dye complex that absorbs blue and emits green light. Both these methods thus allow for continuous quantification of PCR product during the PCR process, typically assessed at the end of each cycle. The most important difference between Taqman and SYBR Green is that the former requires annealing of both primers and Taqman probe for detection and is therefore more specific than SYBR green, which detects all double-stranded DNA in the reaction mixture, including non-specific PCR products. Thus, when using SYBR Green, specific amplification of the target has to confirmed, typically by melting curve analysis showing a single peak and gel electrophoresis generating a single band of the expected size.

Real-time PCR is quantitative because data is collected during the exponential (rather than the subsequent linear and plateau) phase of the reaction when the quantity of PCR product is directly proportional to the amount of starting cDNA template. In qPCR, each sample is ultimately assigned a threshold cycle (C_T) value corresponding to the amplification cycle when the fluorescent signal reaches a set threshold. The same threshold is used for all samples and is typically set somewhere in the middle of the exponential phase. Calculation of expression values is done either using absolute quantification by a standard curve, or relative quantification calculating target gene expression relative to an endogenous/house-keeping gene, which is ideally expressed at similar levels by all cells (e.g. 18S ribosomal RNA, β-actin, and GAPDH). Relative expression can be calculated using the $2^{-\Delta\Delta C_T}$ method, which requires that the amplification efficiencies of the target gene and endogenous gene to be approximately equal (Livak and Schmittgen, 2001).

4.6 β-GALACTOSIDASE STAINING

A powerful approach used to uncover the function of individual components of a signaling pathway is homologous recombination to create transgenic knock-out or knock-in mice (Manis, 2007). This thesis exploited a *LacZ* knock-in mouse. *LacZ*, encoding the bacterial enzyme β-galactosidase, is commonly used as a reporter gene because it can easily be located using the artificial substrate X-gal. X-gal turns into a blue substrate when incubated with and cleaved by β-galactosidase in the cell cytosol, thereby enabling cells that express *LacZ* to be identified.

The animal model used in Paper II to the study the Ihh/PTHrP system in postnatal growth plate was a *Gli1-LacZ* Swiss Webster mouse, which was genetically engineered to express *LacZ* in every cell that *Gli1* is also expressed (Bai et al., 2002). As mentioned in section 2.2, *Gli1* expression is self-induced by its product Gli1 transcription factor that acts downstream of Ihh signaling (di Magliano and Hebrok,

2003; Koziel et al., 2005; McMahon, 2000). Thus, β-galactosidase staining using X-gal of any tissue in the *Gli1-LacZ* mouse effectively serves as readout for Ihh activity.

4.7 MICROSURGICAL MANIPULATION

In order to test the hypothesis that growth factors in the microenvironment regulates chondrocyte differentiation into either growth plate or articular cartilage, a surgical model was devised to switch the local microenvironments of growth plate and articular cartilage and subsequently observe for changes in histology and gene expression.

4-week-old New Zealand White rabbits were initially chosen as the animal model because their size makes surgical manipulations easier. Since growth plate height declines with age, fusing by 8 months in this species (Hunziker et al., 2007), 4 weeks was deemed most suitable based on growth plate size and remaining growth potential. Osteochondral grafts containing articular cartilage, epiphyseal bone, and growth plate cartilage (cylindrical plugs approximately 1.067 mm in diameter and 10 mm in length) were extracted from the intercondylar/trochlear groove of distal femurs using bone marrow biopsy needles and then re-inserted either in original orientation (sham/placebo surgery control) or inverted orientation. The intercondylar groove was chosen as the site of operation because it is a non-weight bearing articular surface, thus reducing the confounding effect of mechanical stimulation. Animals were euthanized at 1, 2, and 4 weeks after surgery.

Growth plate cartilage transplanted to the articular surface appeared to structurally remodel into articular cartilage-like structures. First, hypertrophic chondrocytes at the articular surface appeared to shrink and start proliferating, and over time hypertrophic chondrocytes disappeared altogether. However, as the transplanted cartilage became increasingly integrated with the surrounding articular cartilage, it was difficult to distinguish donor from recipient cartilage. In order to distinguish original growth plate chondrocytes from surrounding articular chondrocytes, an animal model was devised to trace growth plate chondrocytes at the articular surface. Surgical manipulations were performed as previously described except that 4-week-old inbred Lewis rats expressing enhanced green fluorescent protein (EGFP) under the ubiquitous CAG promoter (Lew-tg(CAG-EGFP)1Ys) were used as donors and 4-week-old inbred Lewis rats without the EGFP transgene (LEW/SsNHsd) were used as recipients. Experimental end points were chosen as postoperative week 1, 2, 4, and 8 to reach skeletal maturity in this species (Hughes and Tanner, 1970; Kember, 1973). Detection of EGFP by fluorescent microscopy was confounded by chondrocyte autofluorescence. Immunohistochemistry targeting EGFP was therefore implemented.

4.8 *IN SITU* HYBRIDIZATION

In situ hybridization is a technique that enables specific nucleic acid sequences to be detected in morphologically preserved chromosomes, cells, or tissues (Gall and Pardue, 1969; John et al., 1969). Therefore, *in situ* hybridization can correlate histological information with genetic activity on the DNA and mRNA levels. In this thesis, non-radioactive digoxigenin (DIG) RNA-RNA *in situ* hybridization (Kessler et al., 1990) was used to study gene expression in postnatal epiphyseal cartilage.

Variations of *in situ* hybridization protocols exist to tailor to different types of

tissue. Most protocols for non-radioactive DIG RNA-RNA *in situ* hybridization include the following basic steps [experimental notes based on trial and error in this thesis are in brackets]: 1) design and synthesize riboprobes containing DIG-conjugated uracil for genes of interest [this step is critical and may need to take into account different splice variants in different tissues, a probe length of 300-500 bp is optimal for penetration and signal strength if using a single probe, longer probes up to 1000 bp can provide greater coverage of a gene and can be fragmented by alkaline hydrolysis to ease penetration, and a cocktail of shorter probes can also be used for better gene coverage and a stronger signal]; 2) collect tissue samples in either paraffin [epiphyseal cartilage samples must be fixed in paraformaldehyde to preserve histology and mRNA and decalcified to allow higher quality sectioning] or frozen blocks; 3) section samples onto glass slides [bone and cartilage tissues, like brain tissue, are notorious for not sticking well onto slides, thus Superfrost +/+ slides and preheating were used to improve tissue adherence but yielded variable results, and so far TruBond 380 slides seem to be a promising alternative]; 4) antigen retrieval either by heat treatment or enzymatic digestion is required for fixed tissues [this step is critical and needs to be optimized for each tissue, insufficient antigen retrieval will prevent probe penetration and over retrieval will distort tissue histology, and proteinase K digestion was used in this thesis and success depended on enzyme concentration, incubation time, and temperature (**Fig. 6**)]; 5) treat with acetic anhydride to prevent non-specific binding of negatively charged probes to positive charges on the tissue section and glass slide; 6) hybridize probes to gene of interest by overnight incubation in specialized hybridization buffer [100 ng of probe in 100 µl of hybridization buffer was used]; 7) wash slides with increasing stringency, which means decreasing saline-sodium citrate concentration and increasing temperature, in order to remove excess probe sticking to the tissue by non-specific binding; 8) detection of probe by immunohistochemistry targeting DIG, for example using alkaline phosphatase-conjugated anti-DIG antibodies; 9) develop slides using a colored substrate such as NBT/BCIP to visualize where probes hybridize in the tissue section [a 1-3 hour incubation time was sufficient to detect abundantly expressed genes such as genetic markers (**Fig. 7)**; however, in general, the DIG *in situ* hybridization technique was not sensitive enough to detect many less abundantly expressed genes].

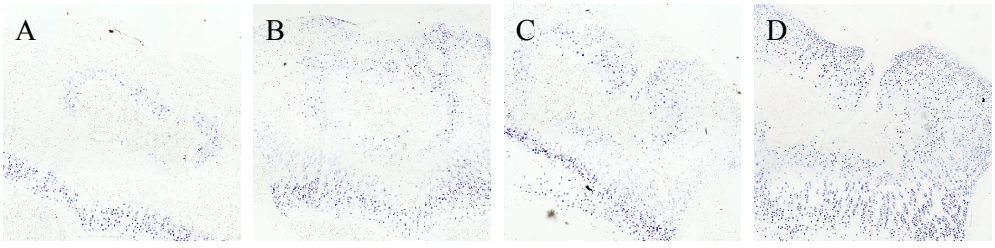

Figure 6. **Optimization of proteinase K antigen retrieval for *in situ* hybridization of postnatal rat epiphyseal cartilage**. *Col2a1*, encoding type II collagen, is expressed by all chondrocytes and thus can gauge whether antigen retrieval is sufficient before testing other genes of interest. The effectiveness of antigen retrieval was found to be primarily dependent on enzyme concentration with 100 µg/ml being optimal and on incubation time with 30 min being optimal (D). Although the maximum activity of proteinase K is at 37°C, activity is greater than 80% of maximum between 20-60°C (Kraus and Femfert, 1976) and room temperature was found to be equally effective. Note that with insufficient antigen retrieval (A) only the hypertrophic zone was properly stained whereas the resting and proliferative zones as well as most of the articular cartilage were negative.

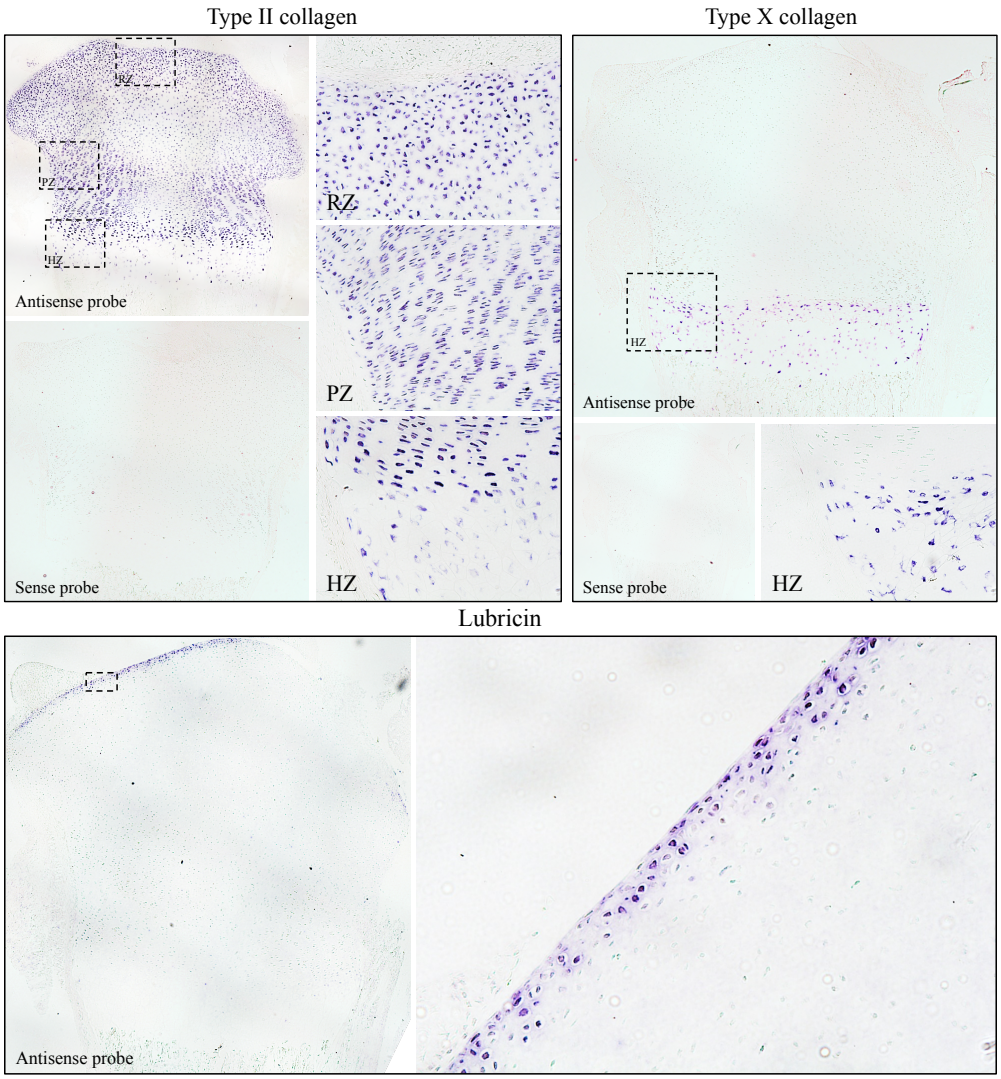

Figure 7. ***In situ*** **hybridization of genetic markers in postnatal rat epiphyseal cartilage**. *Col2a1*, encoding type II collagen, is expressed by all chondrocytes; *Col10a1*, encoding type X collagen, is expressed by hypertrophic chondrocytes; and *Prg4*, encoding lubricin, is expressed by articular cartilage superficial zone chondrocytes. These genes were also detected by *in situ* hybridization of rabbit epiphyseal cartilage (data not shown). Antisense probes target the mRNA of interest, whereas sense probes (negative control) with the same sequence as the mRNA of interest measure non-specific binding.

4.9 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a technique that exploits the property that antibodies bind with high specificity to target antigens (e.g. proteins) (Coons et al., 1941). The technique is widely used in both clinical and research settings, such as for detecting abnormal cells to aid diagnosis of disease and localizing biomarkers or differentially expressed proteins in tissue samples, respectively. Immunohistochemistry was used in this thesis to detect EGFP protein in order to distinguish *EGFP*-expressing cells from *EGFP*-negative cells.

While there are many different immunohistochemistry protocols, the basic steps can be divided into tissue preparation, embedding, sectioning, pretreatment, binding of primary antibody to target antigen, and detection of primary antibody-antigen complex.

In this thesis, tissue preparation involved collecting epiphyseal cartilage, fixation in paraformaldehyde, and decalcification in 10% EDTA. The tissue was then embedded in paraffin, and sectioned (6 μm thick) onto glass slides (superfrost +/+ slides). Antigen retrieval was performed using proteinase K digestion (**Fig. 6**). Heat-induced epitope retrieval in citrate buffer was initially used but it frequently distorted histology. Endogenous peroxidase activity was blocked using hydrogen peroxide to reduce background since the secondary antibody uses a horseradish peroxidase (HRP) enzyme for detection. Non-specific binding of primary and secondary antibody was blocked using serum from the same species as the secondary antibody.

For sample labeling, rabbit anti-rat antibody against GFP was used as the primary antibody for the first incubation with the tissue and goat anti-rabbit antibody was used as the secondary antibody to target the primary antibody during the second incubation with the tissue. To achieve a stronger signal, an additional amplification step using the avidin-biotin system, which is the strongest noncovalent biological interaction known, was opted for. Thus, a biotinylated secondary antibody was chosen, biotinylated HRP was mixed with free avidin in a specific ratio to prevent avidin saturation (avidin has 4 binding sites for biotin) and incubated at room temperature to form unsaturated avidin-biotin-HRP complexes, and the complex was used to target the secondary antibody during the third incubation with the tissue. Finally, DAB chromogenic substrate was subjected to HRP activity to localize EGFP in the tissue section.

The many steps and the sometimes poor binding of antibody to antigen make immunohistochemistry prone to false negative and false positive results. Therefore, is it important to include proper controls including positive and negative tissues, use highly specific primary and secondary antibodies, use a sufficient but not overly harsh antigen retrieval method, block potential non-specific binding sites on the tissue section, block endogenous peroxidase activity if present, block endogenous biotin if present and using the avidin-biotin system for signal amplification, and sufficiently wash the tissue sections between incubations.

5 RESULTS AND DISCUSSION

5.1 PARACRINE REGULATION OF GROWTH PLATE CHONDROCYTE DIFFERENTIATION

Gradients in Bone Morphogenetic Protein-Related Gene Expression Across the Growth Plate (Paper I)

BMPs are part of a complex system of paracrine factors involved in regulating skeletal development. At the level of the growth plate, BMP signaling accelerates longitudinal bone growth by promoting chondrocyte proliferation and hypertrophy and BMP ligands have been implicated to be produced by an endogenous source (De Luca F et al., 2001). In this study, we explored gene expression of BMP-related genes in different layers of microdissected 7-day-old rat growth plate cartilage (**Fig. 8**) using microarray and real-time PCR analysis. We validated the accuracy and reproducibility of manual microdissection by showing upregulated expression of type X collagen and alkaline phosphatase in the proliferative-hypertrophic and hypertrophic zones and by using tissue sections from different animals for microarray (n = 5) as well as real-time PCR (n = 7) analysis.

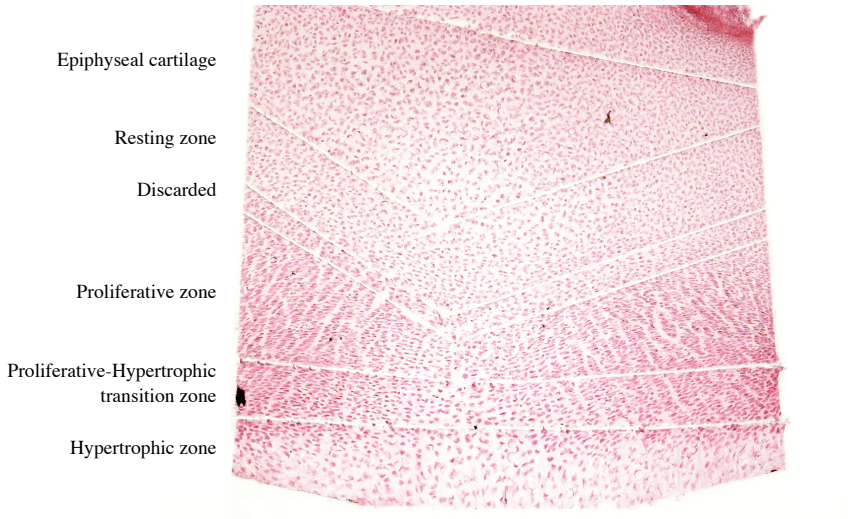

Figure 8. **Growth plate microdissection**. Photomicrograph of a frozen longitudinal section of proximal tibial epiphysis from a 7-day-old rat stained with eosin for microdissection. The section has been cut with a razor blade to separate epiphyseal cartilage, resting zone, proliferative zone, proliferative-hypertrophic zone, and hypertrophic zone. To minimize cross-contamination between the resting and proliferative zone, a segment of cartilage containing the transition from resting to proliferative zone was discarded.

Our findings suggest a gradient in BMP signaling across the growth plate with BMP agonists (BMP-2 and -6) spatially upregulated in HZ and BMP antagonists (Gremlin, Chordin, BMP-3 and -7, and GDF-10) spatially upregulated in RZ and PZ (**Fig. 9**). Furthermore, the BMP receptors BMPR-1a, -1b, and -2 were expressed in all growth plate zones, a finding that is consistent with studies showing that BMPR-1a and -1b have redundant roles in skeletal development (Kobayashi et al., 2005; Yoon et al., 2005).

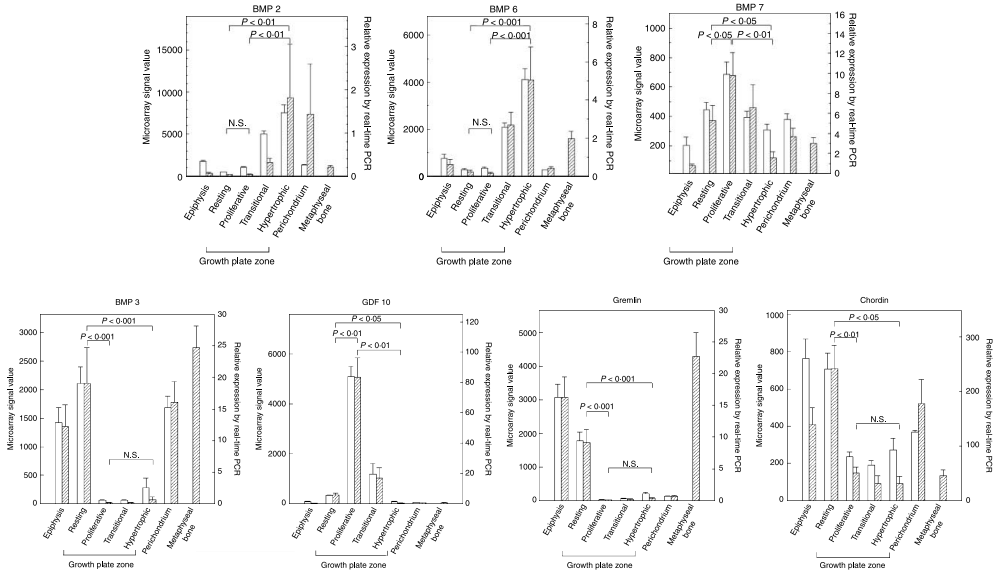

Figure 9. **Expression of genes participating in the BMP signaling system in the growth plate and surrounding tissues**. Growth plate cartilage was microdissected and relative expression of BMP agonists (BMP-2 and -6) and BMP antagonists (Gremlin, Chordin, BMP-3 and -7, and GDP-10) was determined using microarray analysis (n = 5) and real-time PCR (n = 7). Microarray signal values (white bars, left y-axis) were background corrected and normalized. Relative expression values (hatched bars, right y-axis) generated by real-time PCR were normalized to 18S rRNA. Values are mean ±S.E.M. N.S., not significant.

Overall, this study presents a detailed characterization of gene expression related to the BMP signaling system in growth plate cartilage by two independent methods in different sets of animals. These findings are consistent with previous studies suggesting BMPs are produced by hypertrophic chondrocytes and that BMP signaling plays important roles at several steps of endochondral ossification. The findings also suggest a functional BMP signaling gradient that may be a key mechanism responsible for spatial regulation chondrocyte proliferation and differentiation (**Fig. 10**).

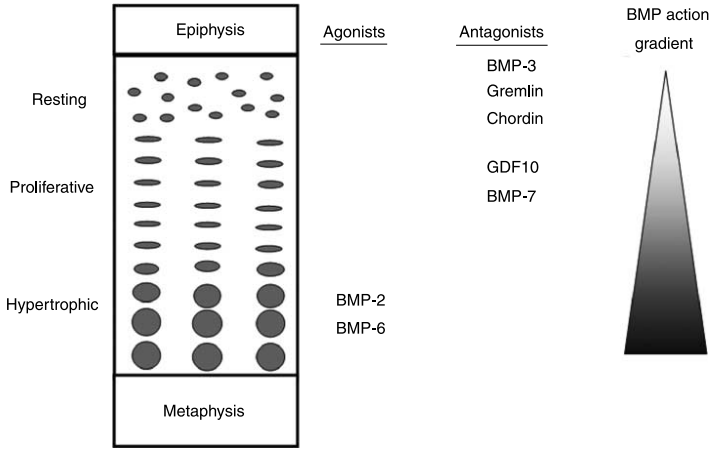

Figure 10. **BMP-gradient hypothesis**. Microarray and qPCR analysis of microdissected growth plate cartilage detected differential expression of BMP-related genes in specific regions, suggesting a BMP signaling gradient across the growth plate that may be important for spatial regulation of chondrogenesis.

Organization of the Indian Hedgehog – Parathyroid Hormone-Related Protein System in the Postnatal Growth Plate (Paper II)

In the fetal epiphysis, Ihh produced by hypertrophic chondrocytes stimulate periarticular chondrocytes to produce PTHrP, which in turn inhibits hypertrophic differentiation and maintains proliferative chondrocytes in the proliferating pool. This feedback loop is a key mechanism that determines the position where proliferative chondrocytes undergo hypertrophic differentiation and thus the height of the proliferative columns (Vortkamp et al., 1996). Postnatally, the epiphyseal region undergoes major structural and functional changes with the formation of a secondary ossification center, which presumably influences the organization of the Ihh/PTHrP system. We therefore first explored Ihh/PTHrP-related gene expression in postnatal rat growth plate cartilage using manual microdissection followed by microarray and real-time PCR analysis as described in Paper I, and then identified chondrocytes exhibiting active Ihh signaling in postnatal growth plate cartilage using a mouse model in which the coding sequences of *Gli1* were replaced with *lacZ*.

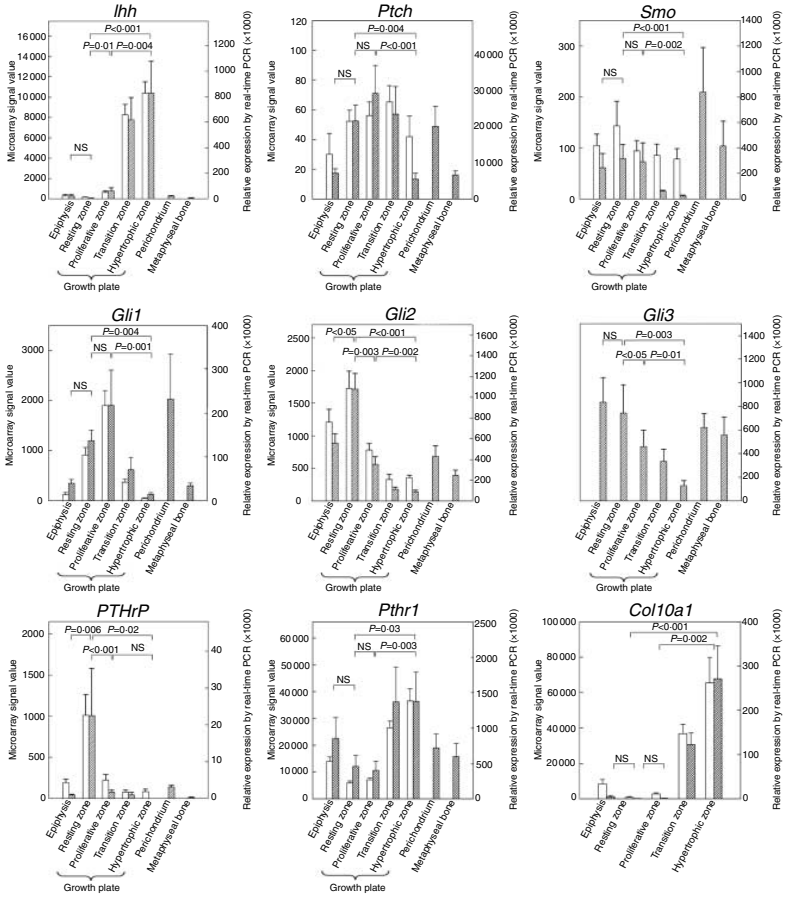

Figure 11. **Expression of genes participating in the Ihh/PTHrP signaling system in the growth plate and surrounding tissues**. Growth plate cartilage was microdissected and relative expression of Ihh, *Patched*, *Smoothened*, *Gli1-3*, *PTHrP*, and *PTHR1* was determined using microarray analysis (n = 5) and real-time PCR (n = 7). Microarray signal values (white bars, left y-axis) were background corrected and normalized. Relative expression values (hatched bars, right y-axis) generated by real-time PCR were normalized to 18S rRNA. Values are mean ±S.E.M. NS, not significant.

We first found that *Ihh*, *Patched, Smoothened, Gli1, Gli2, Gli3,* and *PTHR1* were expressed in regions analogous to the expression domains in prenatal epiphyseal cartilage: *Ihh* was differentially expressed in the prehypertrophic (pre-HZ) and hypertrophic (HZ) zones; *Patched*, the receptor for Ihh, was expressed in the resting (RZ) and proliferative (PZ) zones and perichondrium; *Smoothened*, a second messenger of Ihh signaling, was differentially expressed in RZ, PZ, and perichondrium; *Gli1, Gli2, and Gli3*, transcription factors with activity downstream of Ihh, were differentially expressed in RZ, PZ, and perichondrium; and *PTHR1*, the receptor for PTHrP, was differentially expressed in pre-HZ and HZ. Most notable, however, was that *PTHrP* was differentially expressed in RZ, which is a site that differs from the prenatal source of PTHrP, the periarticular cells (**Fig. 11**).

Since *Gli1* expression is directly regulated by hedgehog signaling (di Magliano and Hebrok, 2003; Koziel et al., 2005; McMahon, 2000), the *Gli1-lacZ* mouse model enabled the use of β-galactosidase staining to visualize Ihh signaling in proximal tibial growth plates pre- and postnatally (**Fig. 12**). We found that, at gestational day 16 (E16), *lacZ* activity was most pronounced in the superficial articular cartilage and perichondrium and gradually dissipated toward a minimum in HZ. At 1 week of age, *lacZ* activity was high in the articular cartilage, RZ, PZ, and perichondrium, whereas expression was low in HZ and minimal in hypertrophic cells located in the middle of the epiphysis where the secondary ossification later forms. At 4, 8, and 12 weeks of age, the *lacZ* activity pattern established at 1 week of age was largely maintained with distinct expression in the articular cartilage, RZ, PZ, and perichondrium, except that the superficial chondrocytes in articular cartilage lost detectable *lacZ* activity.

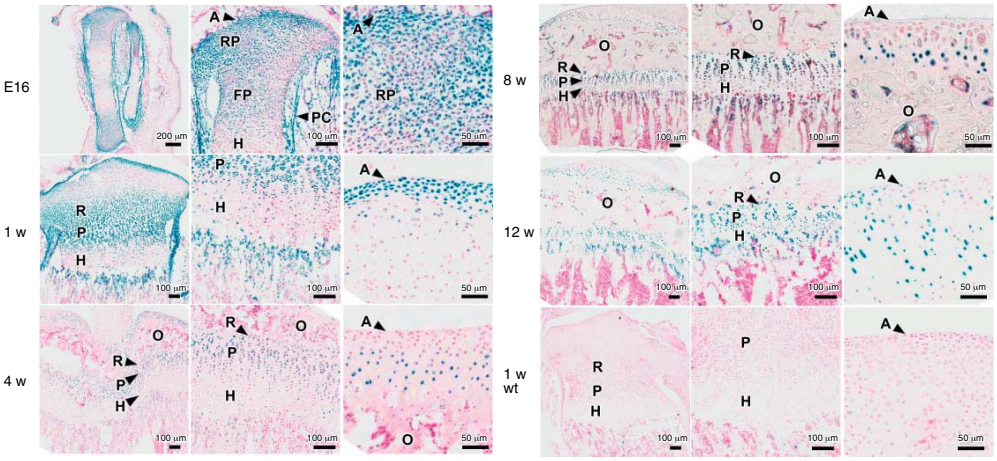

Figure 12. **Ihh signaling represented by *lacZ* expression in proximal tibiae of *Gli1-lacZ* mice at E16, 1, 4, 8, and 12 weeks of age.** A 1-week-old wild-type mouse served as a negative control. Tissue sections were stained for β-galactosidase (blue) and counterstained with nuclear fast red. Representative photomicrographs are shown with increasing magnification from left to right. A, articular surface; RP, round proliferating chondrocytes; FP, flat proliferating chondrocytes; R, resting zone; P, proliferative zone; H, hypertrophic zone; PC, perichondrium; O, secondary ossification center.

Taken together, this study supports the hypothesis that the prenatal Ihh/PTHrP feedback loop is maintained in the postnatal growth plate, except that the source of PTHrP has shifted to the resting zone. Since the number of resting zone chondrocytes decline with age (Schrier et al., 2006), our finding may explain why the height of proliferative columns shortens with age until the entire growth plate disappears at the end of puberty.

5.2 GENOTYPE-PHENOTYPE SIMILARITIES AND DIFFERENCES BETWEEN GROWTH PLATE AND ARTICULAR CARTILAGE

Gene Expression Profiling Reveals Similarities between the Spatial Architectures of Articular and Growth Plate Cartilage (Paper III)

Articular and growth plate cartilage are functionally distinct tissues but arise from a common cartilaginous condensation and have comparable spatial architectures consisting of distinct layers of chondrocytes. To explore the similarities and differences between articular and growth plate cartilage, we analyzed the gene expression profiles of the superficial (SZ) and intermediate/deep zone (IDZ) of articular cartilage and the resting (RZ), proliferative (PZ), and hypertrophic (HZ) zones of growth plate cartilage in proximal tibias of 10-day-old rats using manual microdissection (**Fig. 13**), microarray, and bioinformatics.

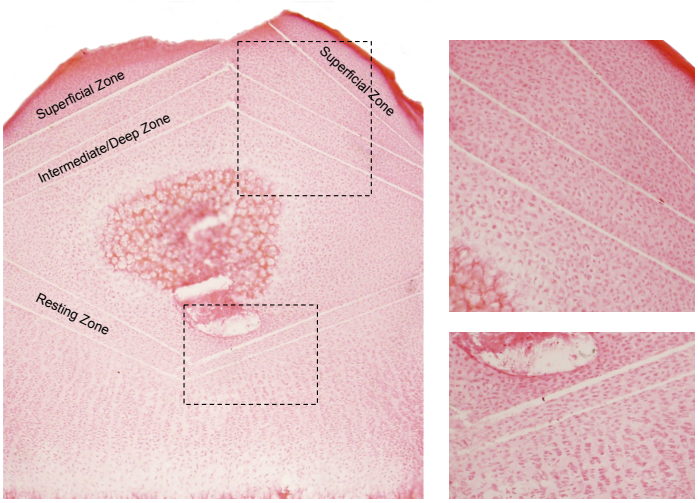

Figure 13. **Articular and growth plate cartilage microdissection**. Photomicrograph of a frozen longitudinal section of proximal tibial epiphysis from a 10-day-old rat stained with eosin for microdissection. The section has been cut with a razor blade to separate the superficial and intermediate/deep zones of articular cartilage and the resting zone of growth plate cartilage. A segment of cartilage containing the transition from the superficial zone to the deep zones of articular cartilage was discarded. Dashed lines delineate the locations of the higher magnification on right.

We first compared the gene expression profiles of SZ, IDZ, and RZ. Principal components analysis displayed closer proximity between RZ and IDZ spheres (**Fig. 14a**), hierarchical cluster analysis showed earlier convergence between RZ and IDZ (**Fig. 14b**), and heat map analysis of genes that were differentially expressed between SZ and IDZ revealed that RZ is more similar to IDZ (**Fig. 14b**). Next, comparing spatially upregulated genes between SZ and IDZ to those between RZ, PZ, and HZ (microarray data from Papers I and II), we found that there was a significant overlap of spatially upregulated genes between SZ and PZ, SZ and HZ, as well as IDZ and RZ, suggesting that IDZ and RZ share transcriptional similarities and SZ has transcriptional similarities to PZ and HZ.

Next, we assessed expression levels of known growth plate cartilage zonal markers (Lui et al., 2010) in SZ and IDZ of articular cartilage and found that RZ markers were significantly overrepresented in IDZ, whereas PZ and HZ markers were significantly overrepresented in SZ, again suggesting IDZ and RZ have similarities in

gene expression and SZ has gene expression similarities to PZ and HZ. We also identified functional biological pathways implicated by the overlapping gene expression patterns between IDZ and RZ, SZ and PZ, as well as SZ and HZ.

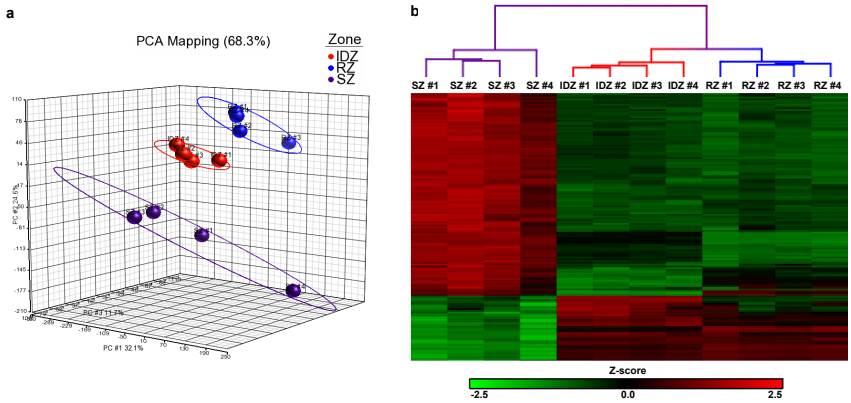

Figure 14. Bioinformatics comparison of articular and growth plate cartilage zones. Bioinformatics was performed on microarray gene expression of SZ and IDZ of articular cartilage and RZ of growth plate cartilage. **(a)** Principal components analysis scatter plot in 3-D retaining 68.3% of original sample variation. **(b)** Dendrogram following unsupervised hierarchical cluster analysis organizing samples by similarity and heat map visualization of RZ gene expression using only genes differentially expressed between SZ and IDZ. Red corresponds to higher gene expression levels represented by z-score. SZ, superficial zone; IDZ, intermediate/deep zone; RZ, resting zone.

Finally, to test for differences between articular and growth plate cartilage, especially the early gene expression changes responsible for their divergence, we identified genes that were differentially expressed between IDZ and RZ. We subsequently identified functional biological pathways that may play roles in the initial separation of articular and growth plate cartilage by the secondary ossification center.

In summary, based on gene expression profiles, the superficial zone of articular cartilage has transcriptional similarities to the proliferative and hypertrophic zones of growth plate cartilage, whereas articular cartilage intermediate/deep zone resembles growth plate cartilage resting zone. Since proliferative and hypertrophic zone chondrocytes derive from resting zone chondrocytes in the growth plate (Abad et al., 2002), these findings suggest that superficial zone chondrocytes differentiate from intermediate/deep zone chondrocytes in articular cartilage (**Fig. 15**).

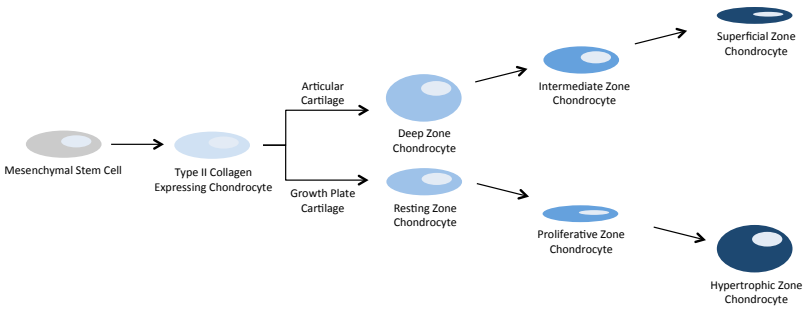

Figure 15. Articular chondrocyte differentiation hypothesis. Bioinformatic analyses revealed gene expression similarities between the intermediate/deep zone of articular cartilage and the resting zone of growth plate cartilage as well as transcriptional similarities between articular cartilage superficial zone and growth plate cartilage proliferative and hypertrophic zones, suggesting that superficial chondrocytes differentiate from intermediate/deep chondrocytes following a program that has similarities to the hypertrophic differentiation program of growth plate chondrocytes.

Growth Plate Cartilage Transplanted to the Articular Surface Remodels into Articular-like Cartilage (Paper IV)

Based on our previous finding that articular cartilage superficial zone and growth plate cartilage hypertrophic zone share many transcriptional similarities despite having marked phenotypic differences, we hypothesized that growth factors in the microenvironment regulate chondrocyte differentiation into either articular or growth plate cartilage. Specifically, the synovial joint microenvironment may inhibit hypertrophic differentiation and/or the metaphyseal bone microenvironment may promote endochondral ossification. To test this hypothesis, we used bone biopsy needles to transplant osteochondral allografts consisting of articular cartilage, epiphyseal bone, and growth plate cartilage from distal femoral epiphyses of inbred rats with ubiquitous EGFP expression to matching sites in inbred EGFP-negative rats, either in inverted or original (sham surgery) orientation, and observed for changes in allograft histology and expression of the hypertrophic chondrocyte marker *Col10a1* using *in situ* hybridization. This surgical manipulation essentially relocated growth plate cartilage to the articular surface.

Recipient animals recovered rapidly after surgery and were able to ambulate on their hind legs immediately after anesthesia wore off. None of the animals developed postoperative infection or any signs of allograft rejection. Grossly and microscopically, allografts appeared to be vital at all experimental end points. The use of inbred donors with ubiquitous EGFP expression (Lew-Tg(CAG-EGFP)YsRrrc) and inbred wild-type recipients (LEW/SsNHsd) enabled tracing of transplanted cells by EGFP immunohistochemistry (**Fig. 17I-L** and **18I-L**). However, immunohistochemical staining of EGFP did not consistently stain all chondrocytes even in cartilage sections from EGFP-positive animals.

On postoperative day 0, growth plate cartilage of donor animals was about twice as thick as articular cartilage of recipient animals (**Fig. 16C and D**). Thus, growth plate cartilage transplanted to the articular surface extended below articular cartilage. A difference in thickness remained on postoperative day 7 (**Fig. 17E-H**), but by postoperative day 28 the thickness of donor growth plate was approximately equal to that of recipient articular cartilage (**Fig. 18E-H**), suggesting substantial structural remodeling occurred.

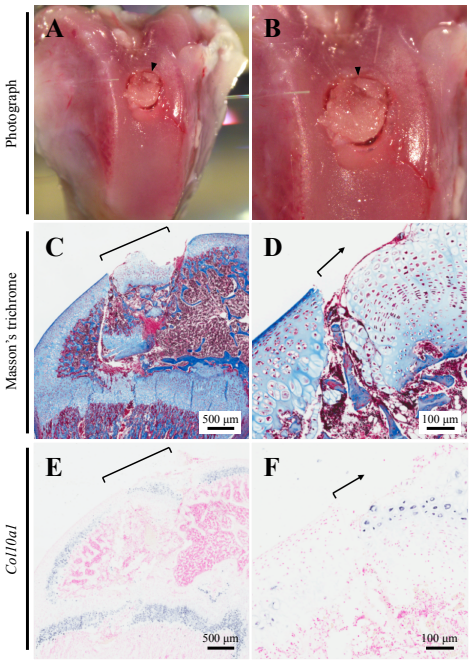

Figure 16. **Postoperative day 0**. Osteochondral allografts consisting of articular cartilage, epiphyseal bone, and growth plate cartilage from distal femur of inbred *EGFP*-expressing rats were transplanted to matching sites in inbred wild-type rats in inverted orientation. Allografts were localized by gross examination (A and B), histology was examined using Masson's trichrome stain (C and D), and *Col10a1* expression was detected by *in situ* hybridization (E and F). Representative photomicrographs are shown with increasing magnification from left to right. Arrowheads and brackets delineate the allografts.

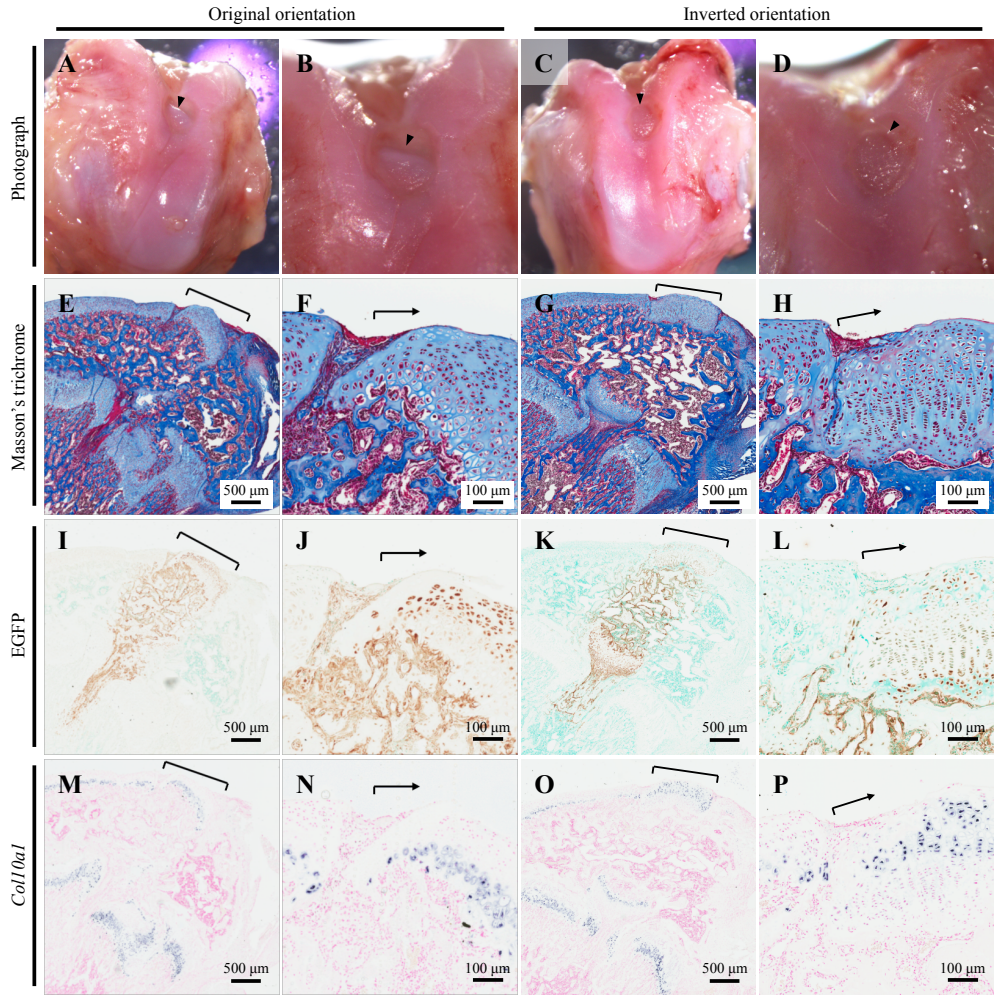

Figure 17. Postoperative day 7. Osteochondral allografts consisting of articular cartilage, epiphyseal bone, and growth plate cartilage from distal femoral intercondylar articular surfaces of inbred *EGFP*-expressing rats were transplanted to matching sites in inbred wild-type rats in original (sham surgery, column 1) or inverted orientation (column 2). Allografts were localized by gross examination (A-D) and EGFP immunohistochemistry (stained with brown DAB substrate and counterstained with nuclear methyl green) (I-L), histology was examined using Masson's trichrome stain (E-H), and *Col10a1* expression was detected by non-radioactive digoxigenin *in situ* hybridization (stained with purple NBT/BCIP substrate and counterstained with nuclear fast red) (M-P). Representative photomicrographs are shown with increasing magnification from left to right. Arrowheads and brackets delineate the allografts.

Growth plate cartilage transplanted to the articular surface also exhibited gradual changes in cell morphology and *Col10a1* expression. Beginning on postoperative day 7, the most superficial cell layer of the transplanted growth plate cartilage consisted of smaller and actively proliferating cells that do not express *Col10a1*, while hypertrophic chondrocytes expressing *Col10a1* remained underneath. This observation suggests that hypertrophic differentiation is inhibited at the articular surface and that hypertrophic chondrocytes placed at the articular surface may even undergo dedifferentiation (**Fig. 17H and P**). Deeper into the allografts, proliferative and resting zone chondrocytes were still identifiable by their characteristic histology (**Fig. 17H**).

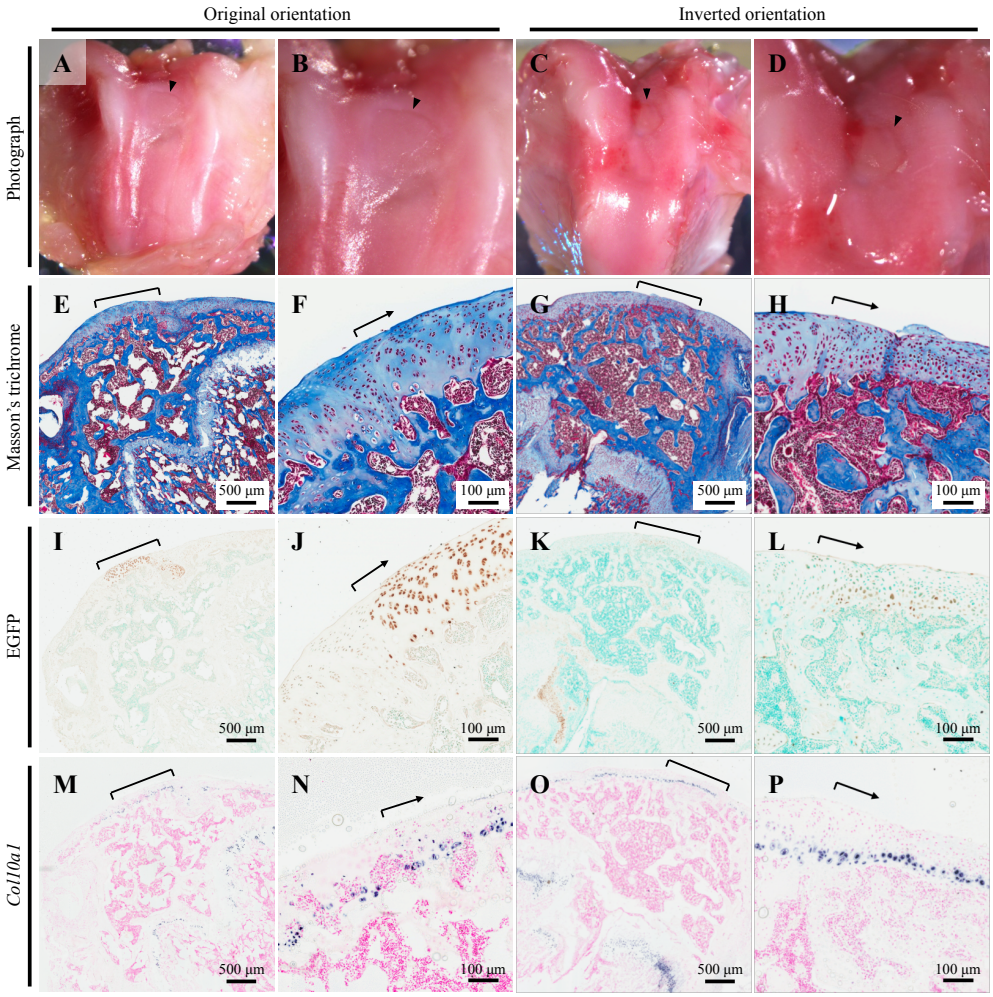

Figure 18. Postoperative day 28. Osteochondral allografts consisting of articular cartilage, epiphyseal bone, and growth plate cartilage from distal femoral intercondylar articular surfaces of inbred *EGFP*-expressing rats were transplanted to matching sites in inbred wild-type rats in original (sham surgery, column 1) or inverted orientation (column 2). Allografts were localized by gross examination (A-D) and EGFP immunohistochemistry (stained with brown DAB substrate and counterstained with nuclear methyl green) (I-L), histology was examined using Masson's trichrome stain (E-H), and *Col10a1* expression was detected by non-radioactive digoxigenin *in situ* hybridization (stained with purple NBT/BCIP substrate and counterstained with nuclear fast red) (M-P). Representative photomicrographs are shown with increasing magnification from left to right. Arrowheads and brackets delineate the allografts.

By postoperative day 28, transplanted growth plate cartilage at the articular surface remodelled into cartilaginous tissue with a structure similar to that of articular cartilage. Hypertrophic chondrocytes were no longer present at the surface of the allografts, but rather smaller chondrocytes that tended to orient parallel to the articular surface were observed (**Fig. 18G and H**). Moreover, the proliferative columns and resting zone chondrocytes initially located in the deeper layers of the allografts were no longer detected. Instead, hypertrophic chondrocytes expressing *Col10a1* were localized in the deep zone of the allografts at the same level as the hypertrophic chondrocytes of adjacent articular cartilage (**Fig. 18O and P**).

Altogether, the changes in histology and type X collagen expression that occurred in growth plate cartilage transplanted to the articular surface demonstrated structural remodeling and cellular differentiation into articular-like cartilage. These findings may suggest that the synovial joint microenvironment inhibits hypertrophic differentiation and promotes articular cartilage formation. Whether the remodeling is due to mechanical loading, chemical milieu, or a molecular growth factor(s) will be explored in future studies.

6 CONCLUSIONS

1. In the growth plate, the BMP antagonists Gremlin, Chordin, and BMP-3 are highly expressed in the resting zone and GDF-10 and BMP-7 in the proliferative zone, whereas the BMP agonists BMP-2 and -6 are highly expressed in the hypertrophic zone, thus suggesting a functional BMP signaling gradient from the resting to hypertrophic zone. BMP action gradients may provide a key mechanism that contributes to the spatial regulation of chondrocyte proliferation and differentiation in growth plate cartilage and thereby also contributing to longitudinal bone growth.

2. The embryonic Ihh/PTHrP feedback loop is maintained in postnatal growth plate cartilage except that the source of PTHrP has shifted to the resting zone. Since the number of resting zone chondrocytes diminishes with age, the resultant decrease in PTHrP production may explain why the height of growth plate cartilage declines with age until eventually disappearing at the end of puberty when the growth plate is completely replaced by bone

3. The intermediate/deep zone of articular cartilage has a gene expression profile more similar to that of the resting zone of growth plate cartilage, whereas articular cartilage superficial zone has a gene expression profile more similar to those of growth plate cartilage proliferative and hypertrophic zones. Since proliferative and hypertrophic chondrocytes derive from resting zone chondrocytes, these findings raise the possibility that superficial zone chondrocytes analogously differentiate from intermediate/deep zone chondrocytes.

4. Growth plate cartilage transplanted to the articular surface remodels into articular-like cartilage as determined by similarities in tissue histology, cell morphology, and down-regulation of the hypertrophic differentiation marker type X collagen. These findings suggest that the synovial joint microenvironment inhibits hypertrophic differentiation and promotes articular cartilage formation.

7 CLOSING REMARKS

Normal skeletal development and maintenance depend largely upon the functions of two relatively small tissues located at the ends of long bones, growth plate and articular cartilage. Over the past decades, considerable effort has been made to help us better understand the cellular and molecular mechanisms underlying the well-coordinated proliferation and differentiation of cells in these tissues. However, much remains to be clarified and discovered regarding articular and growth plate chondrocyte differentiation not only for our basic understanding but also ability to translate scientific knowledge into clinical practice.

This thesis presents the spatial organizations of two important molecular signaling pathways (BMP and Ihh/PTHrP) in growth plate cartilage based on gene expression. It thereby helps explain many previous observations and studies showing the stimulatory effects of BMPs on longitudinal bone growth and how the Ihh/PTHrP feedback loop is maintained in postnatal life as secondary ossification centers form and structurally separate articular and growth plate cartilage. In postnatal animals, we showed that PTHrP is produced in the growth plate by resting zone chondrocytes. Since the number of resting zone chondrocytes decreases with age, this finding may provide insight into a potential key mechanism underlying the normal thinning of growth plate cartilage until fusion at the end of puberty.

In addition, this thesis revealed unexpected spatial similarities between growth plate and articular cartilage based on gene expression profiles. This finding suggests that the cellular differentiation program of articular cartilage follows a pattern that has similarities to the hypertrophic differentiation program of growth plate cartilage and directs away from the secondary ossification, leading us to hypothesize that microenvironment accounts for the marked phenotypic differences between the two tissues. Indeed, we found that growth plate cartilage transplanted to the articular surface remodels into articular-like cartilage. This finding indicates the existence of a novel mechanism responsible for articular cartilage formation.

In future studies, we will consider mechanical loading, chemical milieu, and molecular growth factors as potential mechanisms promoting articular cartilage formation. If the mechanism is related to cell signaling, we plan to identify the putative factor, study its effects on skeletal development using genetic manipulations to knock out or overexpress the gene encoding the putative factor, and test the ability of the putative factor to stimulate differentiation of primary chondrocytes, adult mesenchymal stem cells, or chondrocyte cell lines into articular chondrocytes. It is foreseeable that such a regulatory mechanism can be used to refine current techniques in articular cartilage tissue engineering and orthopaedic surgical procedures such as ACI.

8 ACKNOWLEDGEMENTS

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