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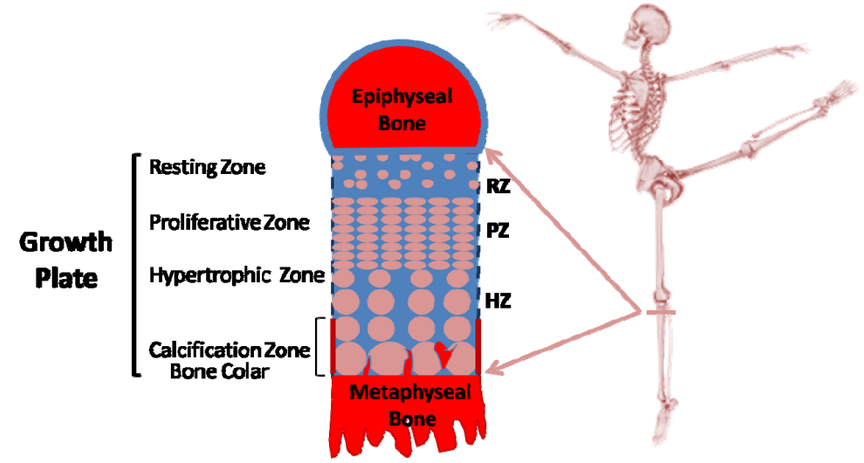
LOCAL REGULATION OF GROWTH PLATE CHONDROCYTES: *Molecular Mechanisms and Implications for Longitudinal Bone Growth*



Anenisia Coelho de Andrade

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From Department of Women's and Children's Health
Karolinska Institutet, Stockholm, Sweden

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"All my life through, the new sights of Nature made me rejoice like a child."

(Marie Curie)

To my beloved parents (Ademar and Neusa), and siblings (Aderivaldo, Artemaria, Adeneusa and Miguel).

Thank you for the inconditional support.

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ABSTRACT

The regulation of linear growth is a complex and 'intriguing' process that has the growth plate cartilage as its final target. Growth plate is a specialized cartilage localized in the end of the long bones between the epiphyseal and metaphyseal bone. It consists of 3 layers (resting, proliferative and hypertrophic zones) with distinct cellular morphology and function, where the continuous process of endochondral ossification takes place. This process is tightly regulated and relies on the interaction between systemic and local action of several hormones and growth factors. Although height is a true multigenetic trait and much has been discovered on the control of skeletal growth; there is still a long way to go to understand the intrinsic control of growth plate chondrogenesis that regulates postnatal bone growth and determines individual height.

In order to explore the molecular mechanisms implicated in the spatial and temporal control of growth plate, we first used microdissection of postnatal rat growth plate combined with microarray and real-time PCR analyses. Bioinformatic analysis of global gene expression implicated novel biological functions, molecular pathways, transcription factors and potential markers for spatially-associated chondrocyte differentiation and temporally-associated growth plate senescence (Paper I).

We next explored some of these molecular pathways implicated in the control of postnatal chondrogenesis. The Wnt/β-catenin signaling was one of the most strongly implicated pathways in the developmental program of growth plate senescence revealed in our microarray analysis. We found that six Wnts were expressed in growth plate chondrocytes, of which Wnts-2b, -4 and -10b act through the canonical β-catenin pathway and Wnts -5a, -5b and -11 acts through the non-canonical Calcium pathway. We observed that all the expressed Wnts exhibited a similar pattern of expression in the growth plate, showing low expression in resting zone, increasing expression as the chondrocytes differentiated into the proliferative zone, and then decreasing expression as the chondrocytes underwent hypertrophic differentiation. In addition, all identified Wnts persisted at similar levels with age. Altogether, our findings suggest that Wnts modulate growth plate senescence and chondrocyte hypertrophy through the canonical β-catenin and non-canonical calcium pathways (Paper II). We then, characterized the expression profile of a growth-regulated network of imprinted genes implicated in embryonic growth of soft tissues. We found that the expression pattern of the network is modified in growth plate cartilage compared both to soft tissues and to bone. In particular, developmental changes in the expression of growth-promoting genes (Mest, Dlk1, Gtl2), and growth-inhibitory genes (Cdnk1c and Grb10) may contribute to the decline in longitudinal bone growth that occurs with age (Paper III). In addition, by using distinct growth inhibiting conditions, we found functional and structural delay in growth plate senescence markers that indicate that growth plate senescence is not simply a function of time *'per se'* but rather of growth, and that delayed senescence may be a general consequence of growth inhibition (Paper IV).

We also identified unique microRNAs (miRNAs) that are preferentially expressed and age-regulated in growth plate chondrocytes. The role of these miRNAs in growth plate chondrogenesis were tested using *in vitro* system for culture and transfection of murine resting zone chondrocytes, which enabled us to study the role of miRNAs in primary chondrocytes phenotype, simulating *in vivo* conditions more closely. These findings suggest that miRNAs contribute to the developmentally regulated decline in longitudinal bone growth through regulation of chondrocyte proliferation and apoptosis. Altogether, our findings contribute to understand the molecular regulation of growth plate chondrocytes and its implications to postnatal linear growth (Paper V).

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-V)

I. Lui JC, **Andrade AC**, Forcinito P, Hegde A, Chen W, Baron J, Nilsson O. Spatial and temporal regulation of gene expression in the mammalian growth plate. Bone. 2010;46:1380-90.

II. **Andrade AC**, Nilsson O, Barnes KM, Baron J. Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. Bone. 2007 May;40(5):1361-9.

III. **Andrade AC**, Lui JC, Nilsson O. Temporal and spatial expression of a growth-regulated network of imprinted genes in growth plate. Pediatr Nephrol. 2010 Apr;25(4):617-23.

IV. Forcinito P, **Andrade AC**, Finkielstain GP, Baron J, Lui JC, Nilsson O. Growth Plate Senescence Is Not a Function of Time per se but of Growth (Submitted).

V. **Andrade AC**, Adams WC, Caramuta S, Nilsson O. MicroRNA-497, -142-3p, and 125a-3p promote chondrocyte proliferation whereas miR-195 enhances apoptosis during growth plate senescence (Manuscript).

RELATED PUBLICATIONS

I. Lazarus JE, Hegde A, **Andrade AC**, Nilsson O, Baron J. Fibroblast growth factor expression in the postnatal growth plate. Bone. 2007 Mar;40(3):577-86.

II. Emons JA, Marino R, Nilsson O, Barnes KM, Even-Zohar N, **Andrade AC**, Chatterjee NA, Wit JM, Karperien M, Baron J. The role of p27Kip1 in the regulation of growth plate chondrocyte proliferation in mice. Pediatr Res. 2006 Sep;60(3):288-93.

CONTENTS

LIST OF ABBREVIATIONS

AR	Androgen Receptor
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
ER	Estrogen Receptor
EZ	Epiphyseal Zone
FACS	Fluorescence-Activated Cell Sorting
FGF	Fibroblast Growth Factor
GC	Glucocorticoid
GH	Growth Hormone
GHR	Growth Hormone Receptor
GP	Growth Plate
HZ	Hypertrophic Zone
IGF1	Type1 Insulin Growth Factor
IGF1R	IGF1 Receptor
IHH	Indian Hedgehog
MB	Metaphyseal Bone
miRNA	microRNA
MSC	Mesenchymal Cells
PTHrP	Parathyroid Hormone (PTH) related Peptide
PPRs	PTH/PTHrP receptors
PZ	Proliferative Zone
qPCR	Quantitative real-time Polymerase Chain Reaction
RNAi	RNA interference
RUNX2	Runt-related Transcription Factor2
RZ	Resting Zone
SOX9	SRY (sex determining region Y)-box 9
TH	Thyroid Hormone
TR	Thyroid Receptor
VEGF	Vascular Endothelial Growth Factor
WNT	Wingless-type MMTV integration site family

1 INTRODUCTION

1.1 Longitudinal Bone Growth

Growth is one of the most fundamental tasks of childhood development and growth assessment is an important screening tool in the pediatric health evaluation. Although tall stature is also a concern, especially among teenage girls, short stature is a more common cause of concern and one of the main causes of referral to the pediatric endocrinologist. Attaining the potential height for every child may be accompanied by parental expectation and anxiety, and lead to emotional issues in the children and adolescents as well. However, "physiological" factors need to be considered when evaluating a child's growth, such as: inherited growth potential (parental height), "physiological age" (pubertal status and bone age), and "normal" variations in nutritional status.

Human height is a quantitative trait with a clear pattern of family resemblance, consistent with a polygenic model of inheritance (Galton 1886; Visscher 2008). Recently, three genome-wide association and genetic linkage studies with a combined sample size of more than 60,000 individuals have tried to explain how much adult height is due to the polygenic model, searching for loci (presumable genes) that might contribute to the variation in adult height and at what extent. However, the 54 detected loci can only explain a small proportion (3-5%) of height variation among the population, with each locus having a small effect size of 0.3-0.6 cm (Gudbjartsson, Walters et al. 2008; Lettre, Jackson et al. 2008; Weedon, Lango et al. 2008). The results of these studies suggest that height is a true multigenetic trait and that future studies with larger cohorts will detect many more genes with even smaller effect size. It is noteworthy that some of these studies found expected as well as unexpected genes, and implicates the role of micro-RNAs (miRNAs), such as let7 family, whose target genes (e.g. IGF1, IGF1R and COL1A2) are known as important regulators of human stature (Lettre, Jackson et al. 2008). However, there is still a long way to go by using this strategy and much more knowledge is required to understand the role of these genes and mechanisms of interaction between them in order to determine individual height. Since there are so many genes involved and very small size effects for each one of them, it seems unlikely that this approach will reach practical clinical application.

The regulation of linear growth is a complex and 'intriguing' process that has the growth plate cartilage as its final target, and relies on the interaction between systemic and local action of several hormones and growth factors. Throughout life, longitudinal growth constitutes a self-limited and decelerated process. Human growth velocity can be as high as 100cm/year or more in fetal life, 50cm/y at birth, remains around 5cm/y during late childhood, and stops just after the pubertal growth spurt (Karlberg 1987). Any disturbance in growth velocity may be an early indication of a great variety of hormonal and non-hormonal systemic diseases, which might compromise the individual's final height (Simm and Werther 2005).

While the influence of hormonal status has been better established, the local signaling pathways that regulate linear bone growth is not fully understood, although substantial progress in this field has been made (Kronenberg 2003). Skeletal development and systemic hormonal regulation of growth will be briefly commented on. Then, the molecular mechanisms intrinsic to the growth plate that regulate proliferation and differentiation of chondrocytes within distinct zones and throughout age, as well as their repercussion on postnatal bone growth will be described in more detail.

1.2 Skeletal Development

The vertebrate skeleton is built from condensations of mesenchymal cells (MSC) that adhere through the expression of adhesion molecules and, under a selective regulation of genes, differentiate into either two processes: intramembranous or endochondral ossification (Hall and Miyake 2000; Kronenberg 2003). Intramembranous ossification is mainly responsible for the formation of the flat bones of the skull and medial clavicles; and result from direct differentiation of MSC into bone cells (osteoblasts), which lay down a matrix rich in type I collagen. Transcription factor such as RUNX2, previously named Cbfa1, is a key regulator of osteoblast differentiation (Ducy, Zhang et al. 1997; Komori, Yagi et al. 1997), as well as the Wnt-β/Catenin signaling pathway has been shown to be essential to direct MSC into osteogenic program (Day, Guo et al. 2005). However, most of the skeleton, including the limbs, results from the process of endochondral ossification.

1.2.1 Endochondral Ossification (Limb Formation)

Endochondral ossification constitutes a complex process where MSC become chondrocytes (cartilaginous cells), which are used as a template to form bone. During this process, chondrocytes secrete a matrix rich in type II collagen (Kosher, Kulyk et al. 1986) and proteoglycan aggrecan, and express a selective set of genes and transcription factors that direct the formation of bone. Parallel to this process, cells at the border of mesenchymal condensations form perichondrial cells, which will form the perichondrium that surround cartilage of the developing bone (Kronenberg 2003).

Endochondral Ossification (limb formation)

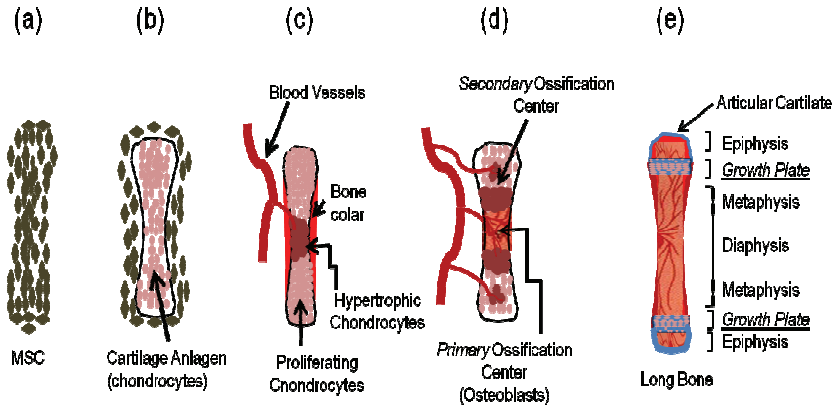

Fig. 1. Endochondral ossification illustrated into sequential steps: A) Mesenchymal cells (MSC) form a condensation, and **B)** undergo differentiation into cartilage cells (chondrocytes) to form the cartilaginous anlagen of the bone. **C)** Chondrocytes in the center of the shaft stop proliferating, undergo hypertrophy and induce blood vessels invasion (primary ossification center) and formation of bone collar. **D)** Then, bone cells (osteoblasts) are formed and originate the primary ossification center (primary spongiosa). Meanwhile, growth consists of chondrocyte proliferation in one dimension, hypertrophy, and mineralization of the surrounding matrix. Secondary ossification centers also form as blood vessels enter near the tips of the bone. **E)** Finally, growth plate cartilage rest between epiphyseal and metaphyseal bones during postnatal life. In the outer part of epiphysis, chondrocytes cells become articular cartilage. *Adapted from Gilber, S.F., 2000.*

Endochondral ossification spreads outward in both directions from the center of the bone (Fig. 1). At the same time that the cartilage mould further enlarges through continued proliferation of chondrocytes, actively dividing osteoblasts arise in regions of the cartilage called ossification centers. As the ossification front get close to the ends of the cartilage mould, the chondrocytes stop proliferating, undergo hypertrophy and are replaced by bone, pushing out the cartilaginous ends of the bone to form the epiphyseal

cartilage (Gilbert 2000). Together with continuous chondrocyte proliferation, hypertrophic chondrocytes becomes the principal engine of bone growth, direct mineralization of their surrounding matrix rich in type X collagen, attract blood vessels trough the production of vascular endothelial growth factor (VEGF) as well as other factors, and attract chondroclasts that digest the matrix that will be later replaced by bone. In addition, hypertrophic chondrocytes direct adjacent perichondrial cells to become osteoblasts, forming a bone collar. Finally, the terminal hypertrophic chondrocytes undergo apoptosis, and the invading osteoblasts that lay down a bone matrix using the calcified cartilage matrix as a scaffold. As consequence, new bone is created progressively at the bottom of the growth cartilage, which results in longitudinal bone growth.

1.3 Postnatal Growth Plate

1.3.1 Structural Organization & Function

The growth plate is the final target organ for endochondral ossification, which allows the elongation of long bones during post-natal life until late adolescence, when growth stops and the growth plates fuse, determining the final height of the individual (Hunziker 1994).

This specialized cartilage is located in the end of the long bones, between the epiphyseal and metaphyseal bone, and consists of three histological and functionally discrete layers that follow an elegant developmental program (Burdan, Szumilo et al. 2009) (Fig. 2). Proximal to the epiphysis (toward the end of the bone), chondrocytes in the resting zone (RZ) are small, round, singly or distributed in pairs within a compact extracellular matrix (ECM), with a low proliferation rate. *In vivo* studies in rabbits show that after removal of proliferative and hypertrophic zones, leaving only RZ in the growth plate bed, a complete growth plate structure is re-established within 1 week, suggesting that RZ chondrocytes act as 'stem-like' cells that continuously renew the pool of proliferative chondrocytes (Abad, Meyers et al. 2002). Furthermore, implantation of growth plate by placing resting zone ectopically, alongside the proliferative columns, induced 90-degree shift in the orientation of nearby proliferative zone chondrocytes, suggesting that RZ chondrocytes may be partially responsible for

the spatial organization of the growth plate into distinct zones of proliferation and hypertrophy as well (Abad, Uyeda et al. 1999; Abad, Meyers et al. 2002).

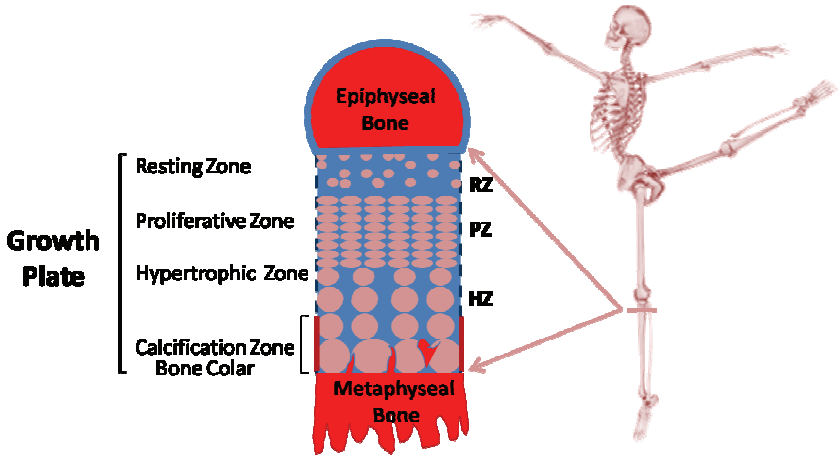

Fig. 2. Structural Organization of the Growth Plate: Placed between epiphyseal and metaphyseal bone of long bones, the growth plate is formed by distinct zones that represent histological and functional stages of chondrocyte differentiation: resting (stem-like), proliferative and hypertrophic zones.

Next, in the proliferative zone (PZ), chondrocytes assume a flattened, discoid shape and form column-like structures, resembling "stacks of coins", that are oriented along to the long axis of the bone, thus directing the lengthening of the bone primarily in one dimension. This layer has a high proliferation rate and produces a matrix (ECM) rich in type II and type IX collagens (Hunziker and Schenk 1989; Nilsson and Baron 2004).

In the hypertrophic zone (HZ), chondrocytes undergo major changes in their phenotype, marked by physical and biochemical changes that occur in a spatial and temporal manner. Hypertrophic chondrocytes lose their capacity to divide, decrease their DNA synthesis, and have a 10-fold increase in their intracellular volume (Hunziker, Schenk et al. 1987). Rather than a passive swelling, this is an active process, marked by an increase in organelles such as mitochondria and the endoplasmic reticulum (Hunziker 1994). Chondrocyte hypertrophy has an important role in the longitudinal growth of the skeleton. The increase in height of chondrocytes is responsible for up to 73% of long bone growth, with the remainder being due to matrix synthesis and chondrocyte proliferation (Wilsman, Farnum et al. 1996). At the end of the cell columns, terminally differentiated hypertrophic chondrocytes, prepare the

surrounding matrix as a "scaffold on which osteoclasts build", and bone gradually replaces the newly formed cartilage (Stump 1925). They produce a calcified matrix rich in type X collagen (Hunziker 1994), and expression of alkaline phosphatase has been related to the widening of the growth plate by increasing phosphate ions required for calcification of the ECM. Finally, chondrocytes undergo apoptosis, and ECM is degraded, followed by invasion of the vascular channels and bone-marrow stromal cells. Essentially, the growth plate is an avascular organ that relies on diffusion of both oxygen and nutrients for cell metabolism from vascular arcades localized on the metaphyseal side of the growth plate (Skawina, Litwin et al. 1994). There, vascular channels are aligned along the longitudinal axis of the bone, and contain an ascending and descending capillary system (Aharinejad, Marks et al. 1995). However, endochondral ossification doesn't occur without vascular invasion of growth plate (Provot and Schipani 2007). It is worth noting that the vascular endothelial growth factor A (VEGFA), expressed solely by the hypertrophic chondrocytes, is a key factor responsible for vascular invasion (Gerber, Vu et al. 1999; Carlevaro, Cermelli et al. 2000; Colnot and Helms 2001). At the same time, hypertrophic chondrocytes undergo apoptosis and vascular invasion occurs; bone collar formation is induced in the surrounding perichondrium (Chung, Lanske et al. 1998). Then, osteoblasts replace the disappearing cartilage with trabecular bone, and bone marrow is formed (Skawina, Litwin et al. 1994; Solomon, Berube et al. 2008).

As long as the growth plate is able to produce chondrocytes, the bone continues to grow. The progression from proliferating to terminal differentiation of hypertrophic cells is the key to the growth of those skeletal elements that are formed by endochondral ossification. Without chondrocyte proliferation and hypertrophy, endochondral bones cannot grow in length, and without hypertrophic cell death and the concomitant vascular invasion, the cartilage model cannot be replaced by bone. The study of molecular mechanisms that regulate chondrocytes proliferation and differentiation is an ongoing process, and there is still a lack of knowledge on how known and unknown mechanisms works together to regulate linear bone growth.

1.3.2 Growth Plate Senescence

Parallel to the observed decline in postnatal growth rate, growth plate undergoes structural and functional changes that appear to be due to intrinsic mechanisms, rather

than caused by changes in hormonal or any other systemic factor (Gafni, Weise et al. 2001; Weise, De-Levi et al. 2001) (Fig. 3).

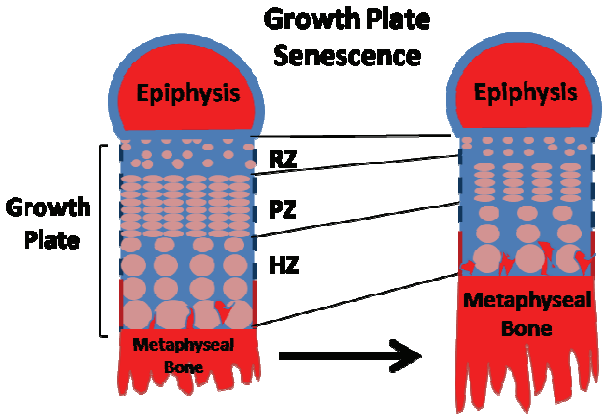

Fig. 3. Structural changes during growth plate senescence. *Adapted from Nilsson O., 2004.*

First, an overall decrease in the total height of the growth plate, as well as of each individual zone, is observed. This phenomenon is accompanied by a decline in the number of proliferative and hypertrophic chondrocytes per column, more widely spaced columns, and smaller size of the hypertrophic cells (Kember 1973). In addition, an age-dependent increase in apoptosis of hypertrophic chondrocytes has been reported, and might contribute to the age-dependent decline in growth rate (Chrysis, Nilsson et al. 2002).

The senescence program of growth plate is primarily marked by a decline of the proliferation rate of chondrocytes (Kember 1979). *In vivo* transplantation of growth plates between rabbits of different ages results in different growth rates in the recipient. The growth rates of transplanted growth plates are dependent on the age of the donor animal, i.e., younger donors lead to a higher growth rate in both young and older recipients (Stevens, Boyer et al. 1999). This study indicates that the age-dependent decline in growth rate is due to a mechanism that is intrinsic to the growth plate rather than to a systemic mechanism. This intrinsic mechanism has been referred to as *'growth plate senescence'* (Baron, Klein et al. 1994).

1.3.3 Growth Plate Fusion & Final Height

In humans, and most mammals, cessation of growth is accompanied by the fusion of the epiphysis and metaphysis, as the growth plate is ossified. Epiphyseal fusion is an active process with its own hormonal control, cellular mechanisms, and structural features. For a long time, epiphyseal fusion was believed to be the cause of cessation of growth (Wilkins 1965; Parfitt 2002). However, it has been clear that the growth plate narrows not because cartilage replacement occurs earlier, but because cartilage addition occurs more slowly as the rate of chondroblast proliferation declines (Walker and Kember 1972). Thus, the growth plate does not begin to disappear until proliferation has stopped altogether. Estrogens are a key component in the control of physis fusion in both sexes (Grumbach 2004). For more detail on its role in growth plate closure, refer to the section of hormonal regulation of growth plate by Sex hormones (section 1.4.1.2).

1.3.4 Catch-Up Growth

Following remission of diverse chronic diseases that affect growth development, "*Catch-up growth*" may occur, which is a phenomenon characterized by height velocity above the normal limits for age. As a result, final height is improved, although this recovery of height might be complete or not (Gafni and Baron 2000). Two principal hypotheses have been proposed to explain the mechanism of catch-up growth. The 'neuroendocrine' hypothesis postulates a central nervous system mechanism that compares actual body size with an age-appropriate set-point and then adjusts growth rate accordingly (Tanner 1963). However, evidences that growth inhibition in a single growth plate is followed by local catch-up do not support the neuroendocrine model (Baron, Klein et al. 1994). Thus, a new hypothesis that places the mechanism within the growth plate itself has been proposed. Accordingly to this model, growth-inhibiting conditions decrease proliferation of growth plate *"stem-like"* cells and, as consequence, preserve their proliferative potential. Experiments analyzing the catch-up growth of animals after treatment of growth-inhibiting conditions, such as hypercortisolism and hypothyroidism, reveal that after the growth-inhibiting condition resolves the growth plates are less senescent and therefore grow more rapidly than normal for age (Gafni, Weise et al. 2001; Marino, Hegde et al. 2008). During catch-up, animals treated from hypothyroidism presented a delayed decline not only in their growth plate function and structure, but in molecular markers of growth plate senescence as well (Gafni, Weise et

al. 2001; Marino, Hegde et al. 2008). Therefore inhibition of growth by different conditions delay the developmental program of growth plate senescence, 'holding' the finite proliferative capacity of growth plate chondrocytes on *'standby'* state, which, at least partly, explain the phenomenon of catch-up growth. The molecular mechanisms controlling the program of growth plate senescence is just starting to unravel, but will explain the observed decline in growth rate of growth plate cartilage, and may also give insight to the simultaneous decline in growth rate of other tissues during postnatal growth.

1.4 CONTROL OF GROWTH & DEVELOPMENT

1.4.1 Hormonal Regulation of Growth Plate Cartilage

A network of hormones is required for normal growth and development during childhood and adolescence. In healthy children, these hormones are produced in appropriate amounts. Only when their circulating levels are too low or to high for prolonged periods will there be an adverse effect on height. In this section, we will briefly discuss the systemic control of growth by the major hormones: the growth hormone (GH), thyroid hormones (T3), Glucocorticoids (GCs) and sex hormones (Estrogen and Androgens), but focusing our discussion on their local regulation in the growth plate (Fig. 4).

1.4.1.1 GH-Insulin-like Growth Factors (IGFs) signaling

Growth hormone (GH) is perhaps the most well studied hormone with established effects on longitudinal growth. In an orchestrated network with insulin-like growth factors, IGF1 and IGF2, and their receptors, GH is believed to be the key regulator of overall linear growth. As an example, enhanced GH secretion caused by a pituitary adenoma in childhood leads to gigantism (Sotos 1996; Eugster and Pescovitz 1999); while any defects in its synthesis, release or local action results in severe dwarfism (Rosenfeld 2005). Thus, targeted ablation of the GH receptor (GHR), IGF1, IGF2, or type1-IGF receptor (IGFR1) impairs bone growth (Walenkamp and Wit 2007).

GH acts both directly and indirectly in the growth plate chondrocytes. Gene expression of GH-IGF system in growth plate cartilage by diverse techniques has revealed that

GHR, IGF1, IGF2 and most of IGF binding proteins (IGFBPs) are detected in growth plate chondrocytes in several species (Nilsson, Carlsson et al. 1990; Shinar, Endo et al. 1993; Wang, Wang et al. 1995; Reinecke, Schmid et al. 2000; Parker, Hegde et al. 2007).

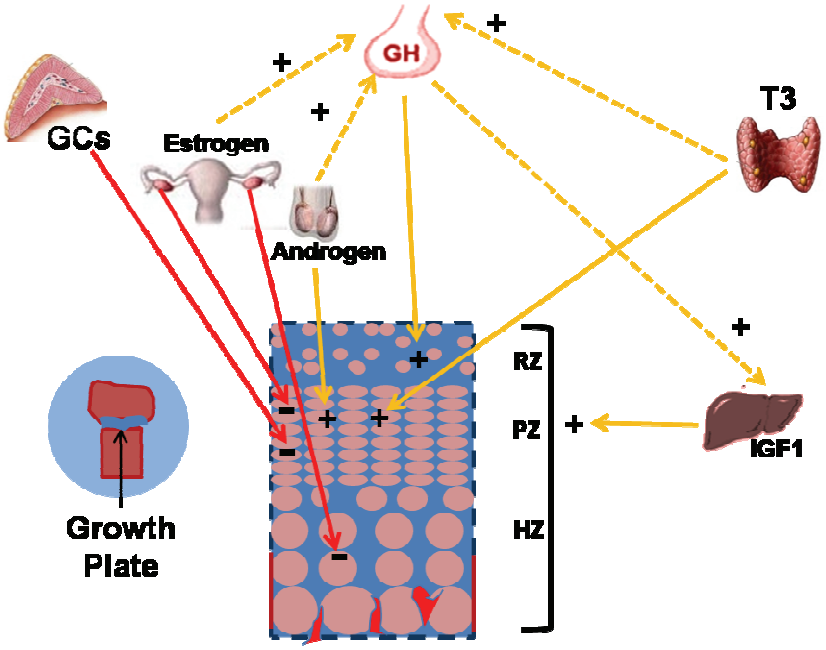

Fig. 4. Hormonal Regulation of Growth Plate Cartilage by major hormones: GH-IGF1, GCs, T3, and sex hormones. Positive (+) and negative (-) effects on target organs are shown. Systemic regulation: orange color (dash lines). Direct effect on growth plate cartilage: orange color (full line), if positive; or red line, if negative effect on chondrocytes proliferation and differentiation.

At the systemic level, pituitary GH acts on the liver to generate IGF1 that acts as an endocrine factor that stimulates longitudinal bone growth. The role of circulating IGF1 has been supported by the two following complementary studies. Liver-specific knockout of IGF1 in mice reduce circulating IGF1 levels by approximately 75%, without appreciable effect on postnatal growth, suggesting that the remaining bioactive IGF1 levels were enough to preserve longitudinal growth (Liu, Yakar et al. 2000). In addition, double knockout of liver-specific IGF1 and acid-labile sub-unit (ALS), a component of the circulating IGF1 complex required for bioactivity of IGF1, further reduces the levels of circulating IGF1, and caused inhibition of linear growth and decreased height of the growth plate (Yakar, Rosen et al. 2002). Furthermore, IGF1

treatment of mice and human with inactivating mutation on GHR markedly improve their linear bone growth, supporting the positive role of circulating IGF1 on linear growth (Guevara-Aguirre, Rosenbloom et al. 1997; Sims, Clement-Lacroix et al. 2000).

Direct injection of GH into the tibial growth plate accelerates longitudinal growth in the injected growth plate compared to the contra-lateral control-tibia, reassuring their direct role in growth plate (Isaksson, Jansson et al. 1982). Parker et al. have detected GHR expression with similar levels in all zones of rat growth plate (Parker, Hegde et al. 2007). At the local level, GH appears to act locally not only in the resting zone of the growth plate, rescuing them into a proliferative state (Ohlsson, Nilsson et al. 1992), but also by stimulating local IGF1 production in proliferative and hypertrophic zones (Schlechter, Russell et al. 1986; Hunziker, Wagner et al. 1994; Wang, Zhou et al. 1999). Interestingly, double knockout of GHR and IGF1 results in mutant mice smaller than single gene knockouts, indicating that GH and IGF1 co-interact positively by promoting longitudinal growth (Wang, Zhou et al. 1999; Lupu, Terwilliger et al. 2001). These findings are consistent with the 'dual effector hypotheses' of GH in the growth plate cartilage. One interesting observation is the absence or very low level of IGF1 mRNA expression throughout all zones of the growth plate, suggesting that local IGF1 may not be produced by growth plate chondrocytes, but most likely come from surrounding tissues, i.e. perichondrium or bone (Parker, Hegde et al. 2007).

On the other hand, IGF2 has been found at high levels in the growth plate, especially in resting and proliferative chondrocytes, suggesting its role in regulation of cell proliferation (Parker, Hegde et al. 2007). IGF2 is a positive regulator of prenatal growth, independent of GH. However, during postnatal growth, its role remains unclear. The finding that IGF2 expression declines by a thousand-fold in growth plate and surrounding tissues during a period of rapidly declining growth rate (Parker, Hegde et al. 2007) is consistent with the finding that IGF2 deficient mice present impaired longitudinal bone growth, primarily early in life (Jehle, Schulten et al. 2003). In addition, IGF2 expression declines in multiple tissues during postnatal development of rodents, which may suggest a regulatory mechanism common to multiple cell types (Brown, Graham et al. 1986; Lund, Moats-Staats et al. 1986; Parker, Hegde et al. 2007).

1.4.1.2 Estrogens

1.4.1.2.1 Pubertal Growth Spurt

The effect of sex steroids, estrogen and androgens, on stature depends on the age at which they are active, and act mostly in the natural growth acceleration during puberty ('pubertal growth spurt') and ultimately in the cessation of growth accompanied by epiphyseal fusion. In the presence of pathological conditions, such as sexual development at a precocious age, increase in sex steroids may therefore result in tallness at the time of steroids production, but because growth also stops prematurely, ultimate stature is likely to be short (Lee 2003). Conversely, when pubertal growth spurt is delayed, stature will fall off compared with that of contemporaries, but growth may continue to an older age and make up for the initial deficit, with a possible outcome of normal or tall stature (Butenandt and Kunze ; Zirilli, Rochira et al. 2008).

The pubertal growth spurt has a better temporal correlation with the increase in estrogen levels than androgen levels (Klein, Martha et al. 1996). A near-normal growth spurt occurs in patients with androgen insensitivity (Zachmann, Prader et al. 1986), whereas little or no growth spurt occurs in patient with aromatase deficiency, an enzyme that converts androgen in estrogen (Rochira and Carani 2009).

Much of the growth acceleration due to estrogen is mediated by estrogen-induced stimulation of the GH-IGF1 axis (Grumbach 2000). The pulses of GH secretion are known to increase in amplitude during puberty. In addition, estrogen treatment results in elevated circulating levels of GH and IGF1, whereas blocking estrogen signaling reduces these levels (Metzger and Kerrigan 1994; Hero, Norjavaara et al. 2005). Estrogens effects are modulated mainly by two nuclear receptors, estrogen receptor-α (ERα) (Green, Walter et al. 1986; Greene, Gilna et al. 1986) and estrogen-receptor-β (ERβ) (Kuiper, Enmark et al. 1996), in a complex that interacts with estrogen response elements (EREs) in the promoter regions of target genes and activate a variety of intracellular signaling cascades. A new membrane-bound G protein-coupled estrogen receptor (GPR30) that rapidly mediates estrogen signaling was recently identified as well (Revankar, Cimino et al. 2005). At the level of growth plate, the local action of estrogens is supported by the expression of both estrogen receptors (ERα and β) in the postnatal growth plate of rats, rabbits, and humans (Nilsson, Abad et al. 2002; Nilsson, Chrysis et al. 2003). GPR30 is also found at high levels in the hypertrophic zone of

growth plate, decrease during puberty, and appear to be required for normal estrogenic responses in the growth plate, contributing to modulate pubertal bone growth (Chagin and Savendahl 2007).

1.4.1.2.2 Epiphyseal Fusion

Estrogens are a key component in the control of physis fusion in both sexes. If deficiency of estrogen occurs, epiphyseal plate can remain unfused long after growth has stopped, followed by normal fusion as long as the missing hormone is replaced (Grumbach 2004). Patients with genetic mutation in either the gene encoding the aromatase enzyme that converts androgen to estrogen (Morishima, Grumbach et al. 1995), or in the gene encoding the estrogen receptor-α (Smith, Boyd et al. 1994) fail to close their physes at the time of sexual maturation, and show evidence of increased height due to longitudinal bone growth into adulthood. Conversely, patients who are exposed to estrogen prematurely close their physes earlier than predicted, such as in precocious puberty.

At the local level, estrogen may affect senescence by acting directly on growth plate chondrocytes. Both types of estrogen receptors (ERα and β) have been detected in postnatal growth plate of rats, rabbits, and humans (Nilsson, Abad et al. 2002; Nilsson, Chrysis et al. 2003). In particular, ERs in resting zone might mediate the long-term estrogenic effect on growth plate senescence and epiphyseal fusion (Nilsson and Baron 2004). The mechanism by which estrogen controls epiphyseal fusion is not fully understood. The current concept is that estrogen does not stimulate the ossification of cartilage directly, but it accelerates the normal process of growth plate senescence, which secondarily causes earlier fusion (Weise, De-Levi et al. 2001). In humans, this hypothesis may explain why the timing of estrogen-mediated fusion depends on the age of the individual. Exposure of estrogen, as seen in precocious puberty, may take up to 10 years if it occurs during early childhood; or only a few months, if it occurs later during adolescence (Sigurjonsdottir and Hayles 1968; Carani, Qin et al. 1997; Bilezikian, Morishima et al. 1998). This temporal association indicates that fusion is triggered when the proliferative potential of growth plate chondrocytes is exhausted (Nilsson and Baron 2004). In a young child, there would be a large proliferative capacity, which requires longer time of estrogen exposure until exhaustion of the replicative capacity of the growth plate has been reached. On the other hand, the proliferative capacity in the growth plate of ‘older individuals’ is far less; and requires a

shorter exposure to estrogen until complete exhaustion is achieved (Nilsson and Baron 2004).

1.4.1.3 Androgens

Androgens also contribute to the pubertal growth spurt, to a lesser extent than estrogens, by mechanisms not fully explained. During puberty, like estrogens, testosterone stimulates the GH-IGF1 axis, whereas non-aromatizable androgens don't, suggesting that androgens 'per se' stimulate pubertal growth through other mechanisms in a GH-independent manner (Keenan, Richards et al. 1993). Local administration of testosterone in growth plate cartilage increases unilateral rat tibial growth plate width, suggesting the local action of testosterone, independent of GH (Ren, Malozowski et al. 1989). However, these local effects may be partially mediated by an increase in local IGF1 expression (Maor, Segev et al. 1999; Krohn, Haffner et al. 2003).

In fact, most of androgen effects on growth are probably due to aromatization into estrogens in peripheral tissues, possibly in growth plate as well. Recent study by Chagin et al. show that oxandrolone does not influence the linear growth of cultures of fetal rat metatarsal bones and support the hypothesis that androgens affect linear growth mainly after being aromatized to estrogen locally in the growth plate (Chagin, Vannesjo et al. 2009). This hypothesis is supported by findings of aromatase P450 (CYP19) (Oz, Millsaps et al. 2001; van der Eerden, Lowik et al. 2004) and androgen receptor (AR) (Abu, Horner et al. 1997; van der Eerden, van Til et al. 2002; Nilsson, Chrysis et al. 2003) expression in the growth plate of different species, including rat and humans.

1.4.1.4 Thyroid Hormones

Thyroid hormones, *thyroxin (T4) and triiodothyronine (T3)* are primarily responsible for proper development and differentiation of all cells in the human body. They are determinant for postnatal somatic growth, including longitudinal bone growth (Williams, Robson et al. 1998). Fundamentally, thyroid hormone signaling results from the interaction of nuclear thyroid hormone receptors (TRs) with specific target gene promoters, a process that can either enhance or repress transcription. The underlying mechanism of these phenomena is deiodination. The iodothyronine deiodinase type II (D2) generates the active form of thyroid hormone T3 via deiodination of T4. Untreated hypothyroidism in children, either congenital or acquired, may result in severe growth retardation and delayed skeletal maturation

(Rivkees, Bode et al. 1988; Leger and Czernichow 1989). In contrast, hyperthyroidism in children is associated with accelerated growth rate and skeletal maturation, and although it is accompanied by tall stature at an earlier stage of the disease, ultimately result in normal predicted stature (Buckler, Willgerodt et al. 1986; Kvistad, Lovas et al. 2004). In hypothyroid rats, the growth plate show a decrease in the heights of both proliferative and hypertrophic zones, accompanied by a decline in chondrocyte proliferation and hypertrophy, vascular invasion and mineralization. Moreover, the normal columnar organization of the growth plate is disrupted (Stevens, Hasserjian et al. 2000).

Thyroid hormone regulates linear growth through different mechanisms. *In vivo* studies suggest that T3 may stimulate longitudinal growth by increasing GH-IGF1 secretion (Kindblom, Gothe et al. 2001). Skeletal abnormalities in TRα1$^{-/-}$ and β$^{-/-}$ are associated with inhibition of the GH–IGF1 axis, and growth retardation is reversed by GH replacement, but it does not rescue the growth plate ossification abnormalities (Kindblom, Gothe et al. 2001). These findings suggest that thyroid hormone also exert direct effects on the skeleton.

The direct effect of T3 on the growth plate and bone is supported by the expression of the α1, α2, β1 and β2 isoforms of T3 receptors (TR) in chondrocytes and osteoblasts (Abu, Bord et al. 1997; Ballock, Mita et al. 1999; Abu, Horner et al. 2000). In rat growth plate, TRs proteins are expressed in resting and proliferating zone chondrocytes but not in the hypertrophic zone (Robson, Siebler et al. 2000; Stevens, Hasserjian et al. 2000). However, in humans, both mRNA and protein of all isoforms of TRs are widely distributed in each zones of the growth plate (Abu, Bord et al. 1997; Abu, Horner et al. 2000). In addition, the expression of iodothyronine deiodinase type II (D2) in the growth plate suggests that local conversion of T4 to T3 by this enzyme may contribute to local effects of T3 in the growth plate (Miura, Tanaka et al. 2002; Shen, Berry et al. 2004; Capelo, Beber et al. 2008). However, the precise localization of T3-responsive cells within the growth plate and their target genes remains unknown.

The generation of transgenic mice lacking different types of TRs has enlightened their functional roles in growth plate and longitudinal growth (O'Shea and Williams 2002). Deletion of all TRα isoforms (TRα$^{0/0}$) result in linear growth retardation, disorganization of the normal columnar architecture, impaired chondrocyte

hypertrophy and delayed mineralization of growth plates chondrocytes (Gauthier, Plateroti et al. 2001), as it is featured in growth plate of hypothyroid mice (Stevens, Hasserjian et al. 2000). On the other hand, selected deletion of all isoforms of TRβ ($TR\beta^{-/-}$) result in no growth retardation or skeletal phenotype, suggesting that TRα, but not TRβ, is the predominant TR governing endochondral ossification (Gauthier, Plateroti et al. 2001).

Surprisingly, the majority of human cases of thyroid hormone resistance are caused by dominant-negative mutations in the TRβ gene. These mutations cause a frame shift in the ligand-binding domain ($TR\beta^{PV}$), resulting in the loss of T3 binding affinity and reduced transcriptional activation (Weiss and Refetoff 2000). In addition, these mutations may affect TRα function as well, with variable skeletal effects (Takeda, Sakurai et al. 1992; Olateju and Vanderpump 2006). Treatment of hypothyroid rats with GC-1, a synthetic analog of TH that selectively binds and activates TRβ over TRα while retaining the same binding affinity as T3, failed to normalize longitudinal growth and structural abnormalities in growth plate (Freitas, Capelo et al. 2005). However, the same study showed normalized differentiation of hypertrophic chondrocytes, type X collagen expression, and growth plate thickness of treated animals, suggesting that some essential effects of thyroid hormone on bone growth may be mediated by TRβ as well (Freitas, Capelo et al. 2005).

1.4.1.5 Glucocorticoids

Glucocorticoids (GCs) excess is one of the commonest conditions associated with growth retardation, but rarely due to pituitary or adrenal disease in childhood. Hypercortisolism is more commonly the result of high dosage GCs treatment of several conditions during growth development, such as juvenile arthritis, chronic asthma and nephrotic syndrome (Allen 1996; Savage, Scommegna et al. 2002). Conversely, familial GC deficiency syndrome is correlated with tall stature, suggesting that GCs act as negative regulator of longitudinal growth at normal levels (Chung, Chan et al. 2010).

Expression of the glucocorticoid receptor (GR) gene has been demonstrated in rat and human growth plate chondrocytes (Abu, Horner et al. 2000; Silvestrini, Ballanti et al. 2000), suggesting their local action as well. Supporting this hypothesis, systemic administration of GC in several species reduces tibial length and growth plate width

(Altman, Hochberg et al. 1992; Silvestrini, Ballanti et al. 2000; Gafni, Weise et al. 2001). Moreover, local infusion of dexamethasone in one growth plate suppresses growth in the infused leg compared to the vehicle-treated contra-lateral leg (Baron, Huang et al. 1992). Smink and collaborators showed that even short term treatment with GCs in mice lead to growth retardation, decreased growth plate width, with decreased rate of chondrocytes proliferation and increased apoptosis in hypertrophic zone, accompanied by a reduction in the local level of IGF1 (Smink, Gresnigt et al. 2003).

Altogether, these observations suggest that the local and suppressive effects of GCs appear to occur mainly through a potent suppression of chondrocyte proliferation (Annefeld 1992; Smink, Gresnigt et al. 2003), and possibly due to an increase in apoptosis of hypertrophic chondrocytes in the growth plate (Chrysis, Ritzen et al. 2003; Smink, Gresnigt et al. 2003). However, altered GH/IGF1 system may not be excluded as a potential additional mechanism.

1.4.2 Local (Paracrine/ Autocrine) Regulation of Growth Plate Cartilage

A multitude of signaling mechanisms are required to maintain spatial organization and optimal balance between chondrocyte proliferation and differentiation within the growth plate cartilage. To date, several signaling pathways that control the endochondral ossification are well-established, especially during early chondrogenesis; since most of the studies have been performed in embryonic models. A growing body of evidence suggests that many of the genes important for embryonic skeletogenesis and still unknown genes, also play an important role during postnatal growth. However, how these distinct molecules interact to coordinate postnatal bone development is not well understood. This section will focus on the study of a number of transcription factors and genes that we are known to regulate endochondral ossification at the level of the growth plate, giving close attention to its regulation during postnatal growth.

1.4.2.1 Transcription Factors

1.4.2.1.1 SOX9

SOX9 (SRY (sex determining region Y)-box 9) is a critical factor for all phases of the chondrocyte lineage, from early condensations to the conversion of proliferating chondrocytes to hypertrophic chondrocytes, and also determines the fate of MSC condensations into collagen type II expressing chondrocytes(Lefebvre and de Crombrugghe 1998; Zhou, Lefebvre et al. 1998; Lefebvre and Smits 2005). In humans, SOX9 mutations cause the rare condition campomelic dysplasia (CD) characterized by severe dwarfism, bowing and angulations of long bones among other skeletal anomalies (Foster, Dominguez-Steglich et al. 1994; Wagner, Wirth et al. 1994). These skeletal anomalies are consequent to a defect in chondrocyte differentiation within MSC condensations and decreased synthesis of cartilage matrix. Selective SOX9 knockout from early limb mesenchyme by using a Cre-loxP strategy, results in the absence of cartilage condensations and increased apoptosis in the mesenchyme (Akiyama, Chaboissier et al. 2002).

At later stages, overexpression of SOX9 in mice results in growth plates with a shorter proliferative zone, decreased chondroblast proliferation and Cyclin D1 expression, which is required for optimal proliferation (Akiyama, Lyons et al. 2004). Furthermore, SOX9 appears to delay the pre-hypertrophy of chondrocytes, since SOX9+/- mouse fetuses feature prematurely mineralized cartilages and expanded hypertrophic zones in their growth plate (Bi, Deng et al. 1999). The molecular mechanism of SOX9 regulation is not fully understood. There is evidence that SOX9 may regulate chondrocyte differentiation, proliferation and maturation by cross-talking with the Wnt/β-Catenin pathway, which is crucial for the differentiation of MSC directly into osteoblast (Akiyama, Lyons et al. 2004).

Interestingly, while SOX9 transcripts are highly expressed throughout the fetal growth plate; during postnatal life, it is mainly expressed in proliferating chondrocytes and switched off in hypertrophic chondrocytes (Wright, Hargrave et al. 1995; Ng, Wheatley et al. 1997). In a recent study, development of transgenic mice misexpressing Sox9 in hypertrophic chondrocytes under the control of a BAC-Col10a1 promoter, shows that newborn mice present an almost complete lack of bone marrow strongly retarded vascular invasion into hypertrophic cartilage and impaired cartilage resorption. These

results in delayed endochondral bone formation and reduced bone growth (Hattori, Muller et al. 2010). In addition, suppression of VEGF expression was observed, which is an important factor for vascularization of terminally differentiated chondrocytes, suggesting that down-regulation of Sox9 in hypertrophic zone of normal growth plates is essential for allowing vascular invasion, bone marrow formation and also endochondral ossification (Hattori, Muller et al. 2010).

1.4.2.1.2 RUNX2 (Runt-related Transcription Factor 2)

RUNX2 (Runt-related Transcription Factor2) , previously named Cbfa1, is expressed in the late condensation stage of chondrogenesis, followed by a substantial decrease in proliferating chondrocytes, with an increased expression again in prehypertrophic and hypertrophic chondrocytes. Although better known by its crucial role in differentiation of MSC into osteoblasts, RUNX2 is also important in the regulation of growth plate cartilage, by promoting differentiation of chondrocytes into hypertrophy (Komori, Yagi et al. 1997; Otto 1997; Inada, Yasui et al. 1999). RUNX2 knockout mice lack most bones and (?) present decreased numbers of hypertrophic chondrocytes, which fail to mineralize their matrix, and also show lower or absent expression of genes commonly expressed in late differentiated chondrocytes, such as osteopontin and matrix metalloproteinase 13 (MMP13) (Komori, Yagi et al. 1997; Otto 1997; Inada, Yasui et al. 1999). In addition, transgenic expression of a dominant-negative form of RUNX2 blocks hypertrophy of all chondrocytes (Ueta, Iwamoto et al. 2001). On the other hand, transgenic expression of RUNX2 accelerates hypertrophy of normal chondrocytes, and reverses the phenotype in knockout mice (Takeda, Bonnamy et al. 2001; Ueta, Iwamoto et al. 2001).

1.4.2.2 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) appears to be a key factor for vascular invasion of the growth plate, a critical step for successful bone formation. VEGF is a protein that targets vascular endothelial cells, expressed exclusively in hypertrophic chondrocytes in the growth plate (Gerber, Hillan et al. 1999; Gerber, Vu et al. 1999; Horner, Bishop et al. 1999). Hypertrophic chondrocytes also express the VEGF receptor, suggesting that this factor may have an autocrine role in these cells (Carlevaro, Cermelli et al. 2000). Inhibition of VEGF function by the use of an oral inhibiting agent (ZD4190) (Wedge and Ogilvie 2000), or by the administration of a

soluble receptor chimeric protein (Flt-(1-3)-IgG) that block activation of the receptor for VEGF (Gerber, Vu et al. 1999), leads to loss of vascular invasion, resulting in profound disturbances in the architecture of the growth plate. Calcified cartilage persists due to a decrease in the resorption of terminally differentiated chondrocytes, resulting in widening of hypertrophic region and in the decrease of trabecular bone formation. Conversely, after cessation of anti-VEGF treatment, the growth plate structure and function return to a normal state, with resumption of capillary invasion, restoration of bone growth, and resorption of hypertrophic chondrocytes.

Several studies show evidence that other factors mentioned previously, such as Ihh, RUNX2, BMPs and Sox9 are implicated in the regulation of terminal differentiation and angiogenesis of the growth plate through the modulation of VEGF expression (Gerber, Vu et al. 1999; Horner, Bishop et al. 1999; Hattori, Muller et al. 2010). Moreover, growth plate specific targeted deletion of a transcription factor known as hypoxia inducible factor (HIF-1α) that regulates VEGF expression in the embryonic growth plate, promoted increased cell death and reduced VEGF expression (Schipani, Ryan et al. 2001).

1.4.2.3 Indian Hedgehog / Parathyroid Hormone-related Peptide (Ihh/PTHrP) Signaling

Indian hedgehog (Ihh) is considered to be a master regulator of chondrocytes proliferation (Kronenberg 2003). Ihh belongs to the 3-member family of hedgehog proteins, including Desert hedgehog and Sonic hedgehog, the latter of which is one of the main regulators of limb outgrowth (McGlinn and Tabin 2006). Expressed mainly by prehypertrophic and early hypertrophic chondrocytes (Vortkamp, Lee et al. 1996), Ihh binds to a receptor called patched (Ptc), which activates Smoothened (Smo), a membrane protein that triggers a cascade resulting in gene activation. Knockout of *Ihh* in mice ($Ihh^{-/-}$) result in normal skeletogenesis at the condensation stage, but is followed by abnormalities of bone growth mainly due to a decrease in chondrocyte proliferation (St-Jacques, Hammerschmidt et al. 1999). In addition, *Smo* knockout also leads to decreased proliferation of chondrocytes (Long, Zhang et al. 2001). Conversely, overexpression of either *Ihh* or *Smo* specifically in cartilage increases proliferation, suggesting that the Ihh signaling pathway is sufficient to promote chondrocyte proliferation (Long, Zhang et al. 2001).

Another unusual feature of *Ihh* null mice is an increase in the population of hypertrophic chondrocytes, which is due to a premature exit of cells from the pool of proliferating chondrocytes. The rate at which growth plate chondrocytes leave the proliferative zone and commit to terminally differentiated hypertrophic cells is controlled by an orchestrated feedback loop involving *Ihh* and parathyroid hormone-related peptide (*PTHrP*), currently named parathyroid hormone-like peptide (*PTHLP*) (Vortkamp, Lee et al. 1996). A constitutive activation of *PTH/PTHrP receptors (PPR*s) reverses the early hypertrophy observed in *Ihh* null mice, but does not reverse the decreased proliferation observed in their growth plates (Karp 2000). These findings indicate that *Ihh* signaling regulates chondrocytes maturation and hypertrophy through *PTHrP*, but may act independently of *PTHrP* with regards to chondrocyte proliferation.

A third striking abnormality in Ihh knockout mice is the absence of osteoblasts in either the primary spongiosa or the bone collar of long bones, suggesting that *Ihh* may also control osteoblasts differentiation. Chimaeric experiments where *PPR* knockout chondrocytes, mixed with wild-type chondrocytes, show ectopic osteoblast formation adjacent to the ectopically positioned hypertrophic chondrocytes. On the contrary, in double *PPR/Ihh*- knockout mice, ectopic bone was absent (Chung, Lanske et al. 1998; Chung, Schipani et al. 2001). Hence, *Ihh* not only controls the differentiation of osteoblasts, but also determines the location where the bone collar is formed, precisely at the transition between chondrocytes proliferation and hypertrophy (Kronenberg 2003).

PTHrP belongs to the calcium-regulating parathyroid hormone (PTH) family. It is expressed mainly by periarticular and early proliferative chondrocytes of the growth plate and plays a crucial role in keeping proliferating chondrocytes in the proliferative stage (Juppner, Abou-Samra et al. 1991; Vortkamp, Lee et al. 1996). On the other hand, its receptor is a G-protein-coupled receptor that is also shared by PTH and is localized primarily in the lower proliferative and prehypertrophic zones (Karaplis 1994; Vortkamp, Lee et al. 1996). In humans, mutations in the *PTH/PTHrP* receptor *(PPR)* that result in a constitutively active PTHrP signal are responsible for *Jansen's metaphyseal chondrodysplasia*, a dwarfing disorder associated with delayed growth plate mineralization and hypercalcemia (Schipani, Langman et al. 1996). Interestingly, knockout of either, *PTHrP* (Karaplis 1994) or *PPR* (Lanske, Karaplis et al. 1996) causes severe dwarfism in mice and accelerated chondrocyte hypertrophy. Conversely,

overexpression of *PTHrP* (Weir 1996) or expression of a constitutively active *PPR* (Schipani 1997) in the growth plate inhibits chondrocyte differentiation.

Altogether, these transgenic models and selected clinical cases confirm the important role of *Ihh* and *PTHrP* in the control of growth plate chondrocyte proliferation and differentiation during fetal life. In summary, it has been proposed that PTHrP secreted from periarticular chondrocytes acts on its receptor (*PPR*) localized on proliferating chondrocytes to keep them proliferating, and thereby delays the production of *Ihh*. As new chondrocytes are formed through proliferation in the upper part of the proliferative zone, chondrocytes in the lower part of the proliferative zone become sufficiently distant from the source of PTHrP to the point where they are no longer stimulated by the protein. Only then do they stop proliferating, start to hypertrophy, and synthesize *Ihh*. *Ihh* subsequently acts on adjacent proliferative chondrocytes to promote proliferation and, through mechanisms not yet fully understood, stimulate the production of PTHrP in periarticular chondrocytes (Kronenberg 2003).

Several studies have shown that *Ihh, PTHrP* and their receptors are also expressed in the postnatal growth plate, indicating that Ihh-PTHrP signaling may continue to be beyond embryonic longitudinal bone growth (Vortkamp, Lee et al. 1996; van der Eerden, Karperien et al. 2000; Farquharson, Jefferies et al. 2001; Nakase, Miyaji et al. 2001). In addition, targeted expression of constitutively active *PPRs* in the growth plates of mice lacking *PTHrP* rescue their growth plate phenotype at birth and result in prolonged postnatal survival of *PTHrP* null mice by up to two months (Schipani 1997). After birth, however, these "rescued" animals grow less than their control littermates, and show premature disappearance of their growth plates and secondary ossification centers (Schipani 1997). These finding indicate that PTHrP is involved in the control of growth plate chondrocyte differentiation both during embryonic and postnatal life.

1.4.2.4 Bone Morphogenetic Proteins (BMPs)

Bone morphogenetic proteins (BMPs) were first identified due to their ability to induce ectopic bone formation, and have later been shown to be essential for multiple stages of skeletal development and endochondral ossification, including early patterning and mesenchymal cell condensation as well as regulation of chondrocyte proliferation and maturation in the growth plate (Urist 1965; Pogue and Lyons 2006). BMPs are multi-functional growth factors that belong to the transforming growth factor β (TGF-β)

Superfamily. To date, around 20 BMP family members have been identified, where only BMPs 2 to 7 belong to the TGF-β superfamily. Three type I receptor transducer BMP signals (type IA, IB, and ALK-2), through the formation of heteromeric complexes with threonine kinase receptors, leading to the activation of the canonical Smad pathway, as well other pathways (Derynck and Zhang 2003).

BMP signaling is required as part of an instructive signal to promote commitment of mesenchymal cells to the chondrogenic lineage. BMP1a and BMP1b receptors knockout mice lack the majority of skeletal elements that form through endochondral ossification (Yoon, Ovchinnikov et al. 2005). Contrarily, mice deficient in the BMP antagonist Noggin have multiple skeletal abnormalities, including enlarged growth plates, presumable due to loss of opposition to BMP signaling (Brunet, McMahon et al. 1998), and overexpression of constitutively active BMP receptors results in the expansion of cartilage (Pizette and Niswander 2000). One potential mechanism is through the maintenance of SOX9 expression in MSC condensations, the earliest known marker for cells committed to chondrogenesis. *In vitro*, BMPs promote the expression of SOX9 in cell cultures, and its expression is required for BMP induced chondrogenesis, as antisense SOX9 oligonucleotides block the ability of BMPs to induce type II collagen (Zehentner, Dony et al. 1999; Fernandez-Lloris, Vinals et al. 2003). *In vivo*, implantation of BMP2 beads near condensed cartilage leads to up-regulation of the Sox protein in condensation, while beads of the BMP antagonist Noggin lead to its severe down-regulation (Chimal-Monroy, Rodriguez-Leon et al. 2003). In addition, overexpression of Noggin blocks MSC condensation, leading to a total absence of cartilage (Capdevila and Johnson 1998).

During later stages of chondrogenesis, BMP signaling appears to promote hypertrophic chondrocyte differentiation (De Luca, Barnes et al. 2001). Expression of different BMPs members have specific pattern in the growth plate (Yoon and Lyons 2004; Nilsson, Parker et al. 2007). BMP-6 and Bmpr1a is highly expressed in hypertrophic chondrocytes in both, the embryonic and postnatal growth plate. BMP-2,-4, and -5 has been detected in the perichondrial cells of embryonic growth plates. BMP-2 was detected at higher levels in the hypertrophic zone of the postnatal growth plate. BMP-7 is mainly expressed in proliferating chondrocytes. On the other hand, expression of gremlin, chordin, and BMP-3, all inhibitors of BMP-induced bone formation, are up-regulated in the resting zone of the postnatal growth plate. The expression pattern in the postnatal growth plate suggests that there is a gradient in BMP-signaling created by the

expression of BMP agonists primarily in hypertrophic zone, and the BMP antagonist in resting as well as proliferative zones in the growth plate (Nilsson, Parker et al. 2007). Altogether, these studies point to a contribution of BMPs in both proliferation and differentiation of the growth plate chondrocytes during pre- and postnatal growth.

The diverse functions of the BMP signaling pathway in the growth plate are directly or indirectly correlated to its interactions with other signaling pathways. Two important interactions are with the Ihh/PTHrP and FGF signaling pathways. BMPs interact with the Ihh/PTHrP pathway by promoting Ihh expression, which maintains BMP levels, indicating the existence of a positive feedback loop between them (Kawai and Sugiura 2001; Minina 2001). However, neither pathway completely mediates the other's functions and both act more in a synergistic way. On the contrary, BMPs and FGFs show functional antagonism in the regulation of chondrocytes. BMP treatment rescues the phenotype of FGF treated growth plates, and FGF treatment neutralizes the effects of BMPs (Minina, Kreschel et al. 2002). The mechanisms underlying this antagonism are not well understood, but may in part be due to opposite effects on Ihh production by hypertrophic chondrocytes (Naski, Colvin et al. 1998; Minina, Kreschel et al. 2002). Further functional studies are needed to clarify the interaction of these factors in the regulation of growth plate cartilage.

1.4.2.5 Fibroblast Growth Factors (FGFs)

Fibroblast growth factors (FGFs) comprise a family of at least 22 secreted proteins that interact with differing affinities to one of the four high-affinity FGF receptors (FGFR) (Ornitz and Marie 2002). Opposite to Ihh/PTHrP and BMP signaling, FGFs provide essential inhibitory signals in the control of chondrocyte proliferation.

Point mutations, G to A (99%) or G to C (1%), at nucleotide 1138 of the FGFR3 gene activate the FGF receptor 3 (FGFR3) and cause achondroplasia (ACH), the most common genetic form of dwarfism in humans, characterized by reduced growth of long bones with proximal elements more severely affected than distal ones (Rousseau, Bonaventure et al. 1994). In mice, an activating point mutation in the FGFR3 decreases the rate of chondrocyte proliferation and lead to shortened and disorganized columns in their growth plates (Naski, Colvin et al. 1998). Conversely, FGFR3 knockout leads to an opposite phenotype, with an increased rate of chondrocyte proliferation and length

expansion of chondrocyte columns (Colvin, Bohne et al. 1996; Deng, Wynshaw-Boris et al. 1996).

Many other human skeletal dysplasias have been attributed to a specific mutation in the genes encoding FGF receptors (Muenke and Schell 1995). As an example, the bony syndactyly resulting from mutations in FGFR2 in Apert syndrome may result from defects in signaling in the mesenchymal condensation. The Apert mutations result in a loss of ligand binding specificity, which allow inappropriate activation of FGFR2 by the ligands (e.g. FGF7) expressed in the MSC condensation, as well as through inappropriate activation of FGFR2b by other ligands such as FGF2, FGF6, and FGF9 (Yu, Herr et al. 2000; Yu and Ornitz 2001).

However, the role of FGF signaling in the condensing mesenchyme is poorly understood. It is possible that FGF signaling induces the expression of SOX9 (Murakami, Kan et al. 2000). Another possible mechanism of FGFs is by interacting with IHH/PTHrP signaling. Knockout of the FGFR3 gene increases Ihh expression and activating mutation of FGFR3 decreases its expression (Naski, Colvin et al. 1998; Ornitz and Marie 2002). Moreover, *in vitro* studies support the idea that FGFs inhibit chondrocyte proliferation by suppressing Ihh expression (Minina, Kreschel et al. 2002). FGFs expression pattern has been shown in chicken, mouse and human limb development (Peters, Werner et al. 1992; Szebenyi, Savage et al. 1995; Delezoide, Benoist-Lasselin et al. 1998). FGFR2 expression is the first observed in the MSC condensation center of the developing limb. At the condensation step, FGFR1 expression is found in the periphery of the mesenchymal condensations. Later, FGFR1 and FGFR2 expression persists in the perichondrium and periosteum. As the epiphyseal growth plate is formed, FGFR1 expression is initiated and maintained mainly in hypertrophic chondrocytes (Deng, Wynshaw-Boris et al. 1994). In contrast, FGFR3 expression is first observed in the condensation center, as chondrogenesis begins, and later, is mainly expressed in the proliferating chondrocytes (Peters, Ornitz et al. 1993).

On the contrary, several of the FGFs ligands are expressed in the developing endochondral bone. Interestingly, expression of FGFs 7, 8, 17, and 18 has been observed in the perichondrium (Mason, Fuller-Pace et al. 1994; Finch, Cunha et al. 1995; Xu, Liu et al. 2000; Liu, Xu et al. 2002), suggesting a possible paracrine signal to the growth plate. However, knockout studies reveal that only the lack of FGF18 results in abnormal chondrogenesis. FGF18 null mice show increased chondrocyte

proliferation, delayed ossification and decreased VEGF expression in the hypertrophic zone and perichondrium, suggesting that FGF18 contributes to the coordinated neovascularization of the growth plate, besides regulating chondrocyte proliferation (Liu, Lavine et al. 2007). In addition, the similarity between the phenotypes of FGF18 and FGFR3 knockout mice suggest that FGF18 may be the natural ligand responsible for inhibition of proliferation through FGFR3. However, the more severe phenotype of FGF18 null mice suggests that FGF18 signals through other FGF receptors. It is possible that the redundancy of ligands (FGFs) combined with the multiple early effects of FGF receptors in bone development may compensate for individual FGFs mutations.

Characterization of FGF and FGFR expression in microdissected growth plate cartilage from postnatal rat using real-time PCR detected high levels FGFR1-3, especially isoform C, whereas most FGFs were not detected or only detected at low levels in growth plate chondrocytes. FGF18 is expressed in perichondrium, and at lower levels in the hypertrophic and resting zones of the post-natal growth plate. (Lazarus, Hegde et al. 2007). These findings thus suggest that FGFs, mainly produced by the perichondrium, act in a paracrine manner on FGFRs expressed on growth plate chondrocytes during postnatal life. Whereas FGFR1 and 3, which are negative regulators of chondrocytes, were expressed at higher levels in the hypertrophic zone; FGFR2 and 4, which are putative positive chondrocyte regulators, were expressed at earlier stages of differentiation, mainly in the resting zone (Lazarus, Hegde et al. 2007). Altogether, these findings point to the importance of FGF signaling in the control of post-natal growth. Interestingly, temporal expression pattern showed down-regulation of FGFR2 and 4 in proliferative zone, and up-regulation of FGF 1, 7, 18 and 22 in perichondrium, suggesting that FGF signaling might play a role in the regulation of growth plate senescence and deceleration of longitudinal growth (Lazarus, Hegde et al. 2007).

1.4.2.6 WNTs (Wingless-type MMTV integration site family)

The importance of Wnt signaling in skeletal development has been widely explored by several *in vivo* and *in vitro* studies (Macsai, Foster et al. 2008). At least 19 Wnts comprise a complex family of secreted cysteine-rich glycoproteins that interact with several receptors called Frizzled (Fzd) as well as co-receptors (Lrp5 and Lrp6). These Wnts are regulated by many antagonists, e.g. Secreted frizzled-related proteins (Sfrps) to finally activate different signaling pathways, including the canonical β-Catenin

pathway, and the non-canonicals calcium-releasing and JNK/planner cell polarity pathways. A key member of the canonical pathway, β-Catenin is an intracellular molecule involved in cell adhesion, which in the absence of Wnt signal is phosporylated and degraded, thus inhibiting gene transcription. Otherwise, activation of Wnt stabilizes β-Catenin, which then accumulates in the cytoplasm, migrates to the nucleus, heterodimerize with TCF/LEF transcription factors and ultimately mediates transcription of Wnt target genes.

During bone formation, Wnt signaling inhibits chondrogenesis and enhances direct ossification, as seen by overexpression of Wnt14 and subsequent activation of the canonical β-Catenin pathway (Guo, Day et al. 2004; Day, Guo et al. 2005). Conversely, conditional inactivation of β-Catenin in undifferentiated mesenchymal cells using different mice models (Dermo1-Cre and Prx1-Cre) result in ectopic chondrocyte formation at the expenses of osteoblast differentiation (Hill, Spater et al. 2005; Hu, Hilton et al. 2005). Interestingly, Wnt signaling is also required during later chondrogenesis. Conditional knockout of β-Catenin in chondrocytes under the control of a type II collagen promoter (Co2a1-Cre) results in mice with short limbs, ectopic cartilage formation, but normal intramembranous ossification (Day, Guo et al. 2005). The generation of Col2a1-ICAT transgenic mice and therefore, decreased in β-Catenin signaling, resulted in viable mice with normal size at birth followed by progressive growth retardion (Chen, Zhu et al. 2008). Col2a1-ICAT transgenic mice showed reduced chondrocyte proliferation and differentiation, and an increase in chondrocyte apoptosis, leading to decreased widths of the proliferating and hypertrophic zones, delayed formation of the secondary ossification center, and reduced skeletal growth. These findings show that Wnt/ β-Catenin canonical pathway is essential throughout all stages of bone formation.

During intramembranous ossification, Wnt signaling in the condensation is high, inhibits chondrocyte differentiation and also promotes osteoblast differentiation. During endochondral ossification, however, Wnt signaling in the condensation is kept low meaning that only chondrocytes can differentiate. Later, Wnt signaling is again up-regulated in the periphery of cartilage, such that osteoblasts will differentiate, in order for the normal endochondral ossification to occur. One of the suggested mechanisms to control Wnt signaling in chondrogenesis is its inhibition by SOX9. Infection of mesenchymal progenitors in micromass culture with SOX9 or Wnt14 using adenovirus result in increased cartilage nodule formation at the outer rim of micromass culture

infected with SOX9 only; whereas Wnt14 inhibits cartilage nodule formation. Interestingly, co-infection with Sox-9 rescued cartilage nodule formation inhibited by Wnt14 to a large extent (Topol, Chen et al. 2009). Additional experiments in the same study show that SOX9 enhance β-catenin phosphorylation and its subsequent degradation, and thus inhibit Wnt signaling (Topol, Chen et al. 2009). In another interesting study, Wnt signaling has been implicated as downstream component of Ihh signaling, a master regulator of chondrocytes differentiation (Day and Yang 2008). Study in double mutant mice using COL2A1-Cre model with floxed alleles for Ptch1 and β-catenin, hedgehog (Hh) signaling is cell autonomously activated, whereas β-catenin is cell autonomously inactivated in developing long bones. Hedgehog (Hh) signaling was increased in the entire skeletal, especially in the perichondrium and articular cartilage, as well as PTHrP expression in the articular cartilage of these mutant mice (Mak, Chen et al. 2006). In the same study, bone formation was blocked in the perichondrium of the double mutant Ptch1; β-catenin mice, as in β-catenin single mutant mice, although Hh signaling was up-regulated (Mak, Chen et al. 2006). Altogether, these findings suggest that Wnt/β-catenin signaling is not required for Hh signaling itself, and acts downstream of Hh signaling in promoting bone formation.

2 AIMS OF THE THESIS

The main goal of this thesis is to identify and explore new genes and intrinsic molecular mechanisms that are important for the spatial and temporal regulation of proliferation and differentiation of growth plate chondrocytes. We therefore aimed to:

1. Identify gene expression changes that occur during the sequential differentiation of growth plate chondrocytes between distinct zones, as well as during growth plate senescence (Paper I);

2. Study genes and pathways identified in previous study in order to explore their importance for temporal and spatial control of chondrocyte differentiation and proliferation within the growth plate (Papers II, III);

3. Verify the intrinsic regulation of growth plate senescence by combining growth-inhibiting conditions and the study of structural, functional and gene expression changes within postnatal growth plate cartilage (Paper IV);

4. Develop an *in vitro* model for growth plate chondrogenesis that allow for transient transfection of primary growth plate chondrocytes (Paper V).

5. Identify developmentally regulated microRNAs and explore their role in growth plate chondrogenesis and senescence (Paper V);

3 ANIMAL & METHODS

The following methods were used and will be briefly discussed. For specific procedures, please consult the individual papers:

- Animal Model (Paper I-V)
- Microdissection of the growth plate (Papers I- V)
- Microarray analysis (Papers I and V)
- Real-time qRT-PCR (Papers I-V)
- *In Situ* hybridization of postnatal growth plate (Paper I)
- Culture of Primary Chondrocytes (Paper V)
- Transient Transfection (Paper V)
- Transfection Efficiency Assessment (Paper V)
- Proliferation assay - BrdU labeling followed by detection using ELISA (photometry) assay (Paper V)
- Apoptosis analysis by using an ELISA-based method (photometry) assay (Paper V)

3.1 ANIMAL MODELS

We have mainly used rodents in the studies included in this thesis. Sprague Dawley rats (Paper I, III and IV) and C57BL/6 mice (Paper II and V) were sacrificed to get their proximal tibial growth plate cartilage. In paper IV, we have used other two models with growth-inhibiting conditions. All studies were approved by local ethic committees.

1. ***Normal and castrated rodents:*** Sprague Dawley rats (Paper I, III and IV) and C57BL/6 mice (Paper II and V) were sacrificed at 1-week-old to get epiphyseal proximal tibia for subsequent manual microdissection of individual zones of the

growth plate. For later time points, to avoid the interference of sex steroids, the animals were castrated before pubertal development starts (van Buul and Van den Brande 1978; Hunziker and Schenk 1989).

2. ***Hypothyroidism induced rats (Paper IV):*** Growth of Sprague Dawley rats were inhibited in newborn male pups by introducing propylthiouracil (PTU) into drinking water of the mother from birth of the pups until 5-weeks-old of age, when we discontinued the treatment and allowed the hypothyroid rats to recover. The PTU treated pups were not weaned during treatment due to the fact that they are not mature enough to support their own nutritional needs during hypothyroidism state. The pups from lactating mothers not receiving PTU were used as controls and weaned at the normal age of 3-weeks-old. In addition, to avoid the interference of sex steroids in growth development, puberty was delayed by depot leuprolide acetate injections every 3 weeks. Depot leuprolide acetate is a long-acting gonadotropin-releasing hormone agonist that down-regulates gonadotropin secretion and thus gonadal steroid production (Ogawa, Okada et al. 1989).

3. ***Tryptophan deficient (Trp^-) rats (Paper IV):*** Sprague Dawley rat growth was inhibited by providing a Trp^- diet to the lactating mother from birth of the pups until they reached 4-weeks of age. Then, treatment was suspended and the animals were allowed to recover. Male pups of not-treated mothers were used as a control.

3.2 MICRODISSECTION OF GROWTH PLATE (PAPERS I-V)

Manual microdissection of growth plate is a reliable and accurate technique that allows the study of individual growth plates of single animals (Nilsson, Parker et al. 2007). Briefly, cartilaginous growth plates of Sprague-Dawley rats or C57BL/6 mice were excised from proximal tibia. To dissect distinct zones of growth plate, 7-days-old animals were used. At this age, the growth plate is relatively tall and dissection of individual zones based on histological characteristics is more accurate and give enough amount of material to extract tRNA for the study of individual zones from a single animal. Frozen longitudinal sections (40-60 μm) of proximal tibial epiphyses were mounted on Superfrost Plus slides (Fisher Scientific, Chicago, IL, USA). Pre-treatment of the slides with fixation and eosin staining was performed. Using an inverted microscope, razor blades, and hypodermic needles, growth plate sections,

under a xylene droplet, were separated based on histological hallmarks into distinct zones (Fig. 5).

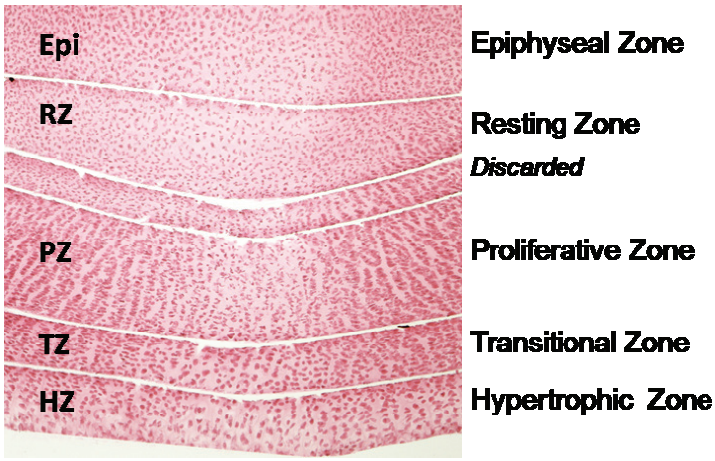

Fig. 5. Growth plate microdissection. Representative photomicrograph of microdissected proximal tibial growth plate from 1-week-old rat. The 60μm-thick longitudinal frozen sections were cut into resting zone, proliferative zone, prehypertrophic region and hypertrophic zone. In the section depicted, cuts were made, but the cartilage was left in place on the microscope slide.

In order to minimize cross-contamination between zones, the uppermost part of one and the lowest part of next zone was discarded. Metaphyseal bone can be collected from a region of trabecular bone beginning approximately 100 μm distal to the hypertrophic zone. For each zone, tissue dissected from both proximal tibias of one animal (18–25 sections) was pooled prior to RNA isolation. 50-200 ng of total RNA was extracted from growth plate of single animals, and then used for microarray or real-time PCR analysis (Paper I and V).

3.3 MICROARRAY ANALYSIS (PAPERS I, V)

Microarray is a high-throughput technique that allows the expression quantification of thousands of genes simultaneously. This thesis included two papers (Papers I and V) that use microarray analysis, which pursue the concept of finding predictive gene groups and signaling pathways from microarray data, which will generate likely hypothesis for the regulation of proliferation and differentiation of chondrocytes

within growth plate. This concept is motivated from the biological assumption that a few gene expression signatures are most accurate for phenotype discrimination. Furthermore, paper V also presents some innovative ideas resulting from the profiling of miRNAs and predicted target genes important for temporal regulation of chondrogenesis.

3.3.1 mRNA Expression Microarray

Expression quantification of thousands of genes is performed simultaneously by measuring the hybridization from the tissue of interest (e.g. growth plate and metaphyseal bone) to probes placed on a small glass slide. We used samples from single animals from the same litter, which minimize the variation of biological components and facilitate the interpretation of the results.

For gene expression analysis (Paper I), we have used Affymetrix platform and the GeneChip 3´ *In Vitro* Transcription (IVT) Expression. Reverse transcription-IVT (RT-IVT) process is considered the gold standard for target preparation for gene expression analysis (Shi, Reid et al. 2006). The GeneChip arrays have a system where Oligonucleotides probes complementary to each corresponding sequence are synthesized *in situ* on the arrays. Poly-A controls (lys, phe, thr and dap) monitor the target labeling process from start to end, as sensitivity indicators of the entire target labeling process. The entire process is based upon linear RNA amplification and employs T7 *in vitro* transcription technology. For visualization of the GeneChip® 3' IVT Express Protocol process, see figure 6.

Briefly, total RNA (50-100ng) undergoes reverse transcription to synthesize first-strand cDNA, which is primed with T7 oligo(dT) primer to synthesize cDNA containing a T7 promoter sequence. This cDNA is then converted into a double-stranded DNA template for transcription. The reaction employs DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second strand cDNA. *In vitro* transcription synthesizes cRNA with IVT labeling and incorporates a biotin-conjugated nucleotide. In the amplification step, Master Mix generates multiple copies of biotin-modified cRNA from the double stranded cDNA templates. The cRNA is then purified to remove unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the biotin-modified cRNA.

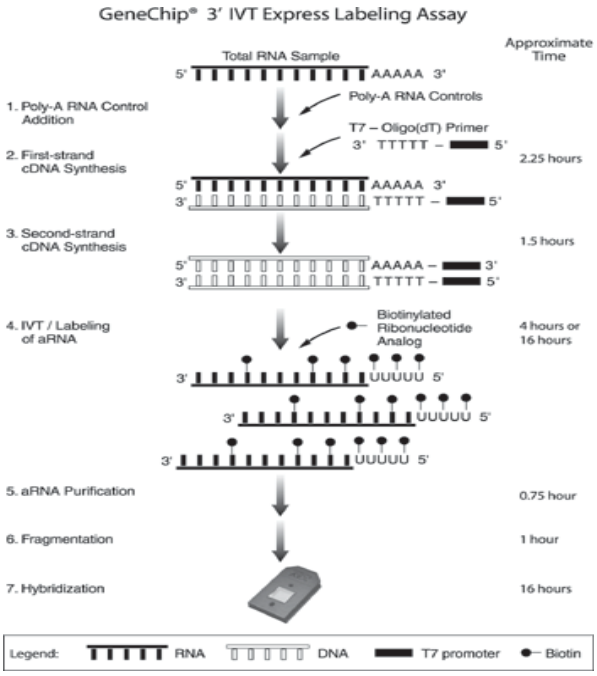

Fig. 6. Overview of the GeneChip 3' OVT Express Kit Labeling Assay

Fragmentation of the biotin-labeled cRNA prepares the sample for hybridization onto GeneChip 3' IVT expression arrays**.** After hybridization, the arrays are washed and stained using a Fluidics Station FS-450 (Affymetrix) following the EukGE-WS2v5 protocol. The distribution of fluorescent material on the processed array is determined using the Affymetrix 3000 GeneArray laser Scanner with the 7G upgrade. Image inspection is performed manually immediately following each scan. All array scanning and data processing on the Affymetrix system is then performed with GeneChip Operating System (GCOS) software.

3.3.2 miRNA Expression Microarray

For miRNA microarray (Paper V), we used Agilent platform (Santa Clara, CA), which contain 20 probes targeting each of 567 mouse and 10 viral miRNAs from the Sanger database v10.1 (miRBase/ TargetScan, 2010). Labeling and hybridization of total RNA samples were performed according to the manufacturer's protocol. 100 ng total RNA was used as input into the labeling reaction, and the entire reaction was hybridized to

the array for 20 hours at 55°C. In combination with the miRNA microarray probe design, Cyanine 3-Cytidine bisphosphate (pCp) reagent selectively labels and hybridizes mature miRNAs. Use of these reagents ensures consistent results. Slides were scanned by using the Agilent DNA Microarray Scanner (Agilent), and results were extracted using Agilent Feature Extraction software (v10.5.1).

3.3.3 Microarray Data Analysis

Data analysis of microarray data is simplified by the use of bioinformatics tools, class prediction tools and higher stringency to select true effect observed by the technique. In the first paper, statistical and bioinformatic analysis of gene expression microarray data were performed by Partek Genomics Suite™ 6.3 (Partek Inc., St. Louis, MO). Probe-level data were pre-processed, including background correction, normalization, and summarization, using robust multi-array average (RMA) analysis (Bolstad, Irizarry et al. 2003; Irizarry, Hobbs et al. 2003). ANOVA analysis was performed for spatial and temporal comparisons on log-transformed data using Partek Pro software (Partek Inc.). In paper V, normalization and data analysis was performed by using GeneSpring software (GX11, Agilent, Santa Clara, CA).

In order to identify functional pathways implicated in the spatial and temporal regulation of growth plate chondrocytes, Ingenuity Pathways Analysis Software 7.0 (Ingenuity Systems Inc., Redwood City, CA) was used. Furthermore, validation of microarray findings was performed by real-time quantitative PCR (paper I and V) and *in situ* hybridization (paper I).

3.4 REAL-TIME qPCR (PAPERS I-V)

Real-time qPCR is a highly sensitive and specific method for detection of gene expression by logarithmic amplification (PCR) and simultaneous quantification of specific cDNA sequences, which reflects the concentration of specific transcripts in the sample analyzed. In this thesis, we have used real-time RT-PCR to study the expression of specific genes (Papers II, III, IV) and miRNAs (Paper V) found to be important in the regulation of growth plate chondrocytes based on previous microarray findings. Briefly, by using a DNA polymerase enzyme that is tolerant to elevated temperatures, mRNA is copied to cDNA by reverse transcriptase and random hexamer oligoprimers. The cDNA is more stable than RNA and furthermore allows amplification by DNA

polymerase. Interestingly, the cDNA mirror the RNA content of the tissue analyzed, and therefore the converted mRNA contains merely exons, since the introns are spliced away in mature mRNAs.

The basic idea of the technique is that the more abundant a particular mRNA is in the sample, and thus cDNA after reverse transcription, the earlier it will reach a threshold during repeated cycles of amplification. In contrast to semi-quantitative PCR that only provide information of amplification product amount at the end of the reaction, q-PCR assess the amount of synthesized amplification product in every amplification cycle and relative abundances of each transcript can be assessed during the logarithmic phase of amplification. The data collection is fluorescence based. Two commonly used chemistries for detection are TaqMan probe and SYBR green. SYBR green directly binds to double-stranded DNA (amplification products) and emits light. As SYBR green dye binds to any double-stranded DNA molecular, including primer-dimers and other non-specific reaction products, it may lead to an overestimate of the PCR product. On the other hand, TaqMan probe is more precise to detect gene expression. Briefly, TaqMan probe are oligonucleotides with a high energy reporter dye on the 5' end and low energy quencher dye at the 3' end. Before reaction, two dyes sit together by which the emission of reporter dye is suppressed by the quencher dye. During PCR when DNA replication reaches to the TaqMan probes bonded on template, the 5'-nuclease of Taq DNA polymerase enzyme cleaves the probe. The release of reporter from quencher increases fluorescent emission of reporter. The signal increases in direct proportion to the amount of PCR product in the reaction.

Since not all tissues samples have the exact same rate of overall gene transcription and the same amount of cell contents, normalization against a constantly expressed housekeeping gene is commonly performed and mRNA relative quantification is analyzed. In our studies, we quantified the relative expression from all samples against the housekeeping gene 18S ribosomal RNA (18S rRNA) for gene expression studies; and the small nucleotide202 (sno202) for miRNA study, which have previously been shown to be constantly expressed in most tissues (Wong L 2007), confirmed in growth plate samples . Real-time qPCR was performed as it is described in each paper.

3.5 *IN SITU* HYBIDRIZATION OF POSTNATAL GROWTH PLATE (PAPER I)

In situ hybridization was performed using frozen sections of growth plate cartilage of 1-week-old rats hybridized to 35S-labeled riboprobes as described by Zhou et al (Zhou, Chin et al. 1991). Briefly, cDNA from growth plate was PCR-amplified by two sequential amplifications using specific primers that contain either a T7 promoter or an SP6 promoter (Divjak, Glare et al. 2002). Next, single stranded ^{35}S-labeled riboprobes for in situ hybridization were produced by in vitro transcription using T7 or SP6 RNA polymerase α-^{35}S labeled nucleotides. T7 polymerase was used for sense probes and SP6 polymerase for antisense probes. Riboprobes were purified by Micro Bio-Spin Columns P-30-Tris-RNase free. ^{35}S incorporation was assessed by comparing radioactivity before and after the column purification, by liquid scintillation counting. Hybridization of pre-treated frozen sections to ^{35}S-labeled riboprobes was performed as described by Zhou et al (Zhou, Chin et al. 1991). The sections were then counterstained with hematoxylin and eosin. Silver grains were visualized by scanning the slides with ScanScope CS digital scanner (Aperio Technologies, Inc.) under bright field microscopy. The corresponding sense riboprobe was used as a negative control for each antisense probe.

3.6 QUANTITATIVE HISTOLOGY (PAPERS IV)

Masson Trichrome-stained sections of paraffin embedded samples of growth plate from proximal tibia were used for quantitative histology using a ScanScope CS digital scanner (Aperio Technologies, Inc. Vista, CA). Measurements were taken as previously described (Marino, Hegde et al. 2008). Height of individual zones and whole growth plate, columns density and the height of the terminal hypertrophic chondrocyte lacuna (the intact lacuna closest to the metaphyseal bone) were assessed by a blinded observer to the age and treatment group. Overall growth plate height was measured from the margin of the metaphyseal bone to the margin of the epiphyseal bone; resting zone height, measured from the edge of the margin of the epiphyseal bone to the first cell of a proliferative column; hypertrophic zone height, measured from the margin of the methaphyseal bone to the last cell bigger or equal to 10 μm; proliferative

zone height, measured from the first cell minor to 10 μm to the first resting chondrocytes; the number of resting zone chondrocytes, assessed as the number of cells per 200 μm growth plate width (measured parallel to the epiphyseal margin); column density, assessed as the number of hypertrophic columns per 500 μm growth plate (measured parallel to the metaphyseal margin); and the height of the terminal hypertrophic chondrocyte lacuna (the intact lacuna closest to the metaphyseal bone). Heights were measured parallel to the chondrocyte columns in 3 areas of each growth plate section and averaged. Column density was calculated in 2 areas per growth plate section. The number of proliferative and hypertrophic cells was counted in 3-4 intact columns per growth plate. Hypertrophic chondrocytes were operationally defined by a height ≥10 μm. The terminal hypertrophic cell height was measured in 8-10 different columns per growth plate section. For each animal, 3 different growth plate sections were analyzed and then all measurements from that animal were averaged. 4-5 animals were studied per treatment and time point. All histological measurements were performed in the central two-thirds of the growth plate sections to avoiding regions close to the perichondrium

3.7 PRIMARY CULTURE OF GROWTH PLATE CHONDROCYTES (PAPER V)

The use of primary cultures of growth plate chondrocytes offered important advantages for our studies. We needed an in vitro model that allowed for efficient transfection of cells and with relevance to in vivo growth plate chondrogenesis. Therefore, we opted for primary chondrocytes cultures.. Primary chondrocytes retain, at least partly, their chondrogenic phenotype and signaling pathways involved in its regulation, thereby providing information of relevance to the chondrogenesis, and experimental conditions can be easily controlled. In addition, we have used chondrocytes mainly from the resting zone, aiming to study proliferation and differentiation of young chondrocytes, without the confusing factor of already differentiated hypertrophic chondrocytes. In order to obtain resting zone, we used young mice (3-5 days-old), since their growth plate is taller, the secondary ossification center is absent, and chondrocytes from the epiphyseal and resting zone represent relatively large portion of the entire growth plate. To increase the number of

cells obtained, we dissect cartilage growth plate from proximal tibia, distal femur and distal tibia from each animal. Briefly, growth plates were dissected aseptically and digested in 0.3% collagenase type IA. The released cells were resuspended in Dulbecco's modified eagle medium/Hams F12, 10% fetal calf serum (FCS), 1% penicillin (100 U/ml)/ streptomycin (100 µg/ml), sodium pyruvate (100 µg/ml) and 50 µg/ml ascorbic acid, and then plated at a density of 10^5 cells/cm^2 in 96-well-plates.

3.8 TRANSIENT TRANSFECTION OF miRNAs (PAPER V)

3.8.1 Transfection of Primary Chondrocytes

Transfection is the process of introducing genetic content into a cell by means other than viral transduction, such as via electroporation or liposome-mediated fusion. The technique has been of exceptional value for functional studies in cell lines or primary cells, in which the researcher can modify the expression of a particular gene or miRNA through inhibition or overexpression, and observe possible phenotypic effects on the cell, such as proliferation, cell death and pure morphological changes. For most applications of transfection, it is sufficient if the transfected gene is only transiently expressed. Since miRNA introduced in the transfection process only needs to reach the cytoplasm and their effects on cell phenotype may be analyzed after 24 hours, this type of transfection is adequate for our study. We performed transient transfection of precursor (gain-of-function) or inhibitor miRNAs (knock-down) into primary murine chondrocytes by using Lipofectamine 2000, a lipid-based solution that transfers the construct miRNA into the cells by merging with the cell membrane. Briefly, primary chondrocytes were transfected by incubation of cells in 100nM precursors (pre-miR), inhibitors (anti-miR), or negative control (nonspecific) oligonucleotides miRNAs by using Lipofectamine 2000 (Invitrogen) in serum-free media (Opti-MEM I). This liposome mediated transfection study is highly efficient, with a transfection rate between 80 and 90%, and fairly non-toxic to the primary murine chondrocytes.

3.8.2 *Transfection Efficiency Assessment*

Fluorescence-Activated Cell Sorting (FACS) has a variety of applications, and was used in paper V for assessment of labeled-miRNAs transfected cells. FACS is a form

of flow cytometry, where cells pass through a laser in a hydrodynamically focused jet of fluid. As the cells pass through the light beam, they scatter the light depending on the physical and chemical characteristics of the cell. These characteristics are recorded for each cell by detectors and provide a range of information about the observed cells. Cells marked with fluorescent labels emit light when passing through the beam, and can be sorted into different containers based on the presence or absence of fluorescent signals. Sorting is done though breaking the hydrodynamically focused fluid jet containing the cells into droplets immediately following the measuring station. A charge is placed on each droplet depending on its fluorescent status, and differentially charged droplets are deflected in an electrostatic field, thereby sorting them into different fractions.

Transfection efficiency of Cy-3-labeled negative-controls for pre-miR and anti-miR miRNAs (Ambion) was found to be between 80-90% in repeated experiments. After one wash with PBS, cells were trypsinized, pelleted and resuspended in PBS containing 1% FCS. Cell cytometry was performed using a FACScan cell analyzer. WinMD12.8 was used for analyses of FACS data.

Furthermore, effective delivery and activity of positive control anti-miR and pre-miR was detected by real-time PCR quantification of their respective targets. Endogenous Let-7 miRNA negatively regulates HMGA2, a ubiquitously expressed nonhistone chromatin protein (Lee and Dutta 2007). Pre-miR-1 miRNA precursor effectively down regulates the expression of Twf1 (twinfilin, actin-binding protein 1) at the mRNA level (Lim, Lau et al. 2005). In our cells, transfection with anti-let-7-miR increased HMGA2 mRNA abundance by 100%, while transfection with pre-miR-1 down-regulated Twf1 gene expression by 70%. Altogether, the data confirm that the developed protocol for transfection of primary growth plate chondrocytes results in efficient transfection and that the model is adequate and may be used to explore the functional role of selected miRNAs in growth plate chondrogenesis.

3.9 PROLIFERATION ASSAY (PAPER V)

In paper V, incorporation of bromodeoxyuridine (BrdU, 10uM) into newly synthesized DNA of proliferating chondrocytes was measured by ELISA (photometry) to quantify cell proliferation. Bromodeoxyuridine (BrdU) is an analogue

of thymidine and competes with thymidine for incorporation into the forming DNA. Differences in BrdU incorporation by proliferating chondrocytes is reliably recorded by a microplate ELISA reader and comparable between different experiments.

3.10 CELL DEATH ELISA (PAPER V)

Cell death may occur by two mechanisms: apoptosis and necrosis, and both types have their own specific and distinct morphological and biochemical characteristics. During chondrogenesis, apoptosis is the main mechanism of cell death, especially in the hypertrophic zone. In our study, for detection of apoptosis in cultured transfected chondrocytes, we used Cell Death ELISA plus (Roche® Applied Science, Indianapolis, IN), which detects cytoplasmic histone-associated DNA fragments (mono- and –oligonucleassomes) by photometric enzyme immunoassay. The assay detects nucleasomes (histone complexed DNA fragments) in the cytoplasm of treated cells with the use of monoclonal antibodies against histone and single-stranded DNA.

4 RESULTS AND DISCUSSION

4.1 MARKERS FOR CHONDROCYTES DIFFERENTIATION AND GROWTH PLATE SENESCENCE (PAPER I)

To obtain an overall, unbiased assessment of how gene expression is regulated as chondrocytes undergo spatially-associated differentiation and temporally-associated senescence, we used microdissection of postnatal rat growth plates into their constituent zones combined with microarray analysis. Here, we report the overall results of the microarray study, using bioinformatic approaches to identify functional pathways and explore large-scale patterns in gene expression that may regulate the processes of postnatal chondrogenesis, and therefore identify specific gene products that may be used as molecular markers for the spatial zones and for temporal development.

In the transition from the resting to the proliferative zone, expression of 677 genes was upregulated and expression of 631 genes was downregulated (with $P < 0.01$ by ANOVA, Table1A). Functional pathways most implicated in this transition included: vitamin D receptor / retinoid x receptor (VDR/RXR) activation, platelet-derived growth factor (PDGF) signaling, BMP signaling, and notch signaling (all $P < 0.05$, Table1A). In addition, we detected biological functions most strongly implicated in this step of differentiation, such as: cellular development, cellular growth and proliferation, cell-to-cell signaling and interaction (all $P < 0.0001$, Table1A), all of which are consistent with the known biological changes occurring in this transition. These differences in expression between zones represent the changes in expression that occur as chondrocytes differentiate from the resting to the proliferative and then to the hypertrophic state. BMPs have already been shown to play an important regulatory role in the growth plate (De Luca, Barnes et al. 2001; Pogue and Lyons 2006; Nilsson, Parker et al. 2007), including a role in differentiation of mesenchymal cells into chondrocytes (Solomon, Berube et al. 2008), of proliferative chondrocytes into hypertrophic chondrocytes (Kobayashi, Lyons et al. 2005; Pogue and Lyons 2006; Solomon, Berube et al. 2008) and proliferation of resting zone chondrocytes (De Luca, Barnes et al. 2001). PDGFs have been shown to stimulate proliferation of cultured growth plate chondrocytes (Hiraki, Inoue et al. 1988; Wroblewski and Edwall 1992; Bulman, Iannotti et al. 1995; Olney, Wang et al. 2004; Kobayashi, Lyons et al. 2005), but there is less information about their role *in vivo*. Members of the notch family and

their receptors are expressed by hypertrophic chondrocytes (Hayes, Dowthwaite et al. 2003), but little is known about their role in growth plate chondrocyte differentiation. Vitamin D receptor ablation in mice causes important growth plate abnormalities but this appears to be related to the resulting abnormal mineral homeostasis (Demay 2006), and at least some of the direct effects of vitamin D on the growth plate appear not to be mediated by the nuclear vitamin D receptor.

A)

Resting to Proliferative

Upregulated Genes		
Genes:		**no. of genes**
P<0.01		677
P<0.01, Fold Change ≥ 2.0		153
Biological Functions Implicated:	**p-value**	**no. of genes**
Cellular Development	6,74E-06	29
Cellular Growth and Proliferation	6,74E-06	45
Cell Morphology	1,02E-04	14
Cellular Compromise	3,71E-04	13
Cell Cycle	6,06E-04	14
Downregulated Genes		
Genes:		**no. of genes**
P<0.01		631
P<0.01, Fold Change ≥ 2.0		241
Biological Functions Implicated:	**p-value**	**no. of genes**
Cell-to-cell Signaling and Interaction	1,91E-06	33
Cellular Growth and Proliferation	1,83E-05	73
Molecular Transport	1,41E-04	13
Small Molecule Biochemistry	1,41E-04	24
Cellular Movement	1,76E-04	46
Canonical Pathway		**p-value**
VDR/RXR Activation		1,710E-02
PDGF Signaling		3,027E-02
BMP Signaling		3,155E-02
Notch Signaling		3,184E-02

B)

Proliferative to Hypertrophic

Upregulated Genes		
Genes:		**no. of genes**
P<0.01		1717
P<0.01, Fold Change ≥ 2.0		794
Biological Functions Implicated:	**p-value**	**no. of genes**
Cellular Growth and Proliferation	6,41E-10	234
Cellular Movement	1,26E-09	152
Cell Death	1,87E-09	213
Cell Morphology	3,28E-08	133
Cellular Development	3,44E-08	181
Downregulated Genes		
Genes:		**no. of genes**
P<0.01		1625
P<0.01, Fold Change ≥ 2.0		635
Biological Functions Implicated:	**p-value**	**no. of genes**
Cell Cycle	5,42E-12	112
DNA Replication, Recombination, Repair	1,33E-09	100
Cellular Assembly & Organization	1,05E-08	69
Cellular Movement	2,46E-07	21
Cellular Growth and Proliferation	4,94E-04	49
Canonical Pathway		**p-value**
p53 Signaling		1,726E-03
Cell Cycle: G2/M Regulation		5,140E-03
Cell Cycle: G1/S Regulation		1,832E-02
Ephrin Receptor Signaling		3,311E-02
Oncostatin M Signaling		3,698E-02
BMP Signaling		4,385E-02

Table 1. Bioinformatic analysis of the genes changing between zones, from: **A)** resting zone to proliferative zone, and from: **B)** proliferative zone to hypertrophic zone in the growth plate cartilage of 1 wk old rats using the computer program Ingenuity Pathway Analysis 7.1.

Next, we identified that expression of 1717 genes was upregulated and expression of 1625 genes was downregulated (with $P < 0.01$ by ANOVA, Table 1B) in the transition from the proliferative to the hypertrophic zone. Biological functions most strongly implicated in hypertrophic differentiation included: cellular growth and proliferation, cellular movement, cell death, cell cycle, and DNA replication, recombination, and repair (all $P < 10^{-8}$, Table1B). Functional pathways that were implicated in this transition included: p53 signaling, cell cycle: G2/M regulation, cell cycle: G1/S regulation, ephrin receptor signaling, oncostatin M signaling, and BMP signaling (all $P < 0.05$, Table1B). P53 may play a role in apoptosis of growth plate chondrocytes [24].

Ephrin receptors, which interact with membrane-bound ligands causing bidirectional signaling, have been implicated in bone remodeling [25] but not, to our knowledge, in growth plate biology previously. Similarly, Oncostatin M, a pleiotropic member of the interleukin-6 cytokine family[26], has not, to our knowledge, previously been implicated in growth plate biology.

Changes associated with growth plate senescence were assessed by comparing gene expression in growth plate cartilage of 3-week-old versus 12-week-old rats. With age, expression of 1221 genes was upregulated and expression of 1046 genes was downregulated (with $P < 0.01$ by ANOVA, Table2). Biological functions most strongly implicated in growth plate senescence included: small molecule biochemistry, cell death, cell morphology, cellular growth and proliferation, cellular function and maintenance (all $P < 0.0001$, Table2). Functional pathways that were implicated in growth plate senescence included: eisosanoid signaling, VDR/RXR activation, p38 mitogen-activated protein kinase (MAPK) signaling, and Wnt/β-catenin signaling (all $P < 0.05$, Table2).

Upregulated Genes		
Genes:		**no. of genes**
3vs12wk P<0.01		1221
3vs12wk P<0.01, Fold Change ≥ 2.0		173
Change also consistent at 6wk & 9wk		152
Biological Functions Implicated:	**p-value**	**no. of genes**
Small Molecule Biochemistry	6,29E-06	25
Cell Death	6,84E-06	38
Cell Morphology	8,75E-06	46
Cellular Growth and Proliferation	2,24E-05	57
Cellular Function and Maintenance	4,79E-05	9
Downregulated Genes		
Genes:		**no. of genes**
3vs12wk P<0.01		1046
3vs12wk P<0.01, Fold Change ≥ 2.0		180
Change also consistent at 6wk & 9wk		131
Biological Functions Implicated:	**p-value**	**no. of genes**
Cellular Growth and Proliferation	5,37E-04	17
Cellular Development	7,62E-04	24
Lipid Metabolism	1,48E-03	8
Small Molecule Biochemistry	1,48E-03	12
Cell Cycle	1,90E-03	10
Canonical Pathway		
		p-value
Eisosanoid Signaling		7,063E-03
VDR/RXR Activation		1,033E-02
p38 MAPK Signaling		2,301E-02
Wnt/beta-catenin Signaling		3,846E-02

Table 2. Senescence (3-wk vs 12-wk) of growth plate. Bioinformatic analysis of the genes changing in the proliferative zone from 3 wk to 12 wk old rats using the computer program Ingenuity Pathway Analysis 7.1.

Many of the detected pathways have previously been implicated in growth plate chondrocyte regulation (Vortkamp, Lee et al. 1996; Rosado, Schwartz et al. 2002;

Agoston, Khan et al. 2007; Wang, Shao et al. 2007; Chen, Zhu et al. 2008) generally, but not specifically in the programmed loss of function and other senescent changes that occur during postnatal life. Furthermore, comparison of gene expression changes during chondrocyte differentiation and senescence did not reveal a substantial overlap, thus suggesting that the mechanisms that block proliferation as chondrocytes hypertrophy is different from those that restrict proliferation with increasing age.

In addition, we have identified potential markers that show greater than 10-fold specificity for each of these zones. Some, but not all, of these potential markers have known or suspected roles in chondrocyte biology. For the resting zones, the highest-ranking marker was secreted frizzled-related sequence protein 5 (Sfrp5), which showed mRNA levels in the resting zone that was 15-fold higher than in proliferative zone and 60-fold higher that in the hypertrophic zone (see Fig. 1).

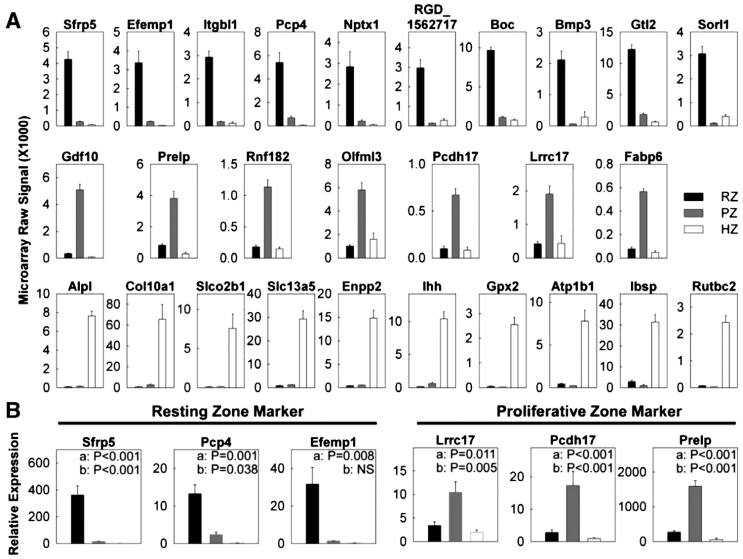

Fig. 7. Spatial Markers for Growth Plate Senescence. Changes in gene expression (mean ± SEM) of spatial markers across the tibial growth plate of 1-week-old rats. (A) The relative expression of mRNA in each zone was measured by expression microarray (Affymetrix Rat Genome Array 230 2.0). (B) The relative expression of *Sfrp5*, *Pcp4*, *Efemp1*, *Lrrc17*, *Pcdh17*, and *Prelp* was verified using quantitative real-time PCR. RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone.

The highest-ranking marker identified for the proliferative zone was growth differentiation factor 10 (Gdf10), which is a member of the BMP family and which showed mRNA levels in the proliferative zone that were 16-fold higher than in the

resting zone and 110-fold higher than in the hypertrophic zone (Fig. 7). And, for the last, the hypertrophic zone, the two highest-ranking markers were tissue non-specific alkaline phosphatase (Alp1) and collagen X (Col10a1), two widely used markers for hypertrophic chondrocytes (Fig. 7). Other candidate markers for all zones are shown in Fig. 7B. To further confirm our findings, the localization of several spatial markers in 1-week-old growth plate was studied by *in situ* hybridization (Fig. 8). The identified mRNAs are likely to prove useful as markers for the resting and proliferative zones in studies using RNA-based methods, as we showed using *in situ* hybridization and real-time qPCR.

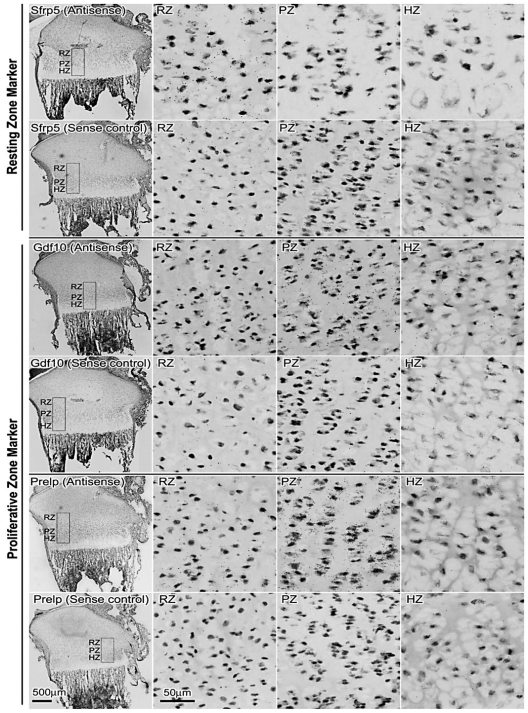

Fig. 8. mRNA expression of *Sfrp5*, *Gdf10*, and *Prelp* in growth plate cartilage of 1-week-old rats by *in situ* hybridization. The left hand panel in each row shows the proximal tibial at low magnification. The other panels show high magnification views of resting zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ) taken from within the rectangular area indicated in the corresponding left hand panels.

In addition to spatial regulation, we identified multiple genes that showed large increases in expression with age and other genes that showed large decreases at either 3-wks (youth markers) or 12-wks (aged markers) (Fig. 9). For the young growth

plate, the highest-ranking marker was insulin-like growth factor 2 (*IGF2*), which showed mRNA levels 82-fold higher at 3-wks than at 12-wks. For the old growth plate, the highest-ranking marker was *Pycard* (PYD and CARD domain containing), which showed mRNA levels increased 9-fold from 3-wks to 12-wks of age (Fig. 3). The expression level of these genes may be useful indicators of the maturational state and residual proliferative capacity of the growth plate chondrocytes, and therefore can be employed to study processes that delay (Marino, Hegde et al. 2008) or accelerate (Weise, De-Levi et al. 2001) growth plate senescence. Whether the proteins encoded by these mRNAs will prove useful as markers using immunohistochemistry or western blot analysis remains to be determined.

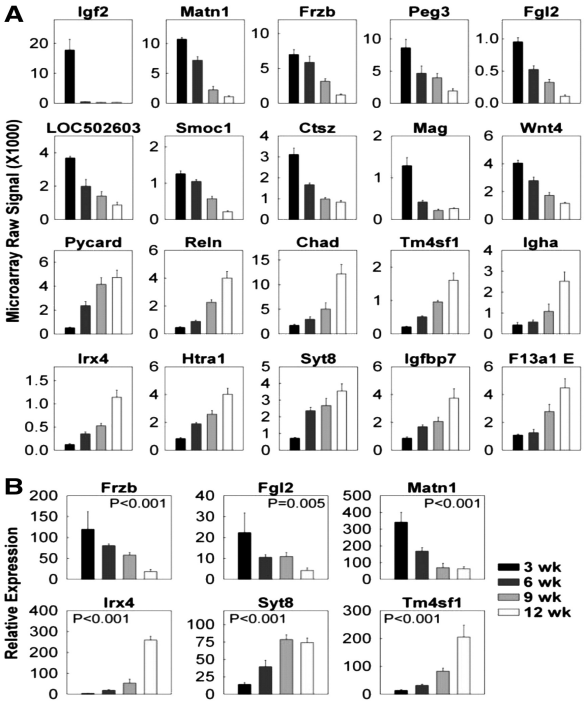

Fig. 9. Temporal Markers for Growth Plate Senescence. Changes in gene expression (mean±SEM) of senescence markers in the proliferative zone of 3-, 6-, 9-, and 12-week-old rats. Proliferative zones of the growth plate cartilage were microdissected. (A) The relative expression of mRNA at different ages was measured by expression microarray (Affymetrix Rat Genome Array 230 2.0). The data was background corrected and normalized using the MAS5 statistical algorithm. (B) The relative expression of *Frzb*, *Fgl2*, *Matn1*, *Irx4*, *Syt8*, and *Tm4sf1* was verified using quantitative real-time PCR

Although bioinformatic analysis of expression microarray findings can identify important functional pathways involved in a physiological, pathological, or pharmacological process, this approach is neither completely sensitive nor specific. Therefore, the findings in the current study can only be considered as hypothesis generating. For some of the novel pathways implicated in growth plate senescence,

such as Wnt signaling, and genes implicated in growth regulation (e.g. imprinted genes, data not shown); additional studies were performed to confirm the expression findings using independent methods and to investigate their functional role in postnatal chondrogenesis.

4.2 ROLE OF WNTs IN POSTNATAL GROWTH PLATE (PAPER II)

Wnt/β-catenin signaling was one of the most implicated pathways in the developmental program of growth plate senescence revealed in our microarray analysis (Paper I). Although the importance of Wnt signaling in chondrogenesis is well established, little is known about which specific Wnts are responsible for these effects. This gap in our understanding is particularly evident in the postnatal growth plate, since most previous work has focused on the embryonic skeleton. We therefore examined the expression profile of Wnt family members in distinct zone of growth plate and metaphyseal bone, by using microdissection followed by real-time qPCR, a quantitative method with greater sensitivity and specificity than previous techniques.

Of the 19 known members of the Wnt family, only six, Wnts -2b, -4, -5a, -5b, -10b, and -11, were clearly detected both in whole growth plate and individual zones (Fig. 10). Although ours is the first study to investigate extensively Wnt expression in the postnatal mammalian growth plate, some of these members of the Wnt family, Wnts -4, -5a and -5b, had previously been implicated in embryonic mammalian cartilage, whereas Wnts -2b, -10b, and -11 had not been implicated in the regulation of the mammalian growth plate at any stage of development. Interestingly, of those six Wnts, three (Wnts -2b, -4, and -10b) signal through the canonical Wnt/β–catenin pathway and three (Wnts -5a, -5b, and -11) signal through the noncanonical, calcium pathway.

In addition, all detected Wnts presented spatial expression remarkably similar (Fig. 10). As the chondrocytes passed into the proliferative state and the prehypertrophic state, expression of these Wnts appeared to increase. As the cells underwent terminal differentiation to the hypertrophic state, Wnt expression tended to decline. This overall pattern of gene expression is broadly consistent with previous studies in the mouse embryo suggesting that Wnts that signal through both the canonical and

noncanonical pathways modulate chondrocyte hypertrophic differentiation. Specifically, Wnts -4, -5a, -5b, -10b, and -11, all showed relatively low expression in resting zone.

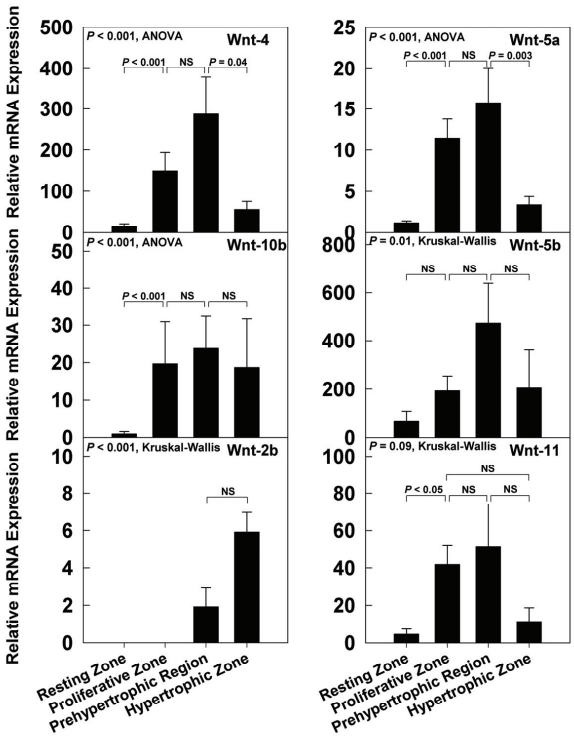

Fig. 10. Temporal Markers for Growth Plate Senescence. Relative mRNA expression of Wnt family members in growth plate zones from 1-week-old mice (n = 6).

Among the Wnt family members that signal through β–catenin, Wnt-4 had the highest mRNA expression in the growth plate. The observed expression pattern is compatible with previous studies showing that Wnt-4 overexpression accelerates hypertrophy of chondrocytes (Hartmann and Tabin 2000). However, mice null for Wnt-4 have been reported to have either no growth plate phenotype (Stark, Vainio et al. 1994) or a slight delay in chondrocyte maturation (Spater, Hill et al. 2006). Our data suggest that this mild phenotype, which contrasts with the more severe delayed hypertrophic differentiation and decreased chondrocyte proliferation of mice lacking β–catenin, may be attributable to redundancy between Wnt-4 and Wnt-10b, which also acts through the β–catenin pathway and shows a similar spatial expression pattern. Consistent with this hypothesized redundancy in growth plate, mice lacking Wnt-10b display decreased

trabecular bone but have not been reported to have abnormal endochondral bone formation at the growth plate (Bennett, Longo et al. 2005). Wnt-2b, although expressed at low levels, may also contribute to redundancy. In our study, Wnt-2b, which is required for limb initiation in zebrafish and chick (Kawakami, Capdevila et al. 2001; Ng, Kawakami et al. 2002), was a partial exception to the overall expression pattern, since it did not seem to decrease in the hypertrophic zone and was absent in resting and proliferative chondrocytes (Fig. 10). There is no study involving ablation or overexpression of Wnt-2b in growth plate to add insights about its role in growth plate regulation. We speculate if Wnt-2b may be important in the process of chondrocyte hypertrophy and terminal differentiation, but additional functional studies are required.

Conversely, the noncanonical calcium pathway can antagonize the canonical Wnt/β–catenin pathway by promoting the degradation of β–catenin (Topol, Jiang et al. 2003; Katoh 2005), and appears to have an important role in growth plate formation and function. Wnt-5a null mice display a severe skeletal phenotype with limb truncation. In growth plates, chondrocyte hypertrophy is delayed, suggesting that Wnt-5a promotes chondrocyte hypertrophy (Yamaguchi, Bradley et al. 1999; Yang, Topol et al. 2003)). Wnt-5b overexpression appears to have different effects, promoting proliferative zone formation and inhibiting cell cycle withdrawal and chondrocyte hypertrophy (Yang, Topol et al. 2003). The expression pattern detected in this study is consistent with the findings from mouse models, described above, suggesting that Wnts -5a and -5b may modulate production of proliferative zone chondrocytes and their conversion to hypertrophic chondrocytes. This expression pattern is also similar to, though perhaps not identical to, the expression pattern observed by in situ hybridization in the embryonic skeleton; Wnt-5a was observed in proliferative and prehypertrophic chondrocytes and Wnt-5b in the region between prehypertrophic and hypertrophic chondrocytes (Yang, Topol et al. 2003). Little is known about Wnt-11 function in the mammalian growth plate. Overexpression of Wnt-11 in the developing chick limb results in slightly truncated limbs and joint fusion but does not appear to delay chondrocyte differentiation (Church, Nohno et al. 2002). Our study, showing similar expression patterns for Wnts -5a, -5b, and -11, all of which signal through the calcium pathway, suggest that these three members of the Wnt family may have redundant, overlapping, or interacting roles in the growth plate. Therefore, ablation of these genes in combinations may reveal a growth plate phenotype that is more severe and perhaps qualitatively different than occurs with ablation of any single one of these genes.

Interestingly, the similarity between the general pattern of Wnt expression and Ihh expression in the growth plates of 1-week-old mice suggests a possible interaction between both pathways. In embryonic mouse long bones, Wnt-9a ablation caused downregulation of Ihh expression and signalling (Spater, Hill et al. 2006). Whether Wnts, either those signalling through the canonical pathway or those signalling through the calcium pathway, interact with Ihh in the postnatal growth plate is unknown and speculative.

Some of the Wnt genes that we found to be expressed in the postnatal growth plate have also been implicated in other models of postnatal chondrogenesis. Human dermal fibroblasts cultured in the presence of chondroinductive demineralized bone powder show increased expression of Wnts -2b, -5b, and -10b (Yates 2004), whereas Wnts -4, -5a and -5b are up-regulated during bone repair in vivo (Hadjiargyrou, Lombardo et al. 2002; Zhong, Gersch et al. 2006).

To rule out the role of the identified Wnts in growth plate senescence, we hypothesized that change in Wnt gene expression might explain the functional changes in growth plate observed with age. We therefore studied Wnt expression in the mouse growth plate at 4 weeks, an age by which longitudinal bone growth has slowed markedly, approximately two-fold, in the mouse (van & Van den 1978). Contrary to our hypothesis, we found that all Wnts that had been readily detected in the growth plates of 1-week-old mice were still expressed in growth plate cartilage of 4-week-old mice, at similar levels (Fig. 10). Because the growth plate height is diminished at this age in mice, we were only able to microdissect the growth plates in two regions, the resting/proliferative and prehypertrophic/ hypertrophic regions. As a result, our comparisons to 1-week-old mice were less precise. However, we can conclude that expression of the Wnts that we detected in growth plate at 1 week persists and remains at similar at levels at 6 weeks, but we cannot exclude modest changes in gene expression of Wnts with age, since the expression analysis from two distinct zones combined may hide or compensate subtle but significant changes expected with age (Fig. 11). In addition, change in expression of Wnt family may include other genes involved in its signaling, such as receptors (e.g. Frizzled), co-receptors (Lrp5, Lrp6) and Wnt-antagonists (e.g. Sfrp).

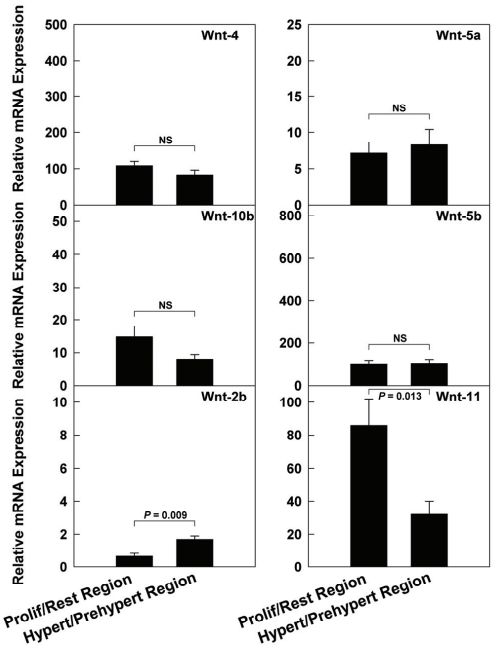

Fig. 11. **Temporal Expression of Wnts in Growth Plate.** Relative mRNA expression of Wnt family members in growth plates of 1-week and 4-week-old mice (n = 6).

4.3 GROWTH-REGULATED NETWORK OF IMPRINTED GENES IN THE POST-NATAL GROWTH PLATE (PAPER III)

In this study, we present data suggesting that a growth regulating imprinted gene network (Cdkn1c, Grb10, Gtl2, Mest, Dlk1, Peg3, Ndn, Slc38a4, H19, and Plagl1/Zac1) previously implicated in the control of embryonic (Varrault, Gueydan et al. 2006) and postnatal growth deceleration of multiple tissues ((Lui, Finkielstain et al. 2008) might show a similar coordinate down-regulation in rat metaphyseal bone, but a more gene-specific pattern of expression in proliferative chondrocytes of rat growth plate cartilage.

Similar to previous findings in kidney, lung and liver, expression of all studied genes, except Gtl2, declined significantly with age in metaphyseal bone (Fig. 12A). In contrast, in growth plate, we found that expression of growth-promoting genes, Mest, Dlk1, and Gtl2 (all $P < 0.001$), decreased with age in PZ, whereas growth-inhibitory genes, Cdkn1c and Grb10 ($P < 0.001$; $P < 0.05$ respectively) increased with age (Fig.

12B). Those findings are thus consistent with the hypothesis that this network of imprinted genes contributes to the developmental decline in the rate of longitudinal bone growth that occurs during postnatal development.

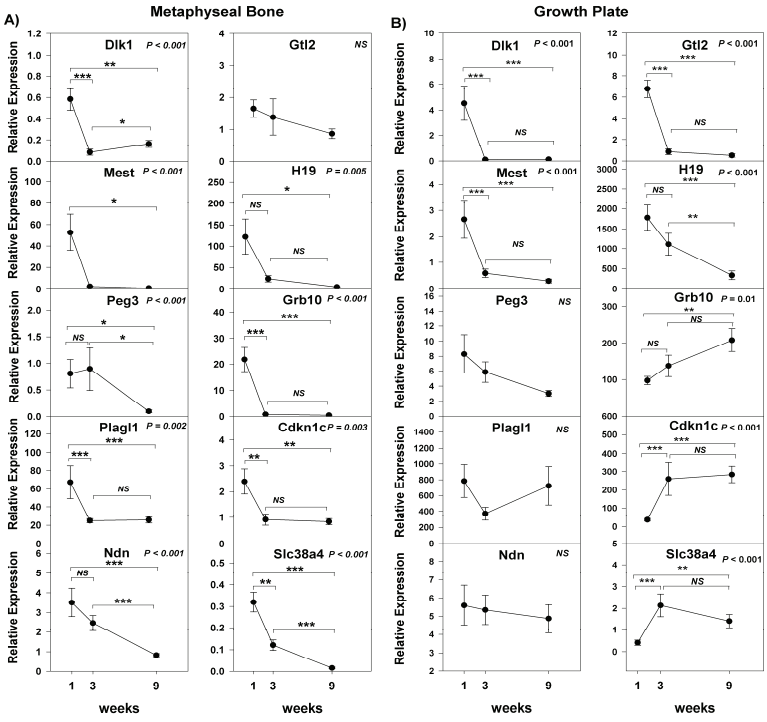

Fig. 12. Temporal mRNA expression of imprinted gene network. A) In metaphyseal bone (MB); and **B)** In growth plate proliferative zone (PZ), from 1-, 3-, and 9-week-old rats (n = 5, all ages). Real-time qRT-PCR normalized to 18S rRNA, and, for convenience, multiplied by 10^6. * P < 0.05; ** P < 0.01; *** P < 0.001.

In order to explore the role of this network of imprinted genes in the regulation of growth plate chondrocyte differentiation, we also characterized its expression in individual zones of the growth plate (Fig. 13). To our knowledge, this is the first report characterizing the expression patterns of these genes, except Igf2, in growth plate cartilage. We have previously shown that Igf2 mRNA expression decrease from RZ to PZ in growth plate cartilage of 1-week-old rats (Parker, Hegde et al. 2007). Expression of other six genes (Mest, Dlk1, Peg3, Grb10, Gtl2 and H19) in the network thus decreases as chondrocytes differentiate from RZ to HZ chondrocytes. Four of these genes (Mest, Dlk1, Peg3, and Gtl2) promote growth, and their expression pattern suggests that they promote proliferation in the RZ and/or PZ. Interestingly, all genes

that were found to decrease with age (Mest, Dlk1, Gtl2, H19 and Igf2) also decreased during chondrocyte differentiation. These findings may suggest that the network not only contribute to the decline in proliferation that occurs during growth plate senescence, but possibly also to the inhibition of proliferation that occurs during hypertrophic differentiation. In contrast, comparisons of global gene expression changes during growth plate senescence and growth plate chondrocyte differentiation do not show substantial overlap (data not shown). Therefore, on a transcriptsome level, growth plate senescence bare little similarity with hypertrophic differentiation, but in regard to the studied network of imprinted genes, the process of growth plate senescence show transcriptional similarities to chondrocyte differentiation.

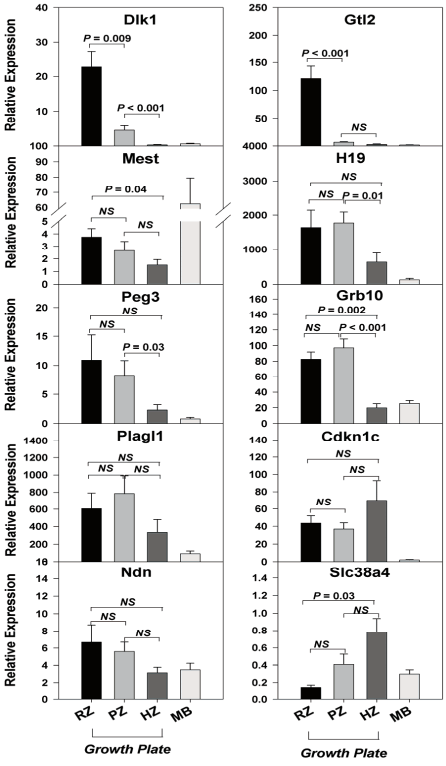

Fig. 13. Spatial mRNA expression of imprinted genes in resting (RZ), proliferative (PZ), and hypertrophic (HZ) zones of growth plate and metaphyseal bone (MB) from 1-week-old rats (n = 5). Quantitative real-time RT-PCR was performed on samples from individual animals, normalized to 18S rRNA, and, for convenience, multiplied by 10^6.

4.4 GROWTH PLATE SENESCENCE IS A FUNCTION OF GROWTH (PAPER IV)

We have shown in the first paper of this thesis that several genes change their expression with postnatal age in the rat PZ, based on microarray data analysis,

suggesting that there is also a developmental program withing growth plate cartilage in addition to the specific changes observed at the functional and structural level. This developmental program has been termed “growth plate senescence” and takes place as longitudinal bone growth decline with age (Nilsson and Baron 2004). Delay in growth plate senescence after transient hypothyroidism in rats (Marino, Hegde et al. 2008) suggests that growth plate senescence is a function of growth rather than age. We reasoned that if this hypothesis is true, then growth plate senescence would be slowed down by any growth inhibiting condition, not only by hypothyroidism. To test this hypothesis, we used another model of transient growth inhibition, providing newborn rats a tryptophan deficient diet (Trp^-) during the first four postnatal weeks.

We found that during recover of the induced growth inhibition, molecular (Fig. 14A), functional (Fig. 14B) and structural markers (Fig. 14C) of growth plate senescence were delayed by prior Trp^- deficiency indicating that the developmental program of senescence had occurred more slowly during the period of growth inhibition. Tibial growth rate, which is a functional marker of growth plate senescence, declined in control animals and was delayed in Trp^- rats compared to controls ($P<0.001$, Fig. 14B). The delay in structural changes was also evident by quantitative histology of growth plate sections, such as shorter growth plate height during tryptophan deficiency compared to control, but significantly taller growth plates after recovery of the growth inhibiting condition (Fig. 14C). Other studies in rabbits with induced-hypercortisolism support this concept as well (Baron, Klein et al. 1994; Gafni, Weise et al. 2001).

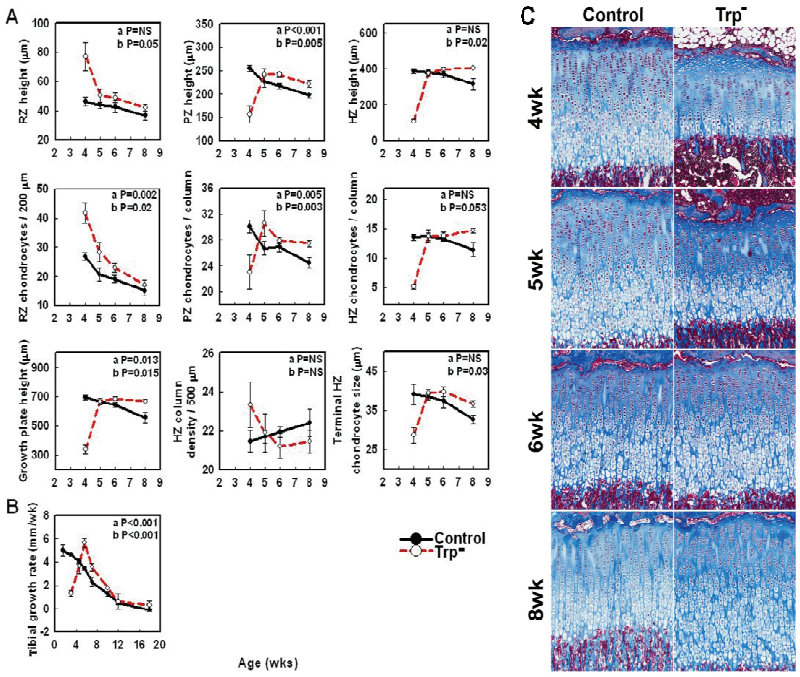

Fig. 14. Structural and functional senescent changes in the growth plate were delayed by prior tryptophan deficiency. **A)** Structural markers at different ages, including resting, proliferative and hypertrophic zones height; number of resting zone chondrocytes per 200 µm of growth plate width; number of proliferative and hypertrophic chondrocytes per column; growth plate height; number of hypertrophic columns or column density per 500 µm of growth plate width; and terminal hypertrophic chondrocytes height. Each experimental group contains 4-5 animals. **B)** Rate of proximal tibia growth per week. **C)** Photomicrographs of Masson-Trichrome stained longitudinal sections of proximal tibial growth plate from control and Trp^- animals at different ages. During the period of growth inhibition (4wk), the overall growth plate height in the Trp^- animals was smaller than the control animals, despite having significantly more resting chondrocytes. At 8-weeks of age, the growth plate height was greater in animals that had previously received a Trp^- diet (N= 4-5 animals). Bar represents 50 µm. a, Effect of age, one-way ANOVA; b, Effect of treatment during the recovery period, two-way ANOVA.

However, the underlying cellular mechanisms by which growth drives senescence and advances the program remain to be elucidated. Postnatal decline in growth rate is observed not only in the long bone, but also in many other organs, suggesting a common and concordant mechanism to maintain body proportion. In transplantation experiments, growth of the transplanted organs generally depends on the age of the donor, supporting this hypothesis (Cooke, Yonemura et al. 1986; Pape, Hoppe et al. 2006).

Based on our microarray data (paper I), we investigated five genes that increase with age and other three genes that significantly decrease with age (Fig. 15). We found that

after catch-up growth, previously Trp$^-$ rats showed a delay in the age-dependent changes in gene expression of *Igfbp7, Calca, Prss11 and Pycard* compared to controls. Because expression of these genes normally rises during senescence, the findings indicate that prior Trp deficiency delayed the normal increase. Conversely, prior Trp deficiency delayed the normal decline of *Igf2* observed in control animals. In addition, hypothyroidism but not tryptophan deficiency caused a significant delay in the developmental decline of *Asb4* expression.

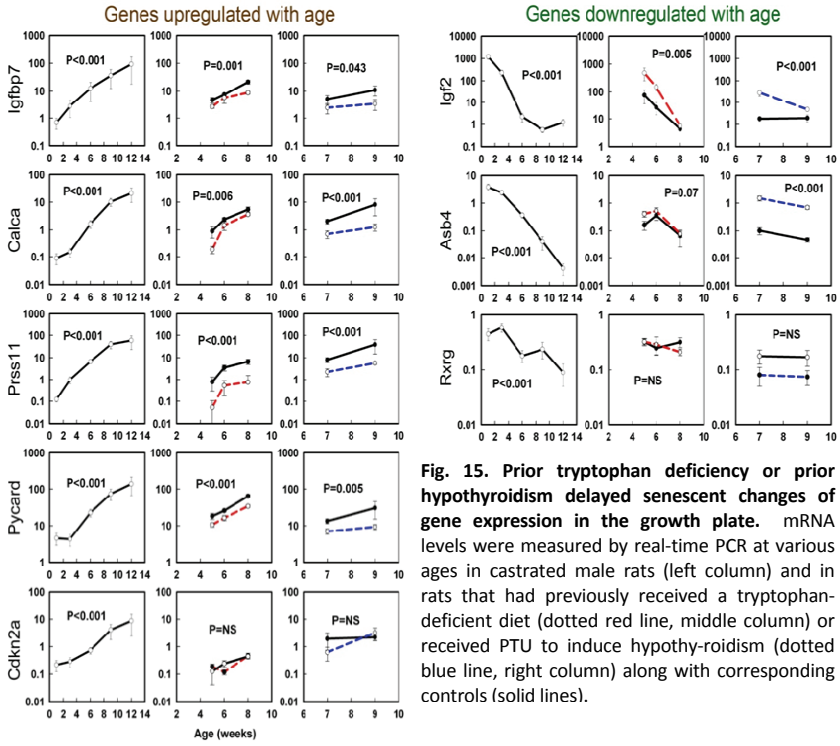

Fig. 15. Prior tryptophan deficiency or prior hypothyroidism delayed senescent changes of gene expression in the growth plate. mRNA levels were measured by real-time PCR at various ages in castrated male rats (left column) and in rats that had previously received a tryptophan-deficient diet (dotted red line, middle column) or received PTU to induce hypothy-roidism (dotted blue line, right column) along with corresponding controls (solid lines).

Finally, our findings also provide important insights into the mechanism of catch-up growth, which may be partly explained by delayed growth plate senescence. In our study, animals previously receiving a Trp$^-$ diet have tibial growth rates higher than the control animals and appear to have a delayed decline in tibial growth of approximately 2 weeks (Fig. 14B). Notably, this delay was similar in magnitude to the delay in structural and molecular markers of the growth plates. Taken together with previous findings during glucocorticoid excess and hypothyroidism (Gafni, Weise et al. 2001; Marino, Hegde et al. 2008), our findings suggest that delayed

growth plate senescence is a general mechanism that contributes to catch-up growth after a variety of growth inhibiting conditions. In summary, our findings support the hypothesis that delayed senescence is a general consequence of growth inhibition, suggesting that growth plate senescence is not a function of time *'per se'*, but rather of growth.

4.5 THE ROLE OF miRNAs IN GROWTH PLATE SENESCENCE (PAPER V)

MicroRNAs (miRNAs) are a new class of small non-coding RNA that regulate gene expression and has been implicated in the control of several biological mechanisms, such as growth proliferation, apoptosis and aging. In order to further characterize growth plate senescence and explore the molecular mechanisms that control the developmental program of growth plate senescence, we used miRNA microarray and real-time PCR analyses to identify miRNA expression in growth plate cartilage and also to detect changes in miRNA expression during growth plate senescence. Here, we report original findings of age-regulated miRNAs in mice growth plate during postnatal growth, and unique miRNAs preferentially expressed in growth plate compared to metaphyseal bone and other tissues. Additional to the microarray profiling, bioinformatic analysis of predicted target genes revealed biological pathways likely involved in the function of age-regulated miRNAs during postnatal bone growth. Furthermore, transfection of selected pre-miRNAs by inhibitory anti-miRNA in primary resting zone chondrocytes show that individual miRNAs may contribute to the developmentally regulated decline in longitudinal growth, such as miR-195 by increasing chondrocytes apoptosis and delaying hypertrophic differentiation.

4.5.1 Developmentally-Regulated miRNAs in Growth Plate Cartilage

Four miRNAs, miR-195, -497, -125a-3p and -142-3p, were found to be at least 10-fold (P<0.01) up-regulated and 14 down-regulated with age in growth plate cartilage (Fig. 16). It has been shown that miR-195 and miR-497 are down-regulated in several cancers, with potential role as tumor-suppressor (Flavin, Smyth et al. 2009). Little is known about the function of the other two miRNAs.

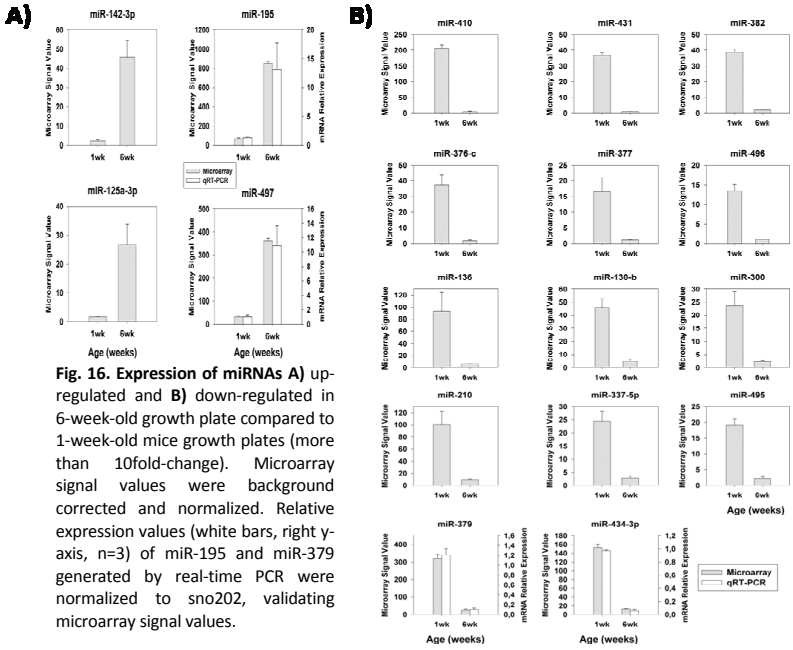

Fig. 16. Expression of miRNAs A) up-regulated and **B)** down-regulated in 6-week-old growth plate compared to 1-week-old mice growth plates (more than 10fold-change). Microarray signal values were background corrected and normalized. Relative expression values (white bars, right y-axis, n=3) of miR-195 and miR-379 generated by real-time PCR were normalized to sno202, validating microarray signal values.

To explore the biological function and mechanisms of action of age-regulated miRNAs in growth plate, first we determined predicted target genes by using TargetScan (Lewis, Shih et al. 2003) and then, analyzed their functional role using Ingenuity pathway analysis (Ingenuity System Inc, Redwood City, CA) (Guerra 2008). The analysis of target genes from both up- (Table 3) and down-regulated miRNAs (Table 4) revealed enrichment of genes involved in the control of cell cycle, cell growth and cell death. It is important to remark that our data analyze the dynamic process of growth plate senescence, in which cell death and cell growth regulation is clearly altered. In addition, canonical pathways most strongly implicated included known pathways involved in chondrogenesis, such as TGF-β and Wnt/β-catenin signaling, corroborating our findings in the previous studies included in this thesis. In special, Wnt/β-catenin has been consistently implicated in postnatal growth plate senescence. In a previous study, we have detected that only six members of Wnt family are expressed in mice postnatal growth plate, and three of them act through the Wnt/β-catenin pathway (Andrade, Nilsson et al. 2007). Among these Wnts, we found that Wnt-4 is targeted by up-regulated miRNAs; whereas Wnt-2b is targeted by down-regulated miRNAs. Wnts -5a, -5b and -11, which act through the non-canonical/Calcium-pathway, are predicted

targets for down-regulated miRNAs. In the postnatal growth plate, the Wnt/β-catenin signaling is necessary for hypertrophic differentiation (Chen, Zhu et al. 2008). Inhibition of β-catenin and TCF (ICAT) inhibit β-catenin signaling but not cell adhesion, resulting in viable *Col2a1*-ICAT transgenic mice, with normal size at birth, followed by progressive growth retardation during postnatal life. Growth plate of *Col2a1*-ICAT transgenic mice present reduced chondrocyte proliferation and increased chondrocyte apoptosis (Chen, Zhu et al. 2008), similar to the phenotype of cartilage-specific Dicer-knockout mice (Kobayashi, Lu et al. 2008). Interestingly, Wnt/β-catenin pathway was also implicated in growth plate senescence in our previous study designed to detect changes in global mRNA expression during growth plate senescence (Lui et al). Altogether, these findings suggest that miRNAs play an important role in the regulation of Wnt signaling during postnatal growth.

miRNAs Up-regulated with age		**no. of miRNAs**
P<0.01		21
P<0.01, Fold Change ≥ 10.0		4
Target Gene (TargetScan database)		**no. of genes**
Predicted targeted genes		803
Top Molecular and Cellular Functions	**p-value**	**no. of genes**
Cell Cycle	5,24E-13 - 6,35E-3	116
Amino Acid Metabolism	1,20E-11 - 6,06E-3	69
Post-Translational Modification	1,20E-11 - 6,06E-3	120
Small Molecule Biochemistry	1,20E-11 - 6,35E-3	87
Cell Death	2,04E-10 - 5,50E-3	210
Canonical Pathway	**p-value**	**Ratio**
Axonal Guidance Signaling	3,25E-06	38/403 (9.4%)
PPARα/RXRα Activation	8,89E-06	22/182 (12.1%)
Insulin Receptor Signaling	9,54E-06	19/140 (13.6%)
TGF-β Signaling	1,14E-05	14/83 (16.9%)
Wnt-β-catenin Signaling	2,63E-05	21/168 (12.5%)

Ratio = number of genes with P < 0.05 / total number of genes in the pathway

Table 3. Target genes regulated by up-regulated miRNAs with age. Bioinformatic analysis of the genes targeted by up-regulated miRNAs (more than 10-fold change) in growth plate chondrocytes with age (6-week vs 1-week-old mice), by using the Ingenuity Pathway Analysis (IPA) software 8.0 (Ingenuity Systems Inc, Redwood City, CA). All *P* values ≤ 0.01 were considered significant.

Target Genes regulated by age-down-regulated miRNAs

miRNAs down-regulated miRNAs with age		**no. of miRNAs**
P<0.01		36
P<0.01, Fold Change ≥ 10.0		14
Target Gene (TargetScan database)		**no. of genes**
Predicted targeted genes		1428
Top Molecular and Cellular Functions	**p-value**	**no. of genes**
Gene Expression	4,78E-28 - 1,22E-3	116
Cell Death	7,92E-14 - 1,22E-3	69
Cellular Development	1,53E-13 - 1,22E-3	120
Cellular Growth and Proliferation	4,78E-11 - 9,99E-4	87
Cellular Movement	9,99E-09 - 1,22E-3	210
Canonical Pathway	**p-value**	**Ratio**
TGF-β Signaling	4,01E-09	24/83 (28.9%)
Wnt-β-catenin Signaling	8,15E-08	35/168 (20.8%)
Factors Promoting Cardiogenesis in Vertebrates	2,73E-07	22/89 (24.7%)
Molecular Mechanisms of Cancer	5,81E-07	54/372 (14.5%)
Axonal Guidance Signaling	4,69E-06	55/403 (13.6%)

Ratio = number of genes with P < 0.05 / total number of genes in the pathway

Table 4. Bioinformatic analysis of the genes targeted by down-regulated miRNAs (more than 10-fold change) in growth plate chondrocytes with age (6-week vs 1-week-old mice), by using the Ingenuity Pathway Analysis (IPA) software 8.0 (Ingenuity Systems Inc, Redwood City, CA). All *P* values ≤ 0.01 were considered significant.

Next, in order to explore the functional role of selected age-regulated miRNAs in growth plate chondrogenesis, *in vitro* gain- and loss-of function experiments in primary chondrocytes were performed. The functional studies in this study were focused on up-regulated and selected highly expressed miRNAs. Among the miRNAs that were up-regulated with age, *in vitro* gain of function experiments showed that miR-195 ($P<0.001$) and miR-497 ($P=0.034$) increase apoptosis of chondrocytes (Fig. 17B). Although miR-195 has been shown to function by promoting cell cycle arrest in other tissues (Flavin, Smyth et al. 2009), our findings suggest that miR-195 controls chondrogenesis mainly by increasing apoptosis, rather than by decreasing proliferation. In addition, gain-of-function of miR-195 in primary cultured chondrocytes also decreased mRNA expression of type 10 collagen, suggesting that miR-195 acts to delay hypertrophic differentiation. Gain-of-function of miR-497 was found to increase apoptosis, presumably by down-regulating expression of anti-apoptotic proteins bcl-2 and bcl-x (Yin, Deng et al.), but did also increase proliferation of primary growth plate

chondrocytes. However, by promoting chondrocyte proliferation together with miR-142-3p (*P*=0.006) and miR-125a-3p *(P*=0.012), miR-497 may contribute to the decline in chondrocyte proliferation observed in growth plate of cartilage-specific dicer-knockout mice (Fig. 17). Among the down-regulated miRNAs, the absence of phenotype observed in this study may be explained by compensatory mechanisms by other miRNAs.

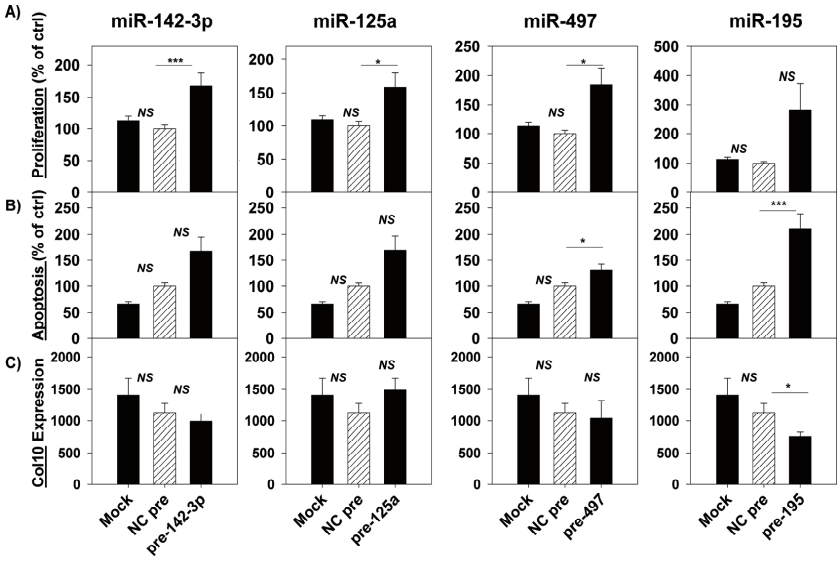

Fig. 17. Gain-of-Function of up-regulated miRNA with age: A) Gain-of-function of miR-125a-3p (*P*=0.012), miR-142-3p (*P*=0.006), and miR-497 (*P*=0.039) increased proliferation of primary chondrocytes. Proliferation was assessed by using BrdU proliferation colorimetric kit (Roche). **B)** Gain-of-function of miR-195 (*P*<0.001) and miR-497 (*P*=0.034) increase apoptosis of chondrocytes compared to negative control. Apoptosis was determined by Cell Death ELISA colorimetric kit (Roche). **C)** Decline in Col10a1 mRNA expression after gain-of-function of miR-195. Results are compared to negative control (non-specific miR) of treated cells (n=3 biological replicates from three independent experiments. Results for proliferation and apoptosis are expressed as percent of negative control, and expressed as mRNA relative expression to 18S by real-time PCR for Col10.

4.5.2 MiRNAs preferentially expressed in growth plate cartilage or metaphyseal bone

Our search for potential cartilage-specific miRNAs revealed that miR-455 (37-fold), miR-503 (69-fold) and miR-379 (29-fold) were preferentially expressed in chondrocytes, in addition to the well known cartilage-specific miR-140 (29-fold) and miR-140* (26-fold) compared to metaphyseal bone (Fig. 18A). The magnitude and

preferential expression of the cited miRNAs compared to metaphyseal bone and other tissues (lung, kidney and heart) suggest that those miRNAs may be necessary for specific chondrocyte phenotype and differentiation during postnatal life.

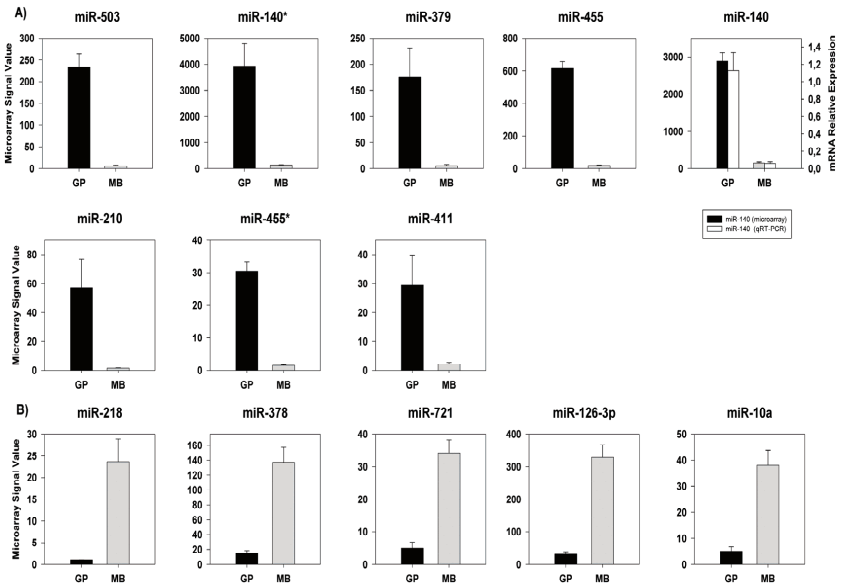

Fig. 18. **MiRNAs preferentially expressed** in **A)** growth plate, and **B)** metaphyseal bone. MiRNA expression was more than 10-fold change between tissues throughout life (1- and 6-week-old mice). Microarray signal values were background corrected and normalized. Relative expression values (white bars, right y-axis, n=3) of miR-140 generated by real-time PCR were normalized to sno202, validating microarray signal values.

Loss-of-function experiments of highly and preferentially expressed miRNAs in growth plate showed that miR-140* (P=0.011) and miR-503 (P=0.012) increase chondrocyte proliferation (FIG. 19A), thus suggesting that they may be negative regulators of longitudinal bone growth. Consistent with our findings, miR-503 has been shown to induce cell-cycle arrest (Forrest, Kanamori-Katayama et al.) and inhibit cell growth (Jiang, Feng et al. 2009) in other cell types. The importance of miR-140 and miR-140* in early chondrogenesis is established, but their functional role remains unknown (Nicolas, Pais et al. 2008; Song, Wang et al. 2009; Tardif, Hum et al. 2009). Inhibition of miR-455, miR-379, and miR-140 using specific anti-miR inhibitors did not cause significant changes in proliferation or apoptosis. Whether this is due to used *in vitro* model or due to a true lack of effect needs to be addressed by further functional studies.

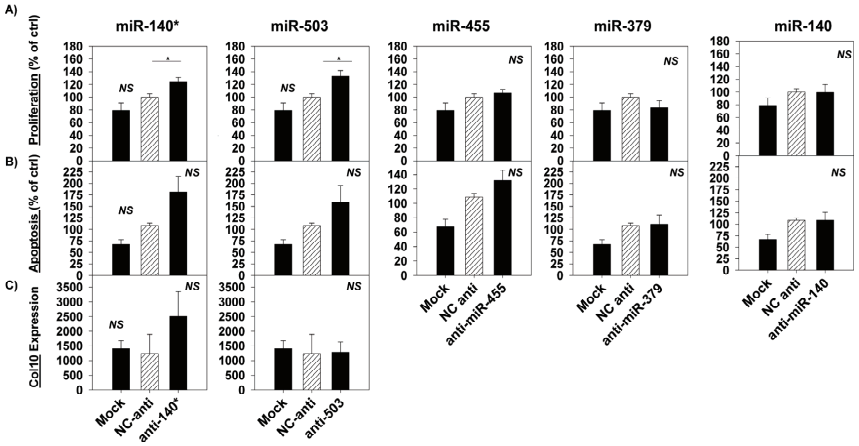

Fig. 19. Loss-of-Function of highly and preferentially expressed miRNAs in growth plate: A) Increase of chondrocyte proliferation 24h after inhibition of miR-140* (*P*=0.011) and miR-503 (*P*=0.012) and other preferentially expressed miRNAs in growth plate chondrocytes. Proliferation was assessed by using BrdU proliferation colorimetric kit (Roche). **B)** Measurement of apoptosis after inhibition of miR-140*, miR-503, miR-455, miR-379 and miR-140. Apoptosis was determined by Cell Death ELISA colorimetric kit (Roche). **C)** Effect of miRNAs in Col10a1 mRNA expression, by inhibition of miR-140* and miR-503. Results are compared to negative control (non-specific miR) of treated cells (n=3 biological replicates from three independent experiments. Results for proliferation and apoptosis are expressed as percent of negative control, and expressed as mRNA relative expression to 18S by real-time PCR for Col10.

Importantly, the identification of miRNAs as potential regulators of chondrocyte differentiation has clinical implications. The control of several genes and pathways important in chondrogenesis makes specific miRNAs attractive therapeutic targets in diseases that affect growth development. Our findings contribute to dissect the role of miRNAs and their predicted target-genes in endochondral ossification. Further functional studies that address the role of individual miRNAs in the regulation of growth plate chondrogenesis are needed.

5 CONCLUSIONS

1. We have found novel biological functions, molecular pathways, transcription factors and markers implicated in the spatially-associated chondrocyte differentiation and temporally-associated senescence of growth plate cartilage, which enlighten the search for novel molecular regulatory mechanisms of postnatal chondrogenesis.

2. We have characterized the spatial and temporal expression pattern of Wnt family and a network of imprinted genes implicated in embryonic growth within postnatal growth plate chondrocytes, suggesting that the identified genes may contribute to the chondrocytes differentiation and the fundamental biological mechanism that causes the linear growth rate to decline with age.

3. We found similar delays in functional, structural and molecular markers of growth plate senescence markers during different growth inhibiting conditions. These findings indicate that growth plate senescence is not simply a function of time '*per se'* but rather of growth, and that delayed senescence may be a general consequence of growth inhibition.

4. We have developed a system for culture and transfection of resting zone chondrocytes, which enabled us to study the role of miRNAs in primary chondrocytes phenotype, simulating more closely *in vivo* conditions.

5. Finally, we identified unique miRNAs that were preferentially expressed and/or age-regulated in growth plate chondrocytes and used bioinformatic approaches to detect signaling pathways that may be developmentally regulated by miRNAs. Functional studies indicate that age-regulated miR-142-3p, miR-125a-3p, and miR-497 positively regulate proliferation and thus act to maintain proliferation during growth plate senescence. Conversely, miR-195 and miR-497 may be important in the control of apoptosis that is increased during growth plate senescence. Our findings contribute to dissect the role of miRNAs and their predicted target genes in the control of endochondral ossification.

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"Amar é querer estar perto, se longe; e mais perto, se perto"

"Love is to wish to be close, if far; and closer, if close"

Vinicius de Morais (Brazilian Poet)

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