The role of PTHrP in chondrocyte differentiation.

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General Introduction

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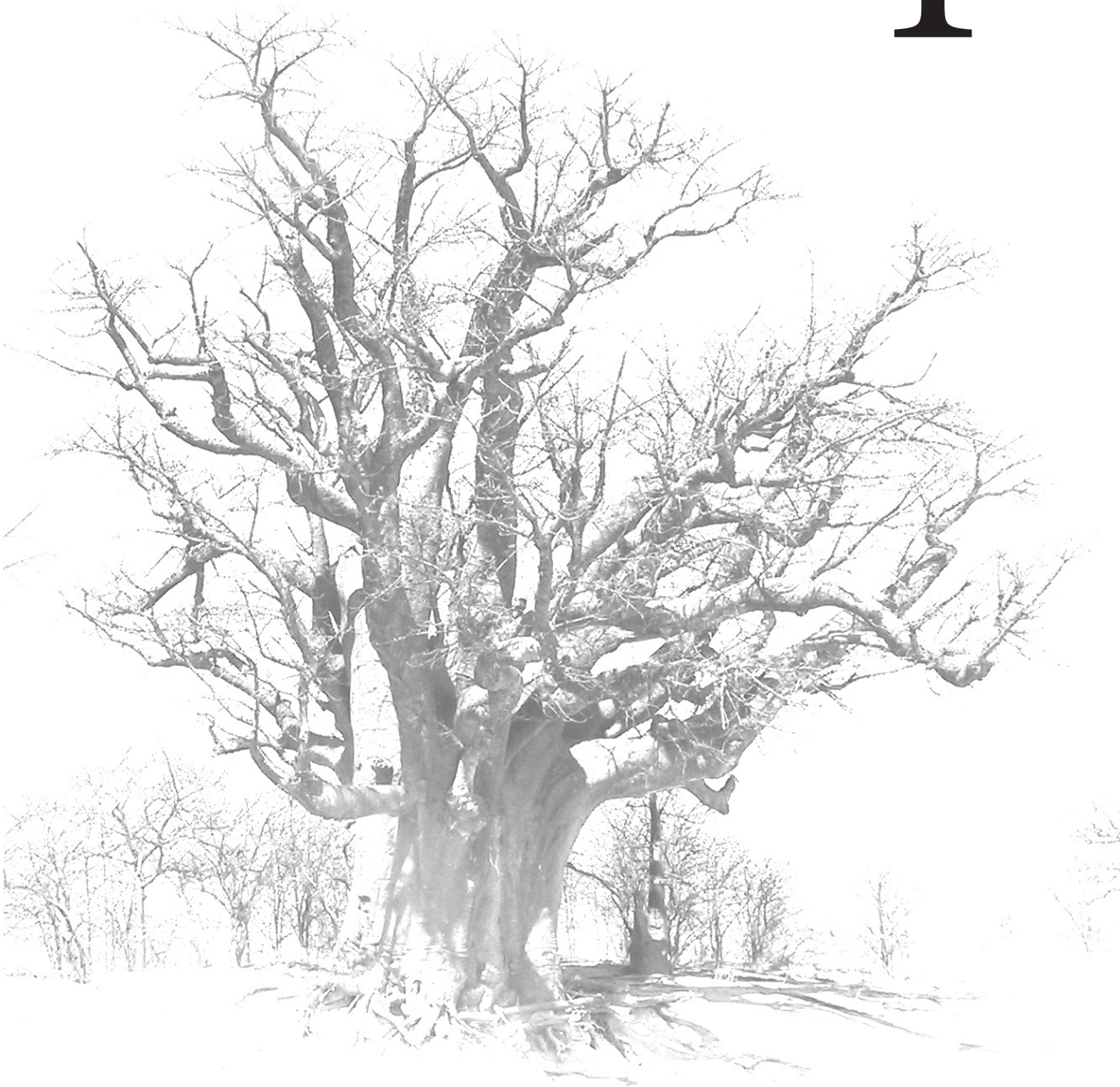

Introduction

Growth is the key characteristic that distinguishes children from adults. Growth of the long bones occurs at the growth plates, which are located at the distal ends of the bones, by a process called endochondral bone formation. This process is governed by a complex network of hormones. The mechanisms by which these hormones affect endochondral bone formation in the growth plate are not completely understood. One of the mechanisms could be by influencing the activity of locally produced growth factors. In this thesis we focus on the role of one of these growth factors, Parathyroid Hormone (PTH) related Peptide (PTHrP) and its receptor, the type I PTH/PTHrP receptor (PTHR1). The main objective of this study is to further elucidate the mechanisms of action of PTHrP and PTHR1 signalling in chondrocyte proliferation and differentiation in the epiphyseal growth plate.

In the following chapter a short overview will be given about the formation of the growth plate and the process of endochondral bone formation. In addition, the involvement in this process of transcription factors is described. Subsequently, the regulation of endocrine and paracrine factors in endochondral bone formation is discussed, followed by a summary about PTHR1 signalling in chondrocytes. Finally, the outline of this thesis will be described.

The growth plate

In the following section the formation and structure of the growth plate is described (fig. 1). During embryogenesis mesenchymal precursor cells reduce in size, become densely packed into condensations and differentiate into chondrocytes[1]. Chondrocytes located at the ends of anlagen will eventually form the cartilage that covers the articular surface of the bone. In contrast, the chondrocytes beneath this zone of resting cells progress through a coordinated program of proliferation, differentiation, maturation and eventually apoptosis. The cartilage matrix is calcified, resorbed, and finally replaced by bone. This process is called endochondral bone formation and takes place in the primary ossification centre (fig. 1A). The primary ossification centre spreads outwards to the ends of the bone. Around birth, a secondary ossification centre is formed in the epiphyseal cartilage at the ends of the bone. The primary and secondary ossification centres remain separated by a layer of cartilage, the epiphyseal growth plate. Within the growth plate, chondrocyte proliferation, hypertrophy and matrix synthesis altogether contribute to longitudinal bone growth. The growth plate can be divided into four anatomic zones, the resting, proliferative, transition, and hypertrophic zone, that go through unique morphological and biochemical stages during the process of endochondral ossification (fig. 1B).

Resting zone

The resting zone is located at the distal end of the growth plate and is characterized by a high ratio of extracellular matrix to cell volume. The resting cells are small, lie as single cells or in clusters, and are in a relatively quiescent state. The cells act as committed progenitor cells that are responsible for the generation of proliferating chondrocytes. This was shown by removal of the growth plate *in vivo* in rabbits, leaving only the resting zone, resulting in a complete regeneration of the growth plate[2]. In addition, the same study showed that the resting zone directs the alignment of chondrocyte columns parallel to the long axis of the bone. Replacing

of the resting zone ectopically alongside the proliferative zone induced a 90-degree shift in the orientation of the nearby proliferating chondrocytes[2]. Furthermore, the resting cells probably produce a morphogen that inhibits hypertrophy of nearby proliferating cells. In this way the resting zone may be partially responsible for the organization of the growth plate into distinct zones of chondrocyte proliferation and differentiation[2].

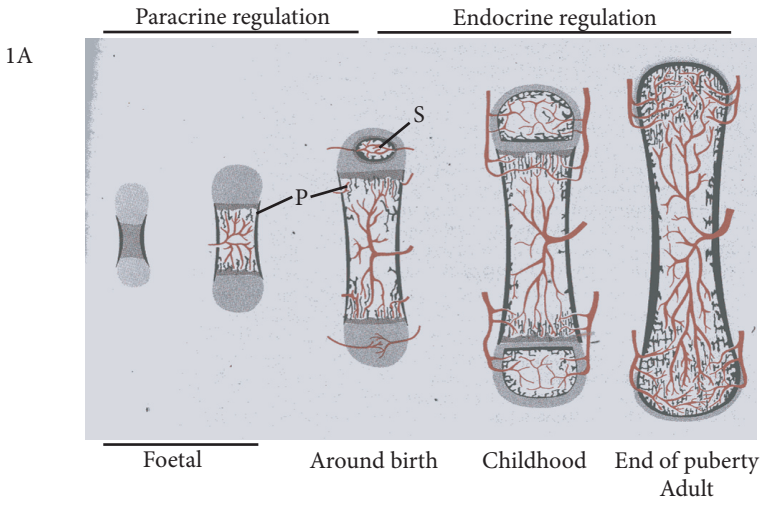

Figure 1. The formation and structure of the growth plate.
A) During foetal development endochondral bone formation takes place in the primary ossification centre (P). Around birth a secondary ossification centre (S) is formed. During childhood longitudinal bone growth increases and the growth plate decreases in size. At the end of puberty growth ceases, due to growth plate fusion. The early development is driven by locally produced growth factors (paracrine regulation). Around birth, when the secondary ossification centre is formed, the regulation switches in favour of endocrine regulation. B) The growth plate is divided into several zones, which are distinguished by biochemical and morphological differences. The resting zone contains stem cell-like chondrocytes. When these chondrocytes start proliferating they enter the proliferative zone. At a certain point, the chondrocyte stop proliferating and start to differentiate in the transition zone. They increase further in size in the hypertrophic zone. Finally, the fully mature chondrocytes go into apoptosis, leaving a scaffold for bone formation. Figure is adapted from the Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, chapter 1, figure 9.

Proliferative zone

Upon an unknown trigger the resting chondrocytes enter into the proliferative zone. The proliferating chondrocytes display flattened shapes, divide, and become organized into longitudinal columns, a characteristic feature of the growth plate. The number of proliferating chondrocytes and thereby the length of the columns contributes to the increase in longitudinal bone growth[3].

Transition zone

After a certain number of cell divisions the chondrocytes stop to divide and start to differentiate into pre-hypertrophic chondrocytes, thereby increasing in size. Both late-proliferative and pre-hypertrophic chondrocytes belong to the transition zone, also referred to as the pre-hypertrophic zone.

Hypertrophic zone

The pre-hypertrophic cells further increase in size to an enlargement of five to ten fold, which contributes to the increase of longitudinal bone growth[3]. The longitudinal septa of cartilage matrix between the columns of hypertrophic chondrocytes eventually become calcified. The terminal differentiated chondrocytes go into apoptosis, leaving a scaffold for bone formation[4]. Together with the invasion of blood vessels from the underlying primary spongiosum, osteoclasts enter and resorb the calcified cartilage matrix. At the same time, osteoblasts enter into the area and produce new metaphyseal trabecular bone.

Extracellular matrix

Within the zones of the growth plate the chondrocytes are embedded in an extracellular matrix, consisting predominantly of collagens and proteoglycans as well as other non-collageneous proteins, most of which are glycoproteins and phosphoproteins (reviewed in[5]). The primary collagen in the growth plate is collagen type 2, which represents 80 to 90% of the total collagen content and is predominantly expressed in the proliferative zone. Other collagens present in relatively small amounts in the cartilage matrix are collagen type 9, type 10 and type 11. Collagen type 9 and type 10 are specifically expressed by pre-hypertrophic and hypertrophic chondrocytes, respectively.

The other major cartilage matrix molecules, the proteoglycans, consist of a core protein to which glycosaminoglycans (GAG) side chains are attached[5]. The GAG group in cartilage consists of chondroitin sulphate, dermatan sulphate, heparan sulphate, keratan sulphate, and hyaluronic acid. Five specific proteoglycans in cartilage are formed with the combinations of the different GAGs: aggrecan, decorin, biglycan, fibromodulin, and collagen type 9. The largest proteoglycan in size and most abundant by weight is aggrecan, a proteoglycan that possesses over 100 chondroitin sulfate and keratan sulfate chains[6]. Together with collagen type 2, aggrecan makes up 90% of the organic matrix.

Degradation and remodelling of the cartilage matrix during endochondral bone formation is regulated by a group of remodelling enzymes, known as matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) (reviewed in[7]). For instance, MMP13, expressed by hypertrophic chondrocytes, degrades preferentially collagen type 2[8]. Furthermore, knockout

models emphasized the important role of MMPs in endochondral bone formation[8-10].

Transcriptional control of growth plate formation and endochondral bone formation

A complete overview of the transcriptional control of growth plate formation and chondrocyte development in the growth plate has recently been reviewed[11]. Here, the actions of members of the Sox family and the runt related transcription factor (Runx) family on growth plate formation and chondrocyte development are discussed in more detail.

The master transcription factor for chondrocyte development is Sox9, a member of the high-mobility-group (HMG)-box DNA-binding domain containing proteins. In the growth plate of long bones, Sox9 is expressed by proliferating chondrocytes, but its expression is completely shut off in hypertrophic chondrocytes. Its essential role in successive steps of the chondrocyte developmental pathway has been emphasized by several approaches in transgenic mouse, including gain as well as loss of function studies. These studies demonstrated that Sox9 positively regulates chondrocyte proliferation and negatively regulates chondrocyte hypertrophy[12-14]. Inactivation of Sox9 in limb buds before mesenchymal condensations in the mouse embryo results in a complete absence of both cartilage and bone[12]. In addition, inactivation of Sox9 after mesenchymal condensation in the mouse embryo results in arrest of condensed mesenchymal cells[12]. In human, heterozygous missense mutations in the Sox9 gene cause campomelic dysplasia, a rare disorder of skeletal development that results in deformities of most of the bones of the body[15]. These malformations are comparable in mice with a haploinsufficiency of the Sox9 gene[14]. Taken together, these data suggest that Sox9 is the first transcription factor that is essential for chondrocyte development and cartilage formation.

Two other members of the Sox family, Sox5 and Sox6, are required for chondrocyte development as well. Both transcription factors are co-expressed with Sox9 during chondrogenesis. In addition, they are absent in limb buds with Sox9 inactivation, indicating that Sox9 is needed for Sox5 and Sox6 expression[12]. Furthermore, like the expression of Sox9, the expression of Sox5 and Sox6 is excluded from hypertrophic chondrocytes. Although individual Sox5 and Sox6 knockout mice are born with mild skeletal abnormalities, double knockouts develop a severe, generalized chondrodysplasia, characterized by a virtual absence of cartilage, due to a defect in chondrocyte proliferation and impairment of cartilage matrix production[16]. These data suggest that, similar to Sox9, Sox5 and Sox6 regulate sequential steps of chondrocyte development in the growth plate.

Other transcription factors regulating chondrogenesis are members of the family of the runt related transcription factors, Runx2 and Runx3. Both factors are expressed by pre-hypertrophic and hypertrophic chondrocytes[17;18]. Beside a transcription factor in chondrocyte hypertrophy, Runx2 is the key transcription factor in osteoblast differentiation. Runx2 knockout mice show a complete arrest in osteoblast differentiation resulting in a total absence of skeletal ossification[19;20]. Homozygous Runx2 mutants and Runx3 mutants as well, show disturbed chondrocyte maturation[17;18]. Analysis of Runx2 and Runx3 double knockout mice demonstrated a complete lack of pre-hypertrophic and hypertrophic chondrocytes[17]. This suggests that Runx2 and Runx3 play an essential role in terminal chondrocyte differentiation.

Furthermore, they partly compensate each other's function in chondrogenesis.
Taken together, 2 families of transcription factors are most important in chondrogenesis. The Sox family of transcription factors regulates the formation of the growth plate and is involved in chondrocyte proliferation, whereas the Runt related family of transcription factors controls chondrocyte hypertrophy.

Endocrine regulation of endochondral bone formation

In contrast to foetal bone growth, which is predominantly regulated by locally produced growth factors and relatively independent of systemic hormones, postnatal bone growth is tightly regulated by systemic hormones (fig 1A), for instance Growth Hormone (GH), Insulin-like Growth Factor-1 (IGF-1), glucocorticoid, Thyroid Hormone, estrogen, androgen, vitamin D, and leptin (reviewed in[21]). From these hormones, GH is the dominant regulator. In addition, estrogen plays a crucial role during puberty. Therefore, the influence of these hormones in endochondral bone formation is discussed in more detail.

Growth hormone and Insulin-like Growth Factor-1

GH is the most essential modulator of longitudinal bone growth after birth, whereas IGF-1 is important in the prenatal growth plate as well. Both are potent stimulators of endochondral bone formation, as shown by several human knockouts. For instance, GH insensitivity due to GH receptor mutations or defects in the GH receptor signalling pathway impairs postnatal growth[22-24]. Severe postnatal growth retardation and delayed bone development are also found in GH knock out mice[25;26].
Homozygous mutations in the human IGF-1 gene or heterozygous mutations in the human IGF-1 receptor gene cause impaired growth in both the pre- and postnatal situation[27-29]. In mice with IGF-1 deficiency, severe pre- and postnatal growth retardation is observed as well[30]. A more severe phenotype is displayed by IGF-1 receptor knockout mice and these mice die early postnatally[30].
Many of GH's actions on the growth plate are likely to be mediated through IGF-1[31]. Systemic IGF-1, as well as locally produced IGF-1, contributes to longitudinal bone growth. However, locally produced IGF-1 has been argued to be of greater importance in the regulation of chondrocyte development than systemic levels of IGF-1[32]. The so-called *dual effector hypothesis* states that GH acts locally on the growth plate to recruit resting chondrocytes into a proliferative state and thereby inducing the local production of IGF-1, which then stimulates proliferation of proliferating chondrocytes[31;33]. The induction of IGF-1 after GH stimulation is mediated via the activation of signal transducer and activator of transcription 5b (Stat5b). Mutations in the Stat5b gene cause GH insensitivity and failure of IGF-1 production[23;24]. Also other members of the Stat family, as Stat1, Stat3 and Stat5a, play a role in GH actions, but cannot compensate for lack of Stat5b (reviewed in[34;35]).

Estrogen

Estrogen is especially important in endochondral bone formation during puberty. The identification of an inactivating mutation in the estrogen receptor alpha (ERα) of a male patient revealed crucial insight in the role of estrogen during growth[36]. This patient lacked

a growth spurt and growth plate fusion did not occur at the end of puberty. An almost identical phenotype was found in two male patients with a mutation in the gene coding for p450 aromatase, which is responsible for the conversion of androgen into estrogen[37;38]. It is believed that low levels of estrogen initiate the pubertal growth spurt and high levels of estrogen causes growth plate fusion at the end of puberty[39]. However, the mechanism of estrogen acting on growth plate chondrocytes remains largely unknown.
Thus, GH regulates the expression of IGF-1 in the growth plate. It is likely, that GH also controls the expression of other locally produced growth factors, for instance components of the growth restraining Indian Hedgehog (IHh)/PTHrP feedback loop (discussed later). Since estrogen receptors are expressed in zones of the growth plate that also express components of the IHh/PTHrP feedback loop and since it has been shown that estrogen affects the expression of PTHrP and its receptor in the rat uterus, it seems possible that the expression of these genes may be regulated by estrogen[40;41].

Paracrine regulation of endochondral bone formation

Endochondral bone formation is not only regulated by endocrine factors, but is also controlled by locally produced growth factors. It is believed that prenatal growth is predominantly regulated by these locally produced growth factors and that it is relatively independent from systemic hormones (fig. 1A). Of the paracrine mechanisms, the IHh/PTHrP negative feedback loop is best studied. Beside this feedback loop other growth factors, like IGFs (discussed in the previous section), Fibroblast Growth Factors (FGFs), Bone Morphogenetic Proteins (BMPs), and members of the Wnt-family play important roles in chondrocyte proliferation and differentiation. Part of these growth factors interact with the IHh/PTHrP negative feedback loop, but can act independently of the feedback loop as well. First, PTHrP and the IHh/PTHrP negative feedback loop will be discussed, which will be followed by a short overview of the actions of other growth factors.

Parathyroid Hormone related Peptide

PTHrP was originally identified as the causative factor for Humoral Hypercalcemia of Malignancy[42]. It shares significant homology with the calcium regulating Parathyroid Hormone (PTH). PTHrP and PTH act through a common receptor, the type 1 PTH/PTHrP receptor (PTHR1). PTHR1 signalling is involved in various phases of embryonic development, such as in the formation of the extra-embryonic endoderm of the parietal and visceral yolk sac, in skin and mammary duct development, in calcium homeostasis and in formation of the skeleton, including osteogenesis and chondrogenesis[43-45].
The crucial role for PTHrP in endochondral bone formation is underlined by a number of studies. Four human conditions exist, in which PTHR1 signalling is disturbed. Two arise from dominant mutations and two arise from recessive mutations in the PTHR1 gene. Dominant mutations have been found in Jansen's type metaphyseal chondrodysplasia (JMC) and in enchondromatosis. Recessive mutations have been described in Eiken syndrome and Blomstrand lethal osteochondrodysplasia (BOCD)[46-54]. BOCD is a lethal skeletal dysplasia and is characterized by skeletal malformations, leading to dwarfism[55-61]. Patients with JMC and Eiken syndrome are viable and these diseases are also characterized by skeletal

malformations resulting in impaired growth[62;63]. Ollier disease is one of the best known enchondromatosis syndromes and is characterized by multiple enchondromas, which are rare benign neoplasms[60;64].
In JMC, four heterozygous mutations have been identified, causing a constitutively activated PTHR1, resulting in decelerated chondrocyte differentiation, finally leading to dwarfism[47;49;54;65]. In addition, a heterozygous mutation has been identified in 2 out of 6 patients with enchondromatosis in one study[53]. The mutation is thought to result in upregulation of the IHh/PTHrP pathway. However, in another study with a larger panel of patients, no mutations were identified in the PTHR1 gene, indicating that the PTHR1 gene is not the main culprit for enchondromatosis[66].
The homozygous mutation identified in a unique family with Eiken syndrome results in a truncated PTHR1, causing abnormal PTHR1 signalling and retarded ossification[48]. A similar phenotype has been identified in mice with a mutation in the C-terminal part of the PTHR1 gene, which is responsible for the activation of the phospholipase C beta (PLCβ) / protein kinase C (PKC) signalling pathway[67]. Finally, BOCD is a lethal skeletal dysplasia, which is caused by an inactivating mutation in the PTHR1 gene, resulting in accelerated chondrocyte maturation[46;50-52]. In comparison with BOCD, striking similarities are found in PTHR1 knockout mice. These mice die around birth and show accelerated chondrocyte maturation[43].
A similar, although less severe, phenotype, perhaps because of the effects of maternal PTHrP or maternal or foetal PTH, is found in mice with homozygous ablation of the PTHrP gene. This also results in accelerated chondrocyte differentiation leading to dwarfism[68]. In addition, ectopic expression of PTHrP causes a delay of chondrocytes differentiation, leading to a smaller cartilaginous skeleton[69]. Until today, no humans lacking PTHrP production have been identified.
Taken together, these data underline the essential role of PTHrP in chondrocyte proliferation and differentiation. An excess of PTHrP, but also a deficiency of PTHrP result in growth plate abnormalities. Therefore, PTHrP expression must be tightly controlled. The protein regulating PTHrP expressing in the growth plate is IHh, together they form the IHh/PTHrP negative feedback loop.

IHh/PTHrP negative feedback loop

The pace of chondrocyte differentiation is regulated by a locally acting growth restraining feedback loop (fig. 2), consisting of IHh and PTHrP, which was first described in 1996, by studying bone explants of PTHrP and PTHR1 knockout mice[43;70]. In the mouse embryonic growth plate pre-hypertrophic chondrocytes express IHh. The IHh signal acts on the perichondrium adjacent to the transition zone where it binds to its receptor complex, consisting of the membrane protein patched 1 (Ptch) and smoothened (Smo), and induces the expression of PTHrP in perichondrial cells and in round chondrocytes at the ends of the bones[71]. PTHrP then binds to its receptor on the late-proliferating and pre-hypertrophic chondrocytes, preventing the transition from proliferating into hypertrophic chondrocytes, and thereby decreasing IHh expression. Further studies revealed that chondrocytes express PTHR1 before differentiating into IHh expressing cell type[70]. The differentiation block induced by IHh appears to occur upstream of IHh expression, which is precisely the target cell type for the IHh/PTHrP negative feedback loop. Taken together, the IHh\PTHrP negative feedback

loop controls the transition of proliferating chondrocytes into hypertrophic chondrocytes.
For many years it has been uncertain how IHh signal reaches the region in which it induces PTHrP expression. Initially it was thought that secondary factors, like Ext, BMPs or TGFβ, mediate the IHh signal. However, recent observations strongly support a model in which IHh acts as a long-range morphogen, directly inducing the expression of PTHrP[72]. Recently, it has been shown that IHh induces PTHrP expression via alleviating the repression of the transcription factor Gli3[73].
After the identification of the IHh/PTHrP negative feedback loop, other growth factors, like BMPs and FGFs, have been implicated as interactors of this feedback loop (fig. 2). IHh induces BMP expression in the perichondrium and in proliferating chondrocytes[74]. In addition, BMP signalling has been shown to regulate chondrocyte proliferation in parallel to IHh. Furthermore, BMPs induce IHh expression in cells that are released from the range of the PTHrP signal and BMPs delay the differentiation of terminal hypertrophic chondrocytes[74]. Another study showed that FGF signalling seems to regulate the same phases of chondrocyte development as BMP signalling, however, with opposite effects[75]. Therefore, it has been stated that BMPs and FGFs act in independent pathways having antagonistic effects on chondrocyte proliferation, IHh expression and chondrocyte hypertrophy[74;75]. By simultaneously regulating proliferation, IHh expression, and terminal differentiation of chondrocytes, the balance of FGF and BMP signalling seem to adjust the process of hypertrophic differentiation to the proliferation rate.
Based on the expression of Ptch, it is believed that in the earlier stages of endochondral bone

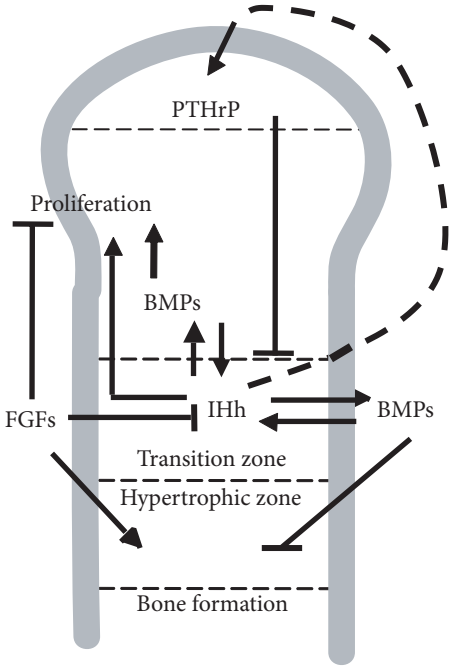

Figure 2. Interactions between growth factors with the IHh/PTHrP negative feedback loop in the embryonic growth plate. For details, see text.

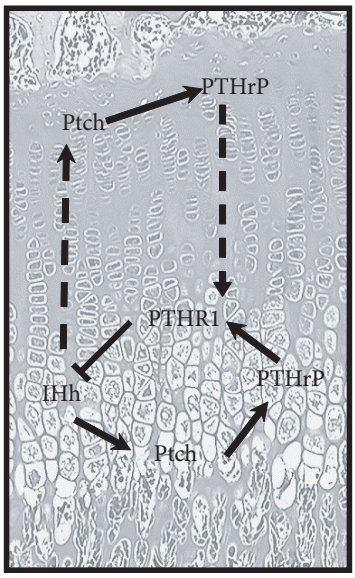

Figure 3. The IHh/PTHrP negative feedback loop in the postnatal growth plate.
For details, see text.

formation the IHh/PTHrP negative feedback loop requires the presence of the perichondrium. During later stages, Ptch is expressed in chondrocytes themselves as well. Thus, in these stages no intermediate perichondrium is required. This is further emphasized in the postnatal growth plate, in which the IHh/PTHrP feedback loop is confined to the growth plate itself (fig. 3). Evidence for this hypothesis is found in the rat growth plate, where all members of the feedback loop are expressed in the transition zone, as well as in the stem cell zone[76]. These data suggest the existence of a second PTHrP/IHh feedback loop in the postnatal growth plate. Beside the PTHrP/IHh negative feedback loop in the transition zone, another loop may be confined to the stem cell zone. It seems feasible that cross talks may occur between the two loops.

Indian Hegdehog

IHh is one of the key regulators of endochondral bone formation controlling at least three different steps. First, IHh regulates the onset of chondrocyte maturation by controlling the expression of PTHrP, which is described above. Second, IHh regulates chondrocyte proliferation by inducing proliferation of resting chondrocytes, which is independent from PTHrP[71;77]. Third, IHh expression is essential for osteoblast development[77]. This essential stimulatory role of IHh in endochondral bone formation is underscored by abnormalities arising from mutation in the mouse and human IHh gene and Gli genes, the downstream mediators of the Hh pathway[77-84]. For instance, homozygous mutations in the IHh gene in mice result in severely reduced chondrocyte proliferation and accelerated chondrocyte differentiation[77;85]. In addition, no bone collar is formed and no cortical and trabecular bone are detected in IHh mutants, indicating the absence of mature osteoblasts[77].

In the growth plate IHh is predominantly produced by pre-hypertrophic and hypertrophic chondrocytes. It binds to its receptor, Ptch, which suppresses the activity of Smo in the absence of IHh. Upon binding to Ptch, the inhibition on Smo is alleviated, resulting in activation of the IHh signalling pathway. Furthermore, IHh signalling is in vertebrates mediated through transcription regulation by the zinc-finger transcription factors, Gli1, Gli2 and Gli3.

Fibroblast Growth Factors

The first functional link between FGF signalling and chondrocyte development was identified with the discovery that achondrodysplasia (ACH), the most common form of skeletal dwarfism in humans, was caused by a missense mutation in FGF receptor 3 (FGFR3)[86]. This mutation resulted in constitutively activation of the FGFR3. Following this initial discovery hypochondrodysplasia (HCH), a milder form of dwarfism, and thanatophoric dysplasia (TD), a more severe form of dwarfism, were also found to result from mutations in FGFR3[87;88]. Beside the chondrodysplasia syndromes, many other human skeletal dysplasias have been attributed to mutations in the three different FGF receptors, FGFR1, 2 and 3[89-91].

FGFR3 and FGFR1 are expressed by proliferating chondrocytes. Pre-hypertrophic chondrocytes express FGFR3 and hypertrophic chondrocytes express FGFR1[92]. FGFR1 and FGFR2 are both expressed by osteoblasts in the underlying trabeculae[92]. Several FGFs are involved in chondrocyte differentiation, of which the role of FGF18, expressed in the perichondrium, is most clear. The phenotype of FGF18 knockout mice resembles that of FGFR3 knockout mice, indicating that FGFR3 acts as a receptor to FGF18 in chondrocyte differentiation[93].

Two pathways are activated by FGFR signalling, the signal transducer and activator of transcription (STAT) pathway and the extracellular signal regulated kinase (ERK) pathway[94;95]. The strong inhibition of chondrocyte proliferation and to a lesser extent the inhibition of chondrocyte differentiation is mediated via Stat1 and probably via other members of the Stat family of transcription factors. Activation of Stat1 leads to the upregulation of the cell cycle inhibitor $p21^{waf1/cip1}$, thereby inhibiting chondrocyte proliferation[75;96;97]. It has been postulated that the balance between ERK and Stat signalling after FGFR activation regulates the effect of FGF in chondrogenesis[98].

Bone Morphogenetic Proteins

The Bone Morphogenetic Proteins are a group of at least 15 proteins and are part of the TGFβ superfamily. They were originally identified as inducers of ectopic bone formation[99]. Nowadays, BMPs are recognized as important regulators of the development of a variety of tissues[100]. Several BMPs and their receptors are expressed in the perichondrium and by chondrocytes in different zones of the growth plate[74;101;102]. They regulate several aspects of chondrocyte development, like inducing chondrogenesis by promoting cell-cell interactions[103]. Furthermore, continuous BMP signalling is required for chondrogenesis by maintaining Sox9 expression[104;105]. In addition, they promote chondrocyte proliferation[74;106-108] and terminal chondrocyte differentiation[107;109-112]. By binding to their cell surface receptors, BMPs activate SMAD proteins, which transmit the signal from the membrane to the nucleus.

Wnt-family

The Wnt-signalling pathway is a complex network of signalling molecules, receptors and downstream mediators, which has been described in detail elsewhere[113;114]. In short, after binding of Wnt to its receptor, Frizzled, and its co-receptor, low-density lipoprotein receptor-related proteins 5 (LRP5) and LRP6, the β-catenin pathway (canonical pathway) and the calcium pathway (non-canonical pathway) are activated. Activation of the canonical pathway leads to the release of β-catenin from a complex of proteins, including Adenomatous Polyposis Coli (APC), Glycogen Synthase Kinase 3β (GSK3β), and Axin. Stabilized β-catenin translocates to the nucleus where it forms a complex with transcription factors of the T-cell factor (TCF) / Leukocyte Enhancer Factor (LEF) family to activate transcription of target genes. Signalling via the non-canonical pathway results in activation of PLCβ and PKC (discussed later).
Wnt signalling is involved in all stages of chondrocyte development (reviewed in[115]). Activation of the canonical pathway prevents the differentiation of progenitor cells into chondrocytes, it inhibits chondrocyte proliferation, but it induces the differentiation of progenitor cells into osteoblasts[13;116]. Furthermore, the canonical pathway is not active in differentiated chondrocytes *in vitro*. Therefore it has been hypothesized that the non-canonical pathway is the predominant pathway active during chondrocyte differentiation[115;117]. Wnt5a and Wnt5b have been shown to control the pace of transition between different growth plate zones independently of the IHh/PTHrP negative feedback loop[118]. Whether other Wnts influence chondrocyte proliferation and differentiation in association with the IHh/PTHrP negative feedback loop is unclear.

Neoplastic growth

Chondrocyte proliferation and differentiation in the normal growth plate is tightly regulated by several growth factors. Growth disorders, like various chondrodysplasias, are caused by disturbed signalling of these growth factors as described in the previous section. In addition, altered growth factor signalling is also considered to be the cause in neoplastic growth, like in chondrosarcomas and enchondromas. The parallels of growth factor signalling between chondrocyte proliferation and differentiation in the normal growth plate and in tumours have become obvious by the identification of various growth factors and their signalling pathways in normal as well as in neoplastic growth, like PTHrP and FGF and mediators of their signalling pathways[119-121]. The elucidation of cartilaginous tumorigenesis requires understanding of the normal regulation of chondrocyte proliferation and differentiation. Vice versa, investigation of cartilaginous tumours could also provide insights into the biology of normal growth plate development[122]. Since cartilaginous tumorigenesis is not the main subject of this thesis, it will not further be discussed.

Species differences

Many animal models are used to study the modulation and modification of endocrine and paracrine factors and the actions of these factors in several processes during development. The most studied organism in genetic and developmental biology is the *drosophila melanogaster*, for instance the actions of the Hh-, FGF-, TGFβ- and Wnt-signalling have extensively been studied in this model[123-126]. Similarities, but also some differences have been identified

between the actions of these factors in invertebrate and vertebrate models[127;128]. To study the role of endocrine and paracrine factors in the regulation of chondrocyte proliferation and differentiation in the growth plate, many mouse models for these factors have been developed. Among them are mouse models to study the actions of PTHrP in chondrocyte proliferation and differentiation as described earlier in this chapter. Whereas in humans at the beginning of puberty an obvious growth spurt occurs and at the end of puberty growth plate fusion, mice do not clearly demonstrate these sexually maturating phenomena. Rabbits are a useful model for studying the actions of endocrine and paracrine factors in chondrocyte proliferation and differentiation in the growth plate, because rabbits demonstrate growth plate closure[129;130]. Practical limitations, however, makes this specie less suitable.

PTHR1 signalling

The main topic of this thesis is the actions of PTHrP in chondrogenesis. Therefore, the characteristics of its receptor, the PTHR1, and the most important PTHR1 signalling pathways in chondrogenesis will be described in this section. The PTHR1 belongs to a distinct group of G protein-coupled receptors termed family B[131]. The typical structure of these receptors is characterized by a relatively long extracellular N-terminus (approximately 160 amino acids), a seven membrane-spanning domain, and an intracellular C-terminus. About 45 amino acid residues, which are distributed throughout the transmembrane domains and in the N-terminal extracellular domain, are conserved in all members of this receptor family, and are likely to have important functions in ligand binding, signal transduction, or both. The human PTHR1 consists of 593 amino acids, including a signal peptide of 25 amino acids, and is encoded by 14 exons (fig. 4)[131]. It binds PTH and PTHrP with equal affinity.

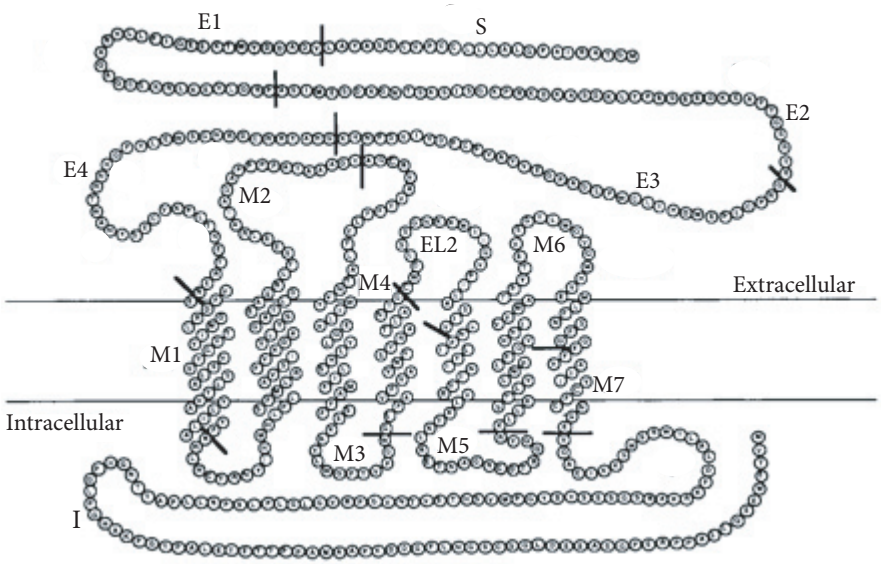

Figure 4. Schematic representation of the structure of the PTHR1.
The PTHR1 consists of 593 amino acids and is encoded by 14 exons. S = signal peptide. E1-4 = extra cellular domain. M1-7 transmembrane domains. EL2: Extracellular domain. I = intracellular domain(109).

Upon binding of PTHrP or PTH, the receptor can activate adenylate cyclase (AC) through Gαs (fig. 5)[132]. Subsequently, cyclic adenosine monophospate (cAMP) is produced by AC and adenosine triphosphate (ATP). cAMP binds then to the regulatory (R) subunits of the inactive protein kinase A (PKA), thereby releasing the catalytic (C) subunits. The free catalytic subunits of PKA can phosphorylate serine and threonine residues of transcription factors.
The PTHR1 can also activate the PLCβ/PKC pathway via Gαq, although this signalling response is generally not as sensitive as the AC/PKA pathway (fig. 4). Activated Gαq stimulates PLCβ, which in turn cleaves phosphoinositol-4,5-biphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Subsequently, IP3 leaves the plasma membrane and diffuses rapidly through the cytosol, to release Ca(2+) from the endoplasmatic reticulum and DAG activates PKC.
While the AC/PKA pathway after PTHR1 signalling is the dominant pathway in chondrogenesis, cells have a mechanism to redirect the PTHR1 signalling to the PLCβ\PKC pathway. This is achieved by direct binding of a scaffold protein, the Na+/H+ exchanger regulatory factor (Nherf), to the PTHR1, at least in kidney cells[133;134]. Two Nherf proteins have been identified, Nherf1 and Nherf2. Binding of Nherf to the PTHR1 switches PTHR1 signalling from Gαs to Gαq, through a psd95, discs large protein, ZO1 (PDZ) domain interaction and by binding of Nherf through another PDZ domain interaction to PLCβ[133;134]. Nherf proteins have also been identified to play a role in the regulation of sodium-hydrogen exchange, and phosphate and calcium transport in kidney cells[135-137].
Recently, chondrocyte specific knockout mice were generated, carrying a mutation in the Gαs[138]. These mice displayed a phenotype comparable to the PTHrP knockout mice, like severe growth plate defects with shortening of the proliferative zone and accelerated chondrocyte differentiation[68]. The opposite was found in mice carrying a mutant form of the PTHR1, which specifically interrupts signalling via the Gαq and signals normally via the Gαs[67]. These mice showed an increase in chondrocyte proliferation and a delay in chondrocyte maturation. Taken together, this indicates that the two pathways have opposite effects on chondrocyte proliferation and differentiation. In addition, these results show that Gαs activation negatively regulates chondrocyte differentiation and that the critical signalling pathway of PTHR1 in growth plate chondrocytes is the AC/PKA pathway.
Known targets of PKA after PTHR1 activation are the transcription factors cAMP responsive element binding protein (CREB) and AP-1, which is a complex formed through interactions between Fos and Jun family members (fig. 5)[132]. CREB is rapidly activated via PKA and it subsequently activates or inhibits the transcription of PTHrP target genes[132;139-142]. One of these targets is c-Fos, thereby enhancing the AP-1 signal[132;142]. The direct activation of CREB and the activation of AP-1 thereafter were established by using dominant negative CREB and dominant negative c-Fos[132]. Since transgenic mice with a dominant negative CREB display a different phenotype compared to PTHrP knockout mice, it is likely that not all the effects of PTHR1 signalling in chondrogenesis are mediated via CREB. PTHR1 signalling probably results in activation of other transcription factors as well. One of these candidates could be the master transcription factor for chondrocyte development, Sox9, which is phosporylated by PKA upon PTHR1 activation[143]. Since Sox9 stimulates chondrocyte proliferation and delays chondrocyte differentiation, Sox9 phosphorylation probably contributes to the actions of PTHrP.

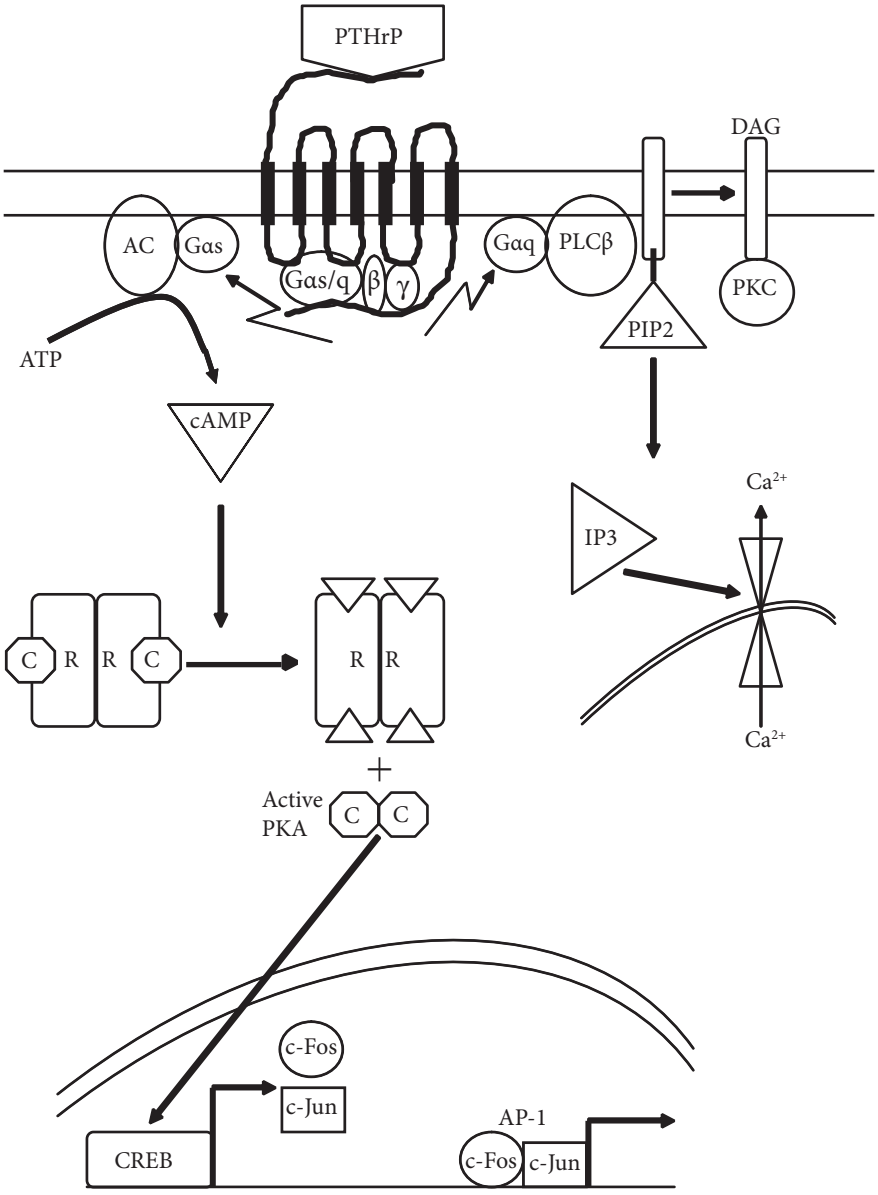

Figure 5. Schematic representation of the PTHR1 signalling pathway.
Upon ligand binding Gαs activates AC, which in turn generates cAMP from ATP. cAMP releases the catalytic subunits (C) from inactive PKA. These catalytic subunits stimulate CREB, which is required for the transcription of c-Fos and c-Jun. These proteins form a complex, AP-1, which activates the transcription of more PTHrP target genes. PLCβ is activated via Gαq and it cleaves PIP2 into DAG and IP3. Subsequently, DAG stimulates PKC and IP3 releases Ca2+ ions from the endoplasmatic reticulum (ER). The combination of the activation of these pathways results in genomic responses.

Upon PTHrP binding, PTHR1 signalling leads to the induction or suppression of mRNA expression of PTHrP target genes, together regulating the biological response to PTHrP. For instance, it has been shown that p57, a member of the CIP/KIP family of inhibitors of cyclin-dependent kinases, is a target gene of PTHrP[144]. Knockout studies with p57 null mice and studies with mice missing both the PTHrP and the p57 gene indicated that suppression of p57 expression is a major mechanism used by PTHrP to maintain chondrocyte proliferation and delay chondrocyte differentiation[144;145]. In addition, PTHrP inhibits the synthesis of the transcription factor Runx2 in chondrocytes[141]. Since Runx2 is a stimulator of chondrocyte differentiation, the suppression of Runx2 mRNA production by PTHrP probably contributes to the delayed differentiation of chondrocytes.

In vitro studies

In this study two *in vitro* models representing endochondral bone formation were used, namely the mouse pre-chondrogenic ATDC5 cell line and the mouse mesenchymal KS483 cell line. ATDC cells are derived from a differentiating culture of the AT805 teratocarcinoma cells[146]. During monolayer culture the cells reproducibly differentiate into chondrocytes in four weeks and they start to produce chondrocyte markers, like collagen type 2, type 9 and type 10[147]. In addition, ATDC5 cells express the PTHR1, however the responsiveness during the first week of culture is low. During differentiation the responsiveness to PTHrP increases[148]. Furthermore, in agreement with *in vivo* studies PTHrP inhibits hypertrophic chondrocyte differentiation[109].

The mesenchymal stem cell line KS483 is a subclone of the KS4 cell line, which is derived from mouse calvariae[149]. Depending on the right culture conditions, the KS483 cells can differentiate into mineralizing osteoblasts, lipid droplets containing adipocytes and into chondrocytes depositing a cartilaginous matrix[150]. The PTHR1 becomes expressed during KS483 osteoblast differentiation. In addition, PTHrP treatment results in inhibition of early and late osteoblast differentiation markers, which is in agreement with *in vivo* studies[151].

Aim and outline of this thesis

The regulation of the development from early chondrocytes into mature chondrocytes has not been fully understood yet. For a complete understanding of the complex regulation of this process, more information is needed on the actions and interactions of endocrine and paracrine regulators. Since PTHrP and its receptor, PTHR1, are key regulators of chondrocyte differentiation, we have focused on the actions of PTHR1 signalling during endochondral bone formation. Therefore, in this thesis the consequences of disturbed PTHR1 signalling were investigated. In addition, to study how PTHrP exert its effect on chondrocyte proliferation and differentiation, *in vitro* models representing endochondral bone formation were used.

In **chapter 2** the underlying causative factors for the heterogeneity in the clinical presentation of BOCD is addressed. The aim of **chapter 3** was first to investigate whether Nherf1 and Nherf2 are expressed during endochondral bone formation, second to elucidate whether Nherf1 or Nherf2 overexpression affects chondrocyte and osteoblast differentiation, and third whether Nherf1 or Nherf2 overexpression influence the effect of PTHR1 signalling in chondrocyte and osteoblasts differentiation. To identify PTHrP early and late response

genes in chondrocytes and to recognize interactions with other regulatory factors, we used the ATDC5 cell line and performed microarray analysis in **chapters 4 and 5.** To calculate p-values and confidence bands in data derived from qPCR analysis, when using many samples, we developed the Double Delta Model (DDM), described in **Chapter 6.** Finally, general conclusions and discussions are described in **chapter 7**.

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