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The role of PTHrP in chondrocyte differentiation

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Nu schijnt ze nog vaag
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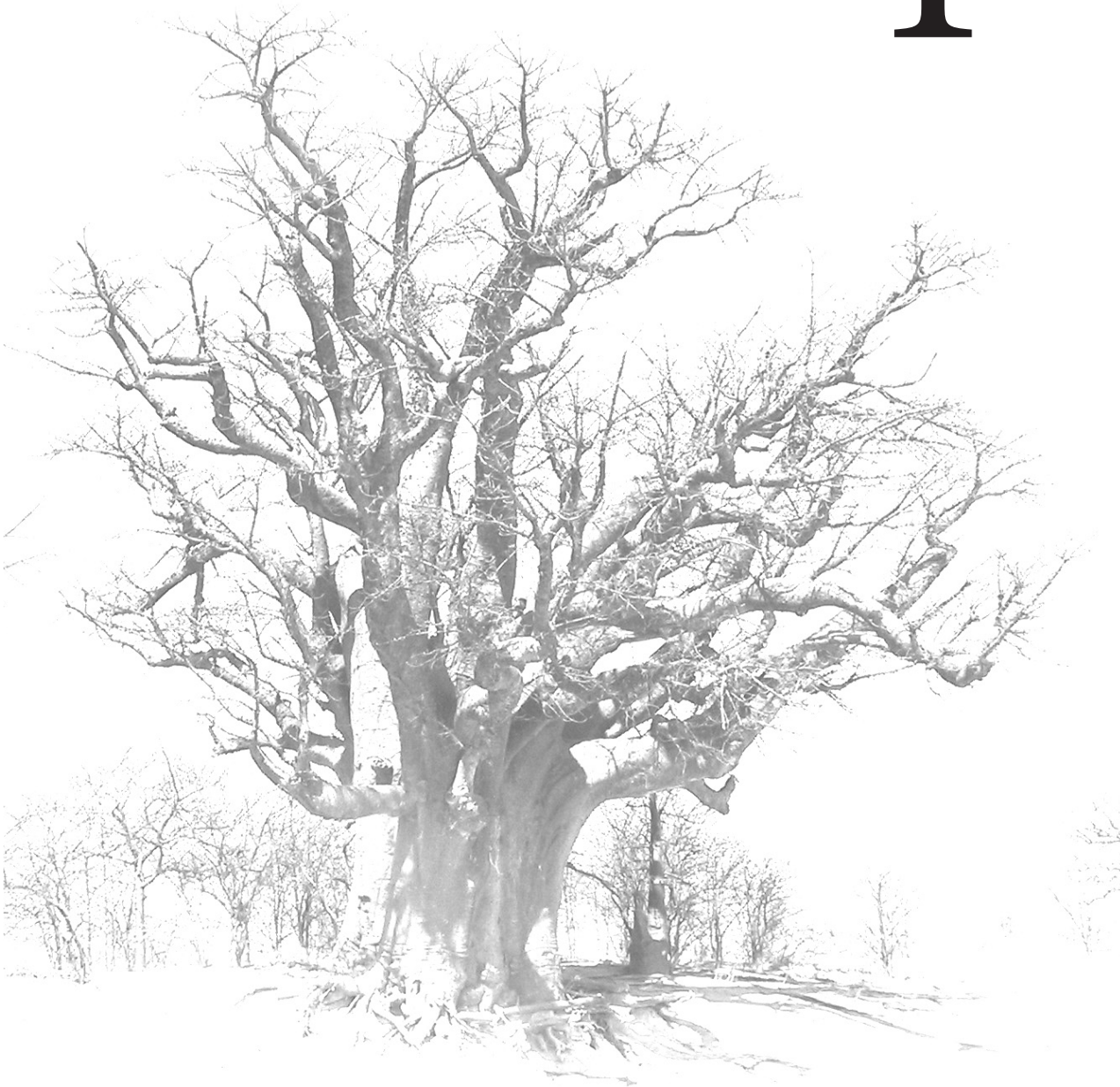

Introduction

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].

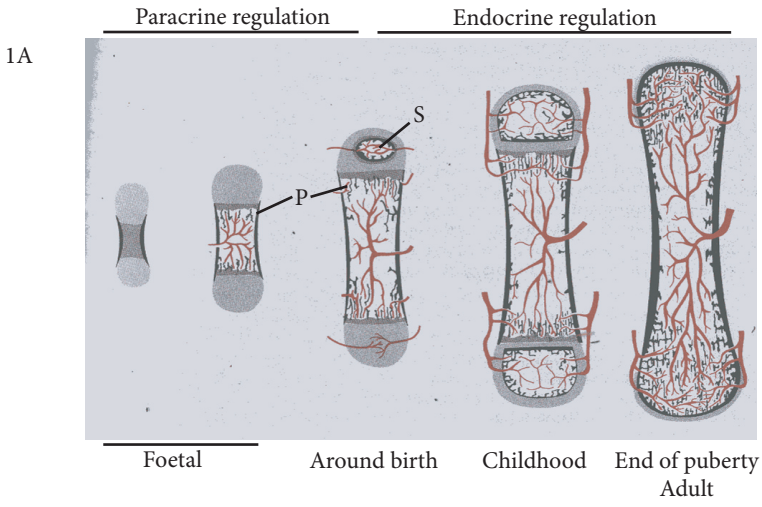

Figure 1. The formation and structure of the growth plate.
A) During foetal development endochondral bone formation takes place in the primary ossification centre (P). Around birth a secondary ossification centre (S) is formed. During childhood longitudinal bone growth increases and the growth plate 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

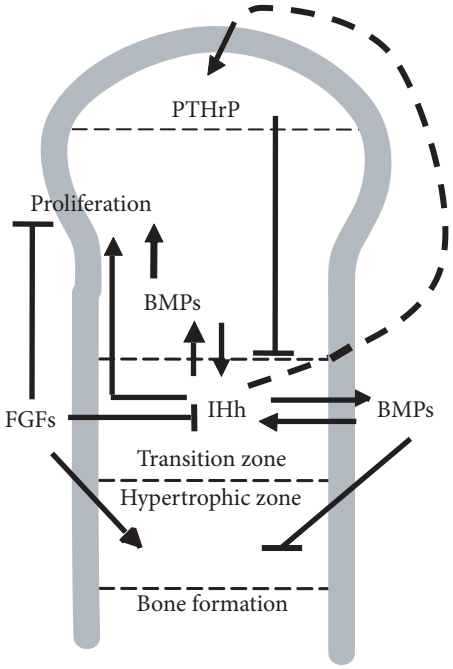

Figure 2. Interactions between growth factors with the IHh/PTHrP negative feedback loop in the embryonic growth plate. For details, see text.

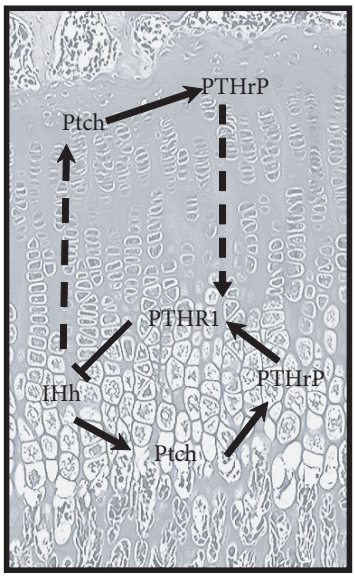

Figure 3. The IHh/PTHrP negative feedback loop in the postnatal growth plate.
For details, see text.

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.

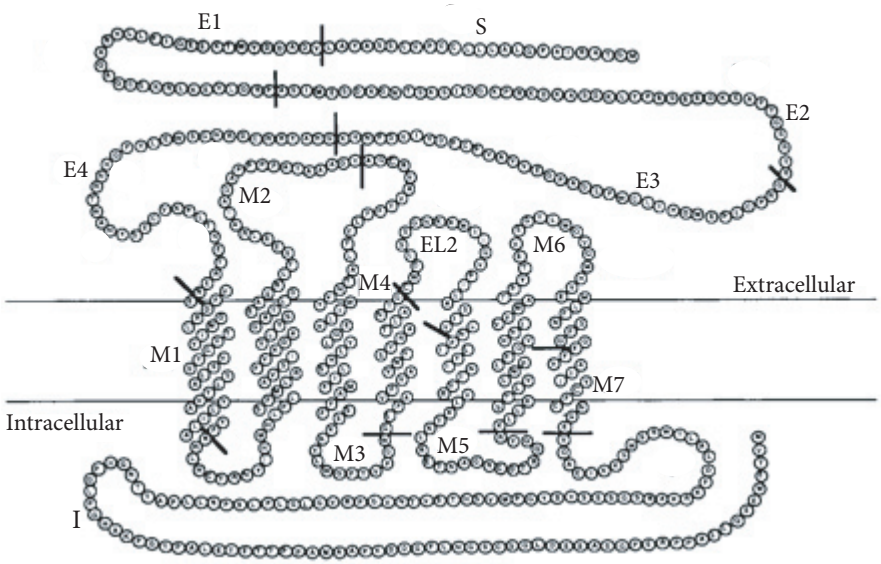

Figure 4. Schematic representation of the structure of the PTHR1.
The PTHR1 consists of 593 amino acids and is encoded by 14 exons. S = signal peptide. E1-4 = extra cellular domain. M1-7 transmembrane domains. EL2: Extracellular domain. I = intracellular domain(109).

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.

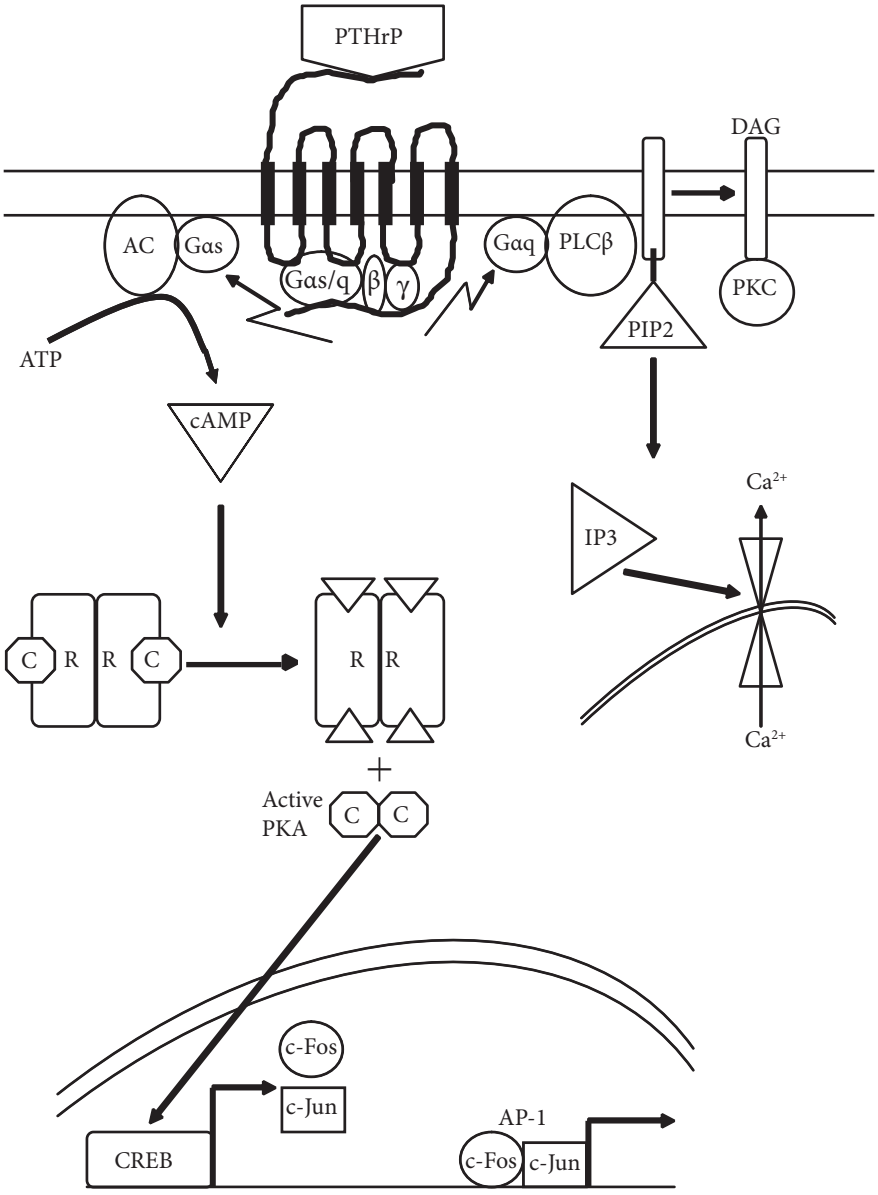

Figure 5. Schematic representation of the PTHR1 signalling pathway.
Upon ligand binding Gαs activates AC, which in turn generates cAMP from ATP. cAMP releases the catalytic subunits (C) from inactive PKA. These catalytic subunits stimulate CREB, which is required for the transcription of c-Fos and c-Jun. 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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Novel mutations in the PTHR1 causing Blomstrand Osteochondrodysplasia type I and II

2

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Abstract

Context: The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor type 1 (PTHR1) plays a key role in endochondral ossification, which is emphasized by diseases resulting from mutations in the PTHR1 gene. Among these diseases is the human variant of the PTHR1 knockout mice, Blomstrand osteochondrodysplasia (BOCD).

Objective: BOCD can be divided into two types, depending on the severity of the skeletal abnormalities. The molecular basis for this heterogenic presentation is unknown.

Design and patients: We performed mutation analysis in 2 families with type I BOCD and in 3 families with the less severe form, type II BOCD. The latter include 1 case that has not been described before.

Results: In one of the type I BOCD cases a nonsense mutation (R104X) was found, resulting in a truncated PTHR1, while in the second type I BOCD case no receptor mutation was identified. A splicing defect (intronM4+27C>T) was demonstrated in one of the type II BOCD cases. The use of the aberrant splice site resulted in a truncated protein. Although at low levels, the wild type transcript remained present. In the other 2 families with type II BOCD a previously identified missense P132L mutation was found. Functional analysis demonstrated a near complete inactivation of the PTHR1 with low residual activity.

Conclusions: In combination with data presented in literature, we conclude that type I BOCD is caused by a complete inactivation of the PTHR1, whereas low levels of residual activity due to a near complete inactivation of the PTHR1 result in the relatively milder presentation of type II BOCD.

Introduction

The parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor type 1 (PTHR1) is a member of a subclass of the G-protein-coupled receptor family. The typical structure of these receptors is characterized by an extracellular N-terminus for ligand binding, a seven-transmembrane domain, and an intracellular C-terminus. The intracellular parts of the transmembrane domains and the C-terminus are responsible for the activation of the two dominant signalling cascades, the adenylate (AC) / protein kinase A (PKA) and the phospholipase C beta (PLCβ) / protein kinase C (PKC) pathways.

Signalling through the PTHR1 plays a key role in calcium and phosphorus homeostasis in foetal and adult life[1]. Recent studies have demonstrated the essential role of PTHR1 signalling in cartilage and bone development in mice. Mice lacking the PTHrP gene die perinatally and show accelerated chondrocyte differentiation, leading to dwarfism[2]. A more severe bone phenotype is caused by loss of the PTHR1 gene. Depending on the genetic background these mice die at various embryonic ages[3].

The essential role for PTHR1 signalling in bone and cartilage development has also been shown in human diseases. Mutations in the PTHR1 can be divided into dominant and recessive mutations. Dominant mutations are found in Jansen´s metaphyseal chondrodysplasia (JMC) and Ollier disease[4;5]. Four different heterozygous mutations have been identified in JMC patients, causing a constitutively activation of the PTHR1, leading to skeletal malformations and dwarfism[4;6-8]. In a paper by Hopyan et al. in two out of 6 patients with enchondromatosis (Ollier and Maffucci diseases), which are common benign tumours of cartilage and bone, a

heterozygous mutation in the PTHR1 gene was identified[5]. The mutation is thought to result in upregulation of the Indian Hedgehog/PTHrP pathway[5]. However, in another study with a larger panel of patients, no mutations were identified in the PTHR1 gene, suggesting that the PTHR1 gene is not the main culprit for enchondromatosis[9].

Recessive mutations have been identified in Eiken syndrome and in Blomstrand osteochondrodysplasia (BOCD)[10-14]. Eiken syndrome, an extremely rare syndrome presently reported in only 1 family, is caused by a homozygous nonsense mutation. This mutation resulted in a truncated protein, only missing a small part of the C-terminus, possibly leading to a disbalance between the activation of the AC/PKA and the PLCβ/PKC signalling pathways[10;15]. BOCD is a rare autosomal recessive disorder characterized by advanced skeletal maturation and premature ossification. In addition, BOCD is also characterized by extra-skeletal manifestations, like hypoplastic lungs, tooth abnormalities, aortic coarctation, and absence of breast development[16;17]. The skeletal and extra-skeletal abnormalities resemble the defects observed in PTHR1 knockout mice[3;18]. It has been proposed, that BOCD presents in two forms, type I and type II[16]. Type I BOCD is the classical and most severe form, characterized by extremely short and malformed bones. Although also lethal, the skeletal manifestations in type II BOCD are less severe compared to type I.

The first case of BOCD was described by Blomstrand et al. in 1985[19]. Until now, 10 other families have been described with the same disorder[16;20-26]. In the majority of the cases parental consanguinity was noted. Only 2 reports have been published in which the affected foetuses were born to non-consanguineous parents[16;23]. In three BOCD cases a mutation in the PTHR1 gene has been described. Jobert et al. reported in 1998 for the first time an inactivating mutation in the PTHR1 gene[13]. In the same year a missense mutation in another BOCD case was reported[11;12]. Thereafter, another study revealed that a frame shift mutation in the PTHR1 gene, resulting in a truncated protein, was the cause of BOCD in the case described by Den Hollander et al.[14;21].

In this study, we address the molecular basis for the heterogenic presentation of BOCD by performing mutation analysis in 2 type I and 3 type II BOCD cases.

Materials and Methods

Cases included in this study

Case 1 is a type I BOCD and was the first reported case of BOCD described in detail by Blomstrand et al.[19]. The female foetus was born after 29 weeks of gestation to consanguineous parents. The second case, a female foetus born to consanguineous Caucasian parents at 32 weeks of gestation, is described in detail elsewhere and is classified as type I BOCD (case I in[16]). Case 3, classified as a type II BOCD, is described for the first time in the present study (for detailed description see next paragraph). Case 4 is one of the three affected siblings born to Asian parents who where first-degree cousins and who had no family history of skeletal dysplasia (case II in[16]). The first two foetuses were described in detail before[16]. Another pregnancy of the same parents resulted in a third affected foetus, showing the same characteristics as its sibs. All three foetuses were classified as type II BOCD. In the fifth case the foetus was obtained from a terminated pregnancy at 26 weeks of gestation (case III in[16]). The Asian parents were non-consanguineous. Post-mortem radiography and osteochondral

histopathology classified the skeletal dysplasia as type II BOCD. Parental consent was obtained for this study.

Case 3 Clinical report and family history
Case 3 was a male infant whose mother presented with polyhydramnion at 32 weeks. Foetal ultrasound at 32 weeks revealed furthermore a relatively large head, a small thorax, lung hypoplasia and very short dense tubular bones. Because of the gross polyhydramnion and the probable lethal prognosis labour was initiated. At birth the infant did not breathe and died within minutes. Birth weight was 1800 gram. The boy showed a short stature (Crown-Rump Length (CRL) 28.5 cm), a large head (Head Circumference (HC) 32 cm) and a hypoplastic viscerocranium (fig. 1A). The face showed typical abnormalities: severe micrognathia with a protruding tongue, a hypoplastic nose, and low-set ears were noted. The infant also had a narrow thorax and no nipples. The limbs were symmetrically shortened. Autopsy revealed hypoplastic lungs and a pre-ductal aortic coarctation. Abdominal and pelvic organs showed no abnormalities. Radiography showed generalised osteosclerosis and advanced skeletal maturation, a small viscerocranium, short ribs, ossification of laryngeal cartilage and patella (fig. 1A). The tubular bones were short with metaphyseal broadening. Most carpal and tarsal bones were ossified. Histology of tubular bones showed a reduction of the resting and proliferative zone of the growth plates (fig.1B). The epiphysis showed irregular columnization of the hypertrophic chondrocytes. The overall picture resembles the clinical manifestations of BOCD. Given the relatively well-developed long bones, a recognizable epiphysis and a normal diaphyseal bone marrow space this case was classified as type II BOCD.
The parents both originated from a 250-person village in Turkey, but they deny any consanguinity. This foetus was the 7th pregnancy of these parents. Two sons are healthy, one child died one week old of unknown cause, one child had been healthy until the age of death at 11 months of unknown cause, one earlier pregnancy had ended in the birth of a stillborn boy at 28 weeks pregnancy and one pregnancy ended in a spontaneous miscarriage at 12 weeks. No medical information on these deceased children is available.

Sequence analysis and site-directed mutagenesis
From cases 1 and 2 only tissue blocks embedded in paraffin were present. Sections from these blocks were deparaffined and hydrated through graded ethanols. Subsequently, DNA was extracted by proteinase K (Invitrogen) digestion and ethanol precipitation. From cases 3, 4 and 5 genomic DNA was isolated from cultured fibroblasts by sequential proteinase K treatment and high salt precipitation.

Primer sets used for the amplification of the coding exons of hPTHR1 were previously described[14;27]. PCR products were sequence verified using an ABI thermal sequencer (PE Applied Biosystems, Foster City, CA, USA).

A mutant PTHR1 receptor cDNA was created by PCR based site directed mutagenesis, using the wild type receptor as a template. The mutant construct was sequence verified and cloned in the pcDNA3.1 expression vector. Primer sets to check for the expression of wild type and mutant PTHR1 transcripts in case 3 are indicated in table 1.

Cell culture, transient transfection assays and cAMP production

At autopsy of cases 3, 4, and 5 a skin biopsy was taken and dermal fibroblast were cultured in αMEM, containing 100 U/ml penicillin (Invitrogen, Breda, The Netherlands), 100 U/ml streptomycin (Invitrogen), and 10 % fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands). Cells were seeded at a density of 15000 cells/cm^2 in a 24-wells tissue culture plate. After 4 days cells were used for intracellular cAMP determination after a challenge with

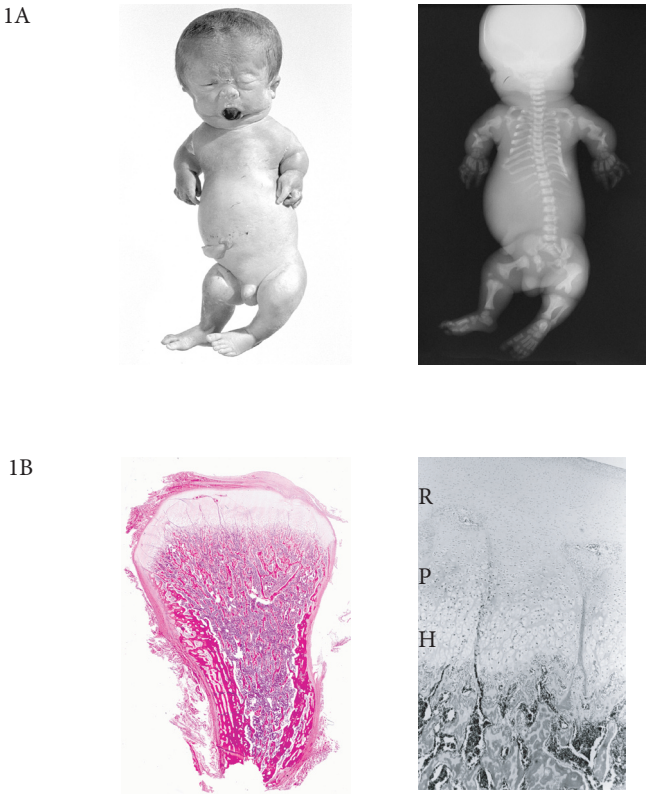


Figure 1. Case 3: phenotypic presentation of type II BOCD.
A) Left panel: external view of a foetus delivered 32 week after gestation, showing short stature and micromelia. Right panel: whole body radiograph, showing advanced skeletal maturation, a small viscerocranium, short ribs, ossification of laryngeal cartilage and patella. The tubular bones are short with metaphyseal broadening. Most carpal and tarsal bones are ossified. B) Histology of a femoral head, showing a reduction in size of the epiphysis. High magnification shows a decrease in resting chondrocytes (R) and a near complete absence of column-wise orientated proliferating chondrocytes (P). Note the irregular border between the growth plate and the primary spongiosum. H: Hypertrophic zone.

Name	Sequence
S8	TGTGGGGCTTCACAGTCTTCG
AS8	AAGTCCCAGCACCCGGTG
AS9	GGATGAAGTTGAGCACAATGG

Table 1: Primer sets used for PCR analysis in case 3.

hPTHrP(1-34) or bNle^{8,18}PTH(1-34) (Bachum Holding AG, Bubendorf, Switzerland), using an enzymimmunoassay (Amersham, Freiburg, Germany), according to the manufacturers protocol, as described before[14]. COS-7 cells were cultured in bicarbonate-buffered DMEM, containing 7.5 % FCS, 100 U/ml penicillin, and 100 U/ml streptomycin. For transient transfection assays, cells were seeded in a 75 cm^2-disk. At 80 % confluence, cells were overnight transfected with 6 µg of the pcDNA3.1 expression vector (Invitrogen), containing either the wild type or mutant human PTHR1 cDNA or no insert (mock) using Fugene-6 (Roche, Indianapolis, IN). The following day, cells were trypsinized and seeded at a density of 15000 cells/cm^2 in a 24-wells tissue culture plate. After 2 days cells were used for determination of intracellular cAMP as described before[14].

RT-PCR analysis

Dermal fibroblasts were cultured as described above. Cells were seeded at a density of 15000 cells/cm^2 in a 56 cm^2 tissue culture disk. After confluence, total RNA was isolated according to the method of Chomczinksy and Sacchi[28]. Next, total RNA was reverse transcribed into cDNA using random hexamer primers (Amersham, Freiburg, Germany). To correct for variations in RNA content and cDNA synthesis between the different samples, cDNAs were equalized on the basis of their content of the housekeeping gene beta-2-microglobulin as described in detail elsewhere[29]. Semi-quantitative PCR was performed for the PTHR1 or for various parts of the PTHR1 under the following conditions: cDNA was denatured at 94°C for 5 minutes, followed by cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and final extension at 72°C for 10 minutes.

Results

Case 1

Sequence analysis of the coding exons and the flanking exon-intron boundaries of the PTHR1 gene revealed a homozygous point mutation (338C>T), causing a premature stop codon at position 104 (R104X) (fig. 2A). The presence of the mutation was verified by restriction fragment length analysis (fig. 2B). DNA corresponding to exon 1 from the foetus was amplified by PCR and was, as expected, resistant to KpnI restriction. Due to the substitution a truncated protein was formed, only consisting of the signal peptide and the first 79 amino acids. This mutant protein lacks all functional domains of the PTHR1, including a large part of the extracellular N-terminus, the transmembrane domains and the intracellular C-terminus, and is, therefore, completely inactivating.

Case 2

Sequence analysis of the PTHR1 gene did not reveal a mutation in the coding exons and flanking exon-intron boundaries (data not shown).

Case 3

The dermal fibroblast of the affected foetus showed no cAMP accumulation after a challenge with a high dose of PTH or PTHrP (fig. 3A). To establish whether the abnormalities were indeed caused by a mutation in the PTHR1, all coding exons and flanking exon-intron boundaries of the PTHR1 gene were sequenced. In none of the coding exons a mutation was found. However, a homozygous point mutation was identified in the intron between exon M4 and exon EL2 (intronM4+27C>T) (fig. 3B). This mutation creates a perfect match with the consensus sequence of an exon-intron boundary with higher homology than the native splice site. RT-PCR analysis of the PTHR1 gene revealed decreased expression and a larger transcript in the fibroblasts from the patient compared to control samples (fig. 3C). To test whether or not the splicing machinery can still use the native splice site, RT-PCR was performed using a reverse primer, spanning the wild type exon M4 and EL2 splice site. This primer can only result in amplification of wild type, but not in mutant transcripts. As shown in figure 3D still a small amount of wild type PTHR1 mRNA was expressed in fibroblasts of the patient compared to control samples, indicating the preferential but not exclusive use of the aberrant splice site. The formation of an aberrant splice site and subsequently the extension of exon M4 resulted in a premature stop codon, thereby creating a truncated protein, lacking the 5th, 6th, and 7th transmembrane domains, the intervening intra- and extracellular domains, as well as the cytoplasmatic tail (fig. 3E).

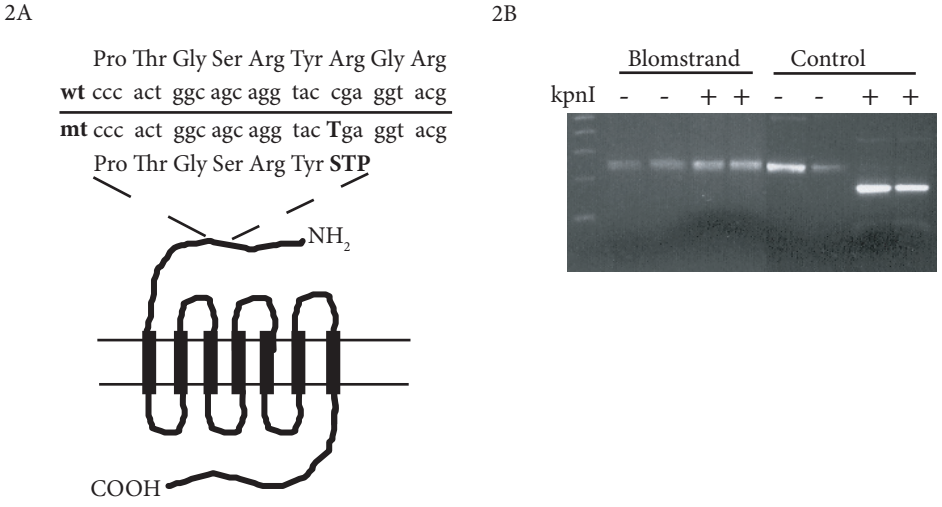
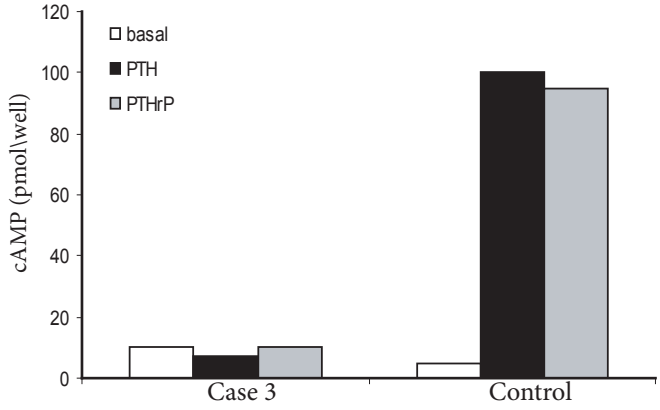
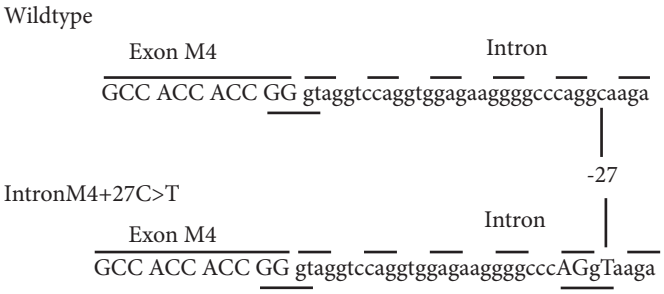


Figure 2. Case 1: premature stopcodon in PTHR1.
A) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. Sequence analysis of PTHR1 gene revealed a 338C>T substitution (capital and bold), resulting in a premature stop codon in the extracellular part of the receptor. The position of the mutation is shown in a schematic representation of the structure of the PTHR1. B) Exon E2 was amplified and subjected to restriction enzyme analysis. The PCR product of the patient but not of a control was resistant to KpnI restriction.

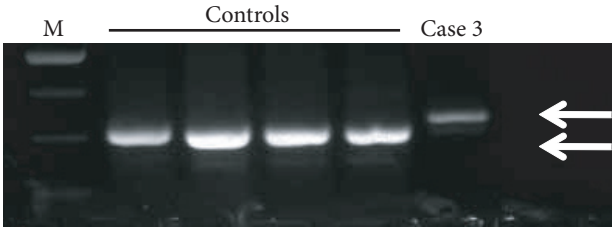
3A


120
100
80
60
40
20
0
cAMP (pmol\well)
basal
PTH
PTHrP
Case 3
Control

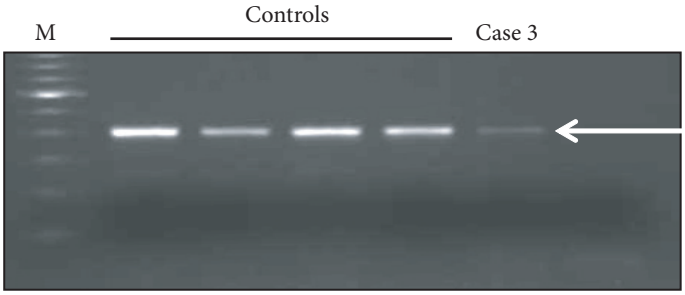
3B


Wildtype
Exon M4
Intron
GCC ACC ACC GG gtaggtccaggtggagaaggggcccaggcaaga
-27
IntronM4+27C>T
Intron
Exon M4
GCC ACC ACC GG gtaggtccaggtggagaaggggcccAGgTaaga

3C


M
Controls
Case 3

3D



3E

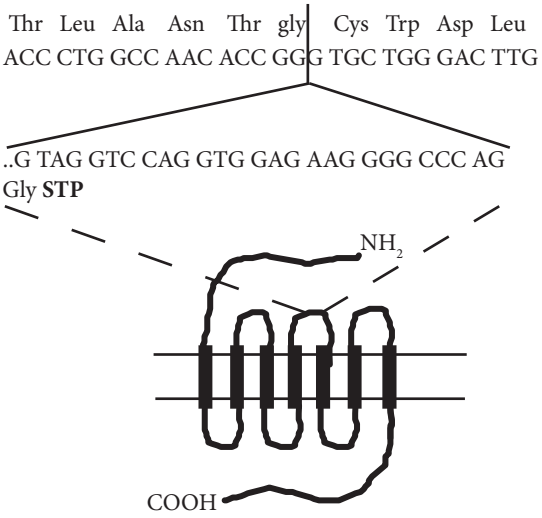
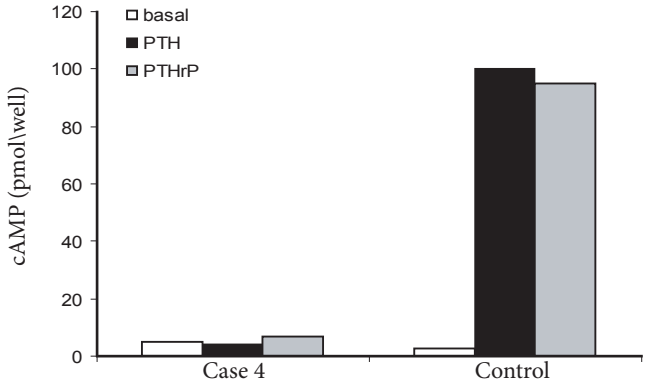


Figure 3. Case 3: Introduction of a novel splice site.
A) Dermal fibroblast of the affected foetus and control dermal fibroblasts were treated with a high dose (10-7 M) PTH(1-34) or PTHrP(1-34) and cAMP accumulation was measured. In contrast to control, dermal fibroblasts of the affected foetus showed no cAMP accumulation. B) A homozygous point mutation was identified 27 bp downstream of exon M4 (intronM4+27C>T), creating a new exon-intron boundary with higher homology to the consensus sequence than the wild type splice site. Native and mutated exon/intron boundaries are underlined. C) RT-PCR of the PTHR1 using a sense primer (S8) located in exon M4 and an antisense primer (AS9) located in the downstream exon EL2 revealed a bigger transcript in case 3 compared to control samples, which is indicative for aberrant splicing. M is marker. D) RT-PCR was performed with a sense primer (S8) specific for exon M4 and an antisense primer (AS8) overlapping the wild type exon-exon boundary of exon M4 and EL2. Only in the case of wild type mRNA this primer combination results in an amplicon. A small amount of wild type PTHR1 mRNA was expressed in the affected sample compared to control samples, indicating the preferential but not exclusive use of the aberrant splice site. E) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. When the alternative splice site is used, the last amino acid of exon M4 will be followed by a stop codon. This results in a truncated protein, lacking the transmembrane domains 5, 6 and 7 and the cytoplasmatic C-terminus. STP: stop codon.

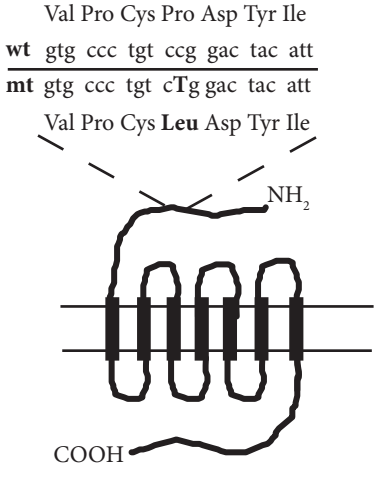
Case 4

Dermal fibroblasts of case 4 showed no cAMP accumulation after a challenge with a high dose of PTH or PTHrP (fig. 4A), indicating abrogated PTHR1 signalling. We subsequently sequenced the coding exons and flanking exon-intron boundaries of the PTHR1 gene. A homozygous point mutation (423C>T) in exon E3 was identified, resulting in a missense mutation at position 132 (P132L) (fig. 4B). The presence of the mutation was verified by restriction fragment length analysis (fig. 4C). DNA corresponding to exon 3 from the foetus was amplified by PCR and was, as expected, resistant to MspI enzymatic activity. The same mutation was identified in the two other sibs with BOCD. Functional analysis by transient transfections of wild type and mutant receptors in COS-7 cells revealed that the mutant receptor could still accumulate cAMP after receptor activation, although to a lesser extent compared to the wild type receptor (fig. 4D).

4A



4B



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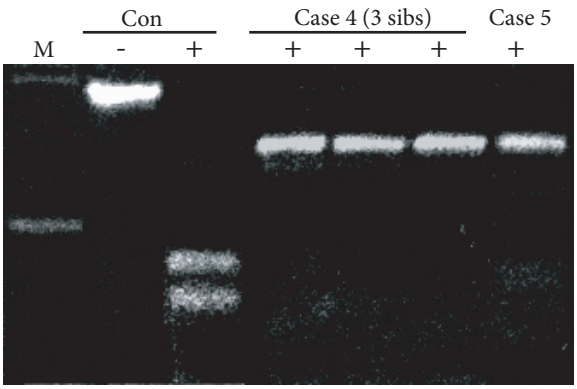

4D

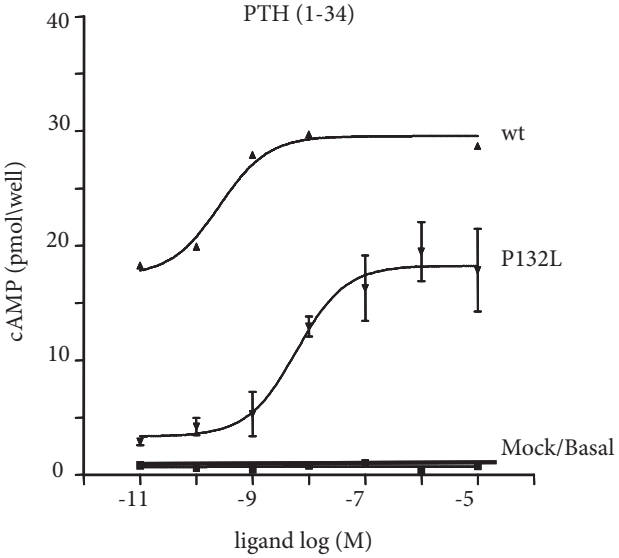

Figure 4. Case 4 and 5: P132L missense mutation.
A) Dermal fibroblast of case 4 and control dermal fibroblasts were treated with a high dose (10-7 M) PTH(1-34) or PTHrP(1-34) and cAMP accumulation was measured. Dermal fibroblasts of the affected foetus showed no cAMP accumulation, whereas in control fibroblasts cAMP accumulation was induced. B) Comparison of the DNA and amino acid sequences of the wild type (wt) and the mutant (mt) receptors. Sequence analysis of PTHR1 gene revealed a homozygous 423C>T substitution (capital and bold), resulting in an amino acid change in the extracellular part of the receptor (P132L). The position of the mutation is shown in a schematic representation of the PTHR1. C) Exon E3 was PCR amplified and subjected to restriction enzyme analysis. The mutation resulted in a loss of an MspI restriction site in case 4 and 5. M is marker. D) COS-7 cells were transiently transfected with an expression vector encoding wild type PTHR1 (wt), mutant P132L PTHR1 or an empty expression vector (mock). Subsequently, cells were treated with a dose range of PTH(1-34) and cAMP accumulation was measured.

Case 5

Sequence analysis also revealed the homozygous mutation 423C>T in the PTHR1 gene as described for case 4, resulting in a P132L substitution. The presence of the mutation was verified by restriction fragment length analysis (fig 4C).

Discussion

In the present study we have performed mutation and functional analysis in 5 cases with BOCD. The presence of inactivating mutations in 4 out of 5 cases further confirms the causative role of the PTHR in BOCD. In one patient (case 2 in this study) we did not identify a mutation in the coding exons and flanking exon-intron boundaries of the PTHR1 gene. Possibly, a mutation is present in an intron or in the promoter region, which inhibits the expression of the PTHR1 gene. Such a mutation probably also underlies the absence of expression of the paternal allele in the case described by Jobert et al.[13]. Another explanation could be the presence of a mutation in another gene, like PTHrP. Unfortunately, we were unable to test this hypothesis, since only a small amount of DNA of poor quality could be isolated from a limited number of paraffin blocks.

Including this study, 5 different mutations have been described in the PTHR1 causing BOCD[11-14, this study]. Each family had its own mutation, with exception of the P132L. This data suggests that this conserved proline residue serves as a hotspot for mutations. Hotspots have already been described in other chondrodysplasias, like the H223R mutation in the PTHR1 causing JMC, and the A380G mutation in the FGFR3 causing achondrodysplasia [4;30]. Another explanation for the relatively high frequency of the P132L mutation could be that the patients originate from the same ancestor. Pedigree analysis revealed no indications for this. However, since the 3 families with P132L live in the same region of England and originate from the same ethnic population, such a founder effect cannot be ruled out.

The topic we addressed was the difference between type I and type II BOCD. Oostra et al. postulated that type I BOCD is caused by a completely inactivating mutation in the PTHR1 gene[16], while the less severe abnormalities in type II BOCD are caused by incomplete inactivation of the PTHR1. We present evidence in concordance with this hypothesis. The mutations in the type I BOCD cases described in this study and in previous studies are all completely inactivating mutations[13;14]. In the three families classified by Oostra et al. as type II BOCD, the P132L mutation was found[11;12;16, this study]. Functional analysis of the P132L mutation, after transient transfections in COS-7 cells, revealed residual activity. Also in the study of Zhang et al. some residual activity was present with respect to activation of the cAMP/PKA pathway, but not to activation of the PLCβ/PKC pathway[12].

The residual activity of the P132L mutation in COS-7 cells contrasts our observation in dermal fibroblasts of case 4, in which no cAMP accumulation was found after stimulation with PTH or PTHrP (not shown). This discrepancy is most likely explained by a substantial difference in the number of receptors present in dermal fibroblasts and transiently transfected COS-7 cells. Only in the presence of high receptor numbers residual activity might become evident. Since chondrocytes and osteoblasts express higher levels of PTHR1 mRNA and, therefore, are more responsive than dermal fibroblasts, the residual activity of the P132L mutation *in vivo* may only become evident in the developing skeleton, resulting in a milder clinical presentation.

An alternative explanation is that the milder phenotype in the three type II BOCD cases is associated with the same ethnic background. It has been described that the phenotype of PTHR1 knock out mice strongly depends on this genetic composition[3]. This latter explanation seems, however, unlikely, since we have identified in this study a new type II BOCD case of distinct ethnic background, caused by a novel mutation (intronM4+27C>T). Like in case 4, dermal fibroblasts of this patient did not reveal any responsiveness to PTH or PTHrP, using intracellular cAMP accumulation as a read out. However, by sensitive RT-PCR we show that the native splice site is also used, although at very low levels. This will result in the presence of low levels of wild type mRNAs. Apparently, in dermal fibroblasts, a cell type with low levels of PTHR1 transcripts, this expression is too low to result in a measurable functional response. In skeletal cells with high levels of mRNA and high responsiveness, low residual activity due to native splicing may become evident, resulting in the milder skeletal manifestations. Taken together, the relatively milder presentation of BOCD type II, particularly in the skeleton, is most likely caused by a partial inactivating mutation of the PTHR1 gene, resulting in low amounts of residual activity during endochondral bone formation.

The heterogeneity in clinical manifestations, depending on the kind of mutation, is not only shown by differences in skeletal abnormalities, but also by differences in extra-skeletal abnormalities. A characteristic of PTHR1 knockout mice is the absence of breast and nipple formation[18]. This finding is confirmed in a patient with BOCD[17]. Also in case 3 reported in this study, absence of nipple formation was noted during obduction. However, in one case (specimen M684 in the study by Oostra et al.), having the typical skeletal characteristics of BOCD, nipple formation is clearly visible (see figure 4 in[20]). We postulate that the presence or absence of nipple formation may also depend on the nature of the mutation in the PTHR1, affecting skeletal development but not nipple formation. Unfortunately, because of long-term preservation (over 100 years in formalin), DNA isolation from this case for sequence analysis was not possible to test this hypothesis.

Heterogeneity in clinical manifestations due to the nature of the mutation in the PTHR1 is not only shown in BOCD families, but also in JMC families. Two missense mutations at the same amino acid position in the PTHR1 (T140P and T140R) have distinct effects on receptor activity. Compared to T140P, the T140R substitution resulted in a less severe form of ligand dependent activation of the AC/PKA pathway[7]. This became clinically evident in a less severe presentation of JMC[8]. The impact of a mutation on receptor function critically determines the clinical presentation.

The mutations in the PTHR1 gene identified in this study and in previous studies are shown in table 2. It summarizes the type and position of the mutations identified in 4 disorders, caused by defective PTHR1 signalling. They are divided into dominant and recessive disorders. Not only the type of the mutation, but also the position of the mutation influences the kind and the severity of the disease. The mutations are evenly distributed over the open reading frame. The striking differences in clinical presentations caused by distinct mutations further underscores the critical role of the PTHR1 in normal endochondral bone formation. Multiple signalling pathways are activated by the PTHR1. It seems feasible that distinct mutations affect only subsets of these pathways, resulting in differences in signalling properties and hence in clinical manifestations. Detailed functional analysis of mutations in the PTHR1 identified in human patients may, therefore, provide novel insights in the role of each of these signalling pathways

in the biological effects of PTHR1.

Acknowledgements

We thank dr. H.J. van der Harten (Department of Pathology, Free University Amsterdam, Amsterdam, The Netherlands) for helpful discussion and dr. L.G. Kindblom (Department of Pathology, Sahlgrenska University Hospital, Göteborg, Sweden) for kindly providing tissue blocks of case 1.

dominant/ recessive	disease	nucleotide change	amino acid change	Type of mutation	Impact on receptor	reference
dominant	JMC	696A>G	H223R	missense	constitutively activating	(6)
		1401T>G	I458R	missense	constitutively activating	(4)
		1256A>C	T410P	missense	constitutively activating	(7)
	JMC (less severe)	1257C>G	T410R	missense	less severe constitutively activating	(8)
	Ollier syndrome	476C>T	R150C	missense	disturbed regulation of Hedgehog pathway	(5)
recessive	Type I BOCD	338C>T	R104X	nonsense	Inactivating	This report
		1122delG	V365fsX505	frameshift	Inactivating	(14)
		1176G>A		splicing defect	Inactivating	(13)
	Type II BOCD	423C>T	P132L	missense	partial Inactivating	This report (11;12)
		intronM4+ 27C>T		splicing defect	partial inactivating	This report
	Eiken syndrome	1656C>T	R485X	nonsense	partial inactivating	(10)

Table 2: PTHR1 mutations.

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Nherf2, but not Nherf1 overexpression, stimulates osteoblast and chondrocyte matrix mineralization

3

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Manuscript in preparation

Abstract

The PDZ domain containing proteins Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2 redirect type 1 Parathyroid Hormone (PTH)/PTH related Peptide (PTHrP) receptor (PTHR1) signalling in renal tubuli. Whether Nherf1 and Nherf2 are also expressed during endochondral bone formation and whether they play a role in osteoblast and chondrocyte differentiation is not known. Furthermore, it is not known whether they modulate PTHR1 signalling in these cells.

We showed that Nherf1 and Nherf2 are expressed by growth plate chondrocytes and by osteoblasts in the bone collar using in situ hybridization. In addition, overexpression of Nherf1 and Nherf2 in KS483 mesenchymal progenitor cells resulted in opposite effects on terminal osteoblast differentiation. Nherf1 overexpression inhibited, while Nherf2 overexpression stimulated matrix mineralization. Cartilage matrix mineralization was increased in the Nherf2, but not in the Nherf1 overexpressing cell line. Overexpression of Nherf1 or Nherf2 had no effect on the production of the early chondrocyte marker, the glycosaminoglycans. PTHrP inhibited osteoblastic matrix mineralization and GAG production in chondrocytes. Overexpression of Nherf1 or Nherf2 did not change the effect of PTHrP treatment on osteoblast and chondrocyte differentiation.

The distinct actions of Nherf1 and Nherf2 are probably due to the recruitment of different target molecules, like transcription factors, receptors and signalling molecules.

Introduction

The Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2 form a family of adaptor proteins and are characterized by the presence of two tandem psd95, discs large protein, ZO1 (PDZ) protein interaction domains. They were first found in the apical membranes of renal epithelial cells, where they are involved in regulating the sodium-hydrogen exchange[1]. Other studies revealed that Nherf proteins also play a role in the regulation of phosphate and calcium transport in kidney cells[2;3]. Beside interaction with ion channels, Nherf1 and Nherf2 also cooperate with transcription factors, signalling proteins, structural proteins, and with G-protein coupled receptors, including the type 1 Parathyroid Hormone (PTH)/PTH related Peptide (PTHrP) receptor (PTHR1)[4;5].

The interactions of Nherf1 and Nherf2 with the PTHR1 are best studied in kidney cells. Immunohistochemical analysis suggests that the PTHR1 co-localizes with Nherf1 and Nherf2 in the tubuli. Binding of PTHR1 to Nherf1 occurs through the PDZ1 domain and binding to Nherf2 through the PDZ2 domain[4;6;7]. Both adaptor proteins bind, beside to the PTHR1, also to the intracellular signalling mediator phospholipase C beta (PLCβ), which results in a switch of PTHR1 signalling from the adenylate cyclase (AC)/protein kinase A (PKA) pathway to the PLCβ/protein kinase C (PKC) pathway[4;6]. It is unclear whether Nherf1 or Nherf2 are also expressed in osteoblasts and chondrocytes, the other main targets of PTH and PTHrP signalling, and whether they play a comparable role in redirecting PTHR1 signalling in these cells.

In chondrocytes and osteoblasts PTHR1 signalling results in the activation of two main pathways, the AC/PKA pathway via the Gαs-protein and PLCβ/ PKC pathway via the Gαq-protein. Activation of the Gαs-protein results in phosphorylation of the plasma membrane

bound enzyme AC, which synthesizes cyclic AMP (cAMP) from ATP. Subsequently, cAMP exerts its effects by activating the enzyme PKA, which finally leads to the activation of cAMP response element binding protein (CREB). In addition, Gαq-protein activates PLCβ, which in turn cleaves phosphatidylinositol 4,5-biphosphate (PIP2) to generate two products: diacylglycerol (DAG) and inositol 1,4,5-triphosphate 3 (IP3). At this step the signalling pathway splits in two branches. First, IP3 leaves the plasma membrane and diffuses rapidly through the cytosol, to release Ca(2+) from the endoplasmatic reticulum. Second, DAG activates PKC, which in turn activates downstream mediators, resulting in genomic responses.
Both pathways are essential in the regulation of chondrocyte proliferation and differentiation. This can be deduced from comparing the bone phenotypes of transgenic mice. PTHR1 knockout mice show a severe inhibition of chondrocyte proliferation and premature maturation of chondrocytes[8]. A similar, but less severe phenotype is observed in PTHrP knockout mice[9]. Chondrocyte specific knockout mice, carrying a mutation in the Gαs-protein, leading to disruption of the AC/PKA pathway, displayed a comparable phenotype to the growth plate phenotype observed in PTHrP knockout mice[9;10]. The opposite was found in mice carrying a mutant form of the PTHR1, which specifically interrupts signalling via the PLCβ/PKC pathway and signals normally via the AC/PKA pathway[11]. These mice showed an increase in chondrocyte proliferation and a delay in chondrocyte hypertrophy and vascular invasion. These data indicate that the PLCβ/PKC pathway regulates the transition from proliferating into hypertrophic chondrocytes. In addition, it suggest that the AC/PKA pathway is the dominant pathway after PTHR1 signalling in chondrogenesis and that PLCβ/PKC signalling opposes the effects of the AC/PKA pathway[11].
Whether Nherf1 and Nherf2 are involved in directing PTHR1 signalling towards the PLCβ/PKC pathway in chondrocytes and osteoblasts is unclear. Therefore, in this study we first addressed whether Nherf1 and Nherf2 are expressed during endochondral bone formation *in vivo*. In addition, the actions of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation *in vitro* were examined. Finally, the influence of Nherf1 and Nherf2 overexpression on the effect of PTHR1 signalling *in vitro* was investigated.

Materials and Methods

In situ hybridization

Nherf1 and Nherf2 full-length cDNA were obtained from Image Consortium and they were sequence verified. For in situ hybridizations, plasmids were linearized with the appropriate restriction enzymes and antisense and sense cRNA probes were generated using T7 or Sp6 RNA polymerase, respectively. The probes were labelled and in situ hybridization was performed as previously described[12].

Generation of stable cell lines

Stable cell lines were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol. In short, first a KS483 Flp-in host cell line was generated by stable introduction of a single copy of an Flp-Recombinase target (FRT) site as an integral part of an antibiotic resistancy gene in the genome of KS483. One clone, KSfrt 4D3, was selected that was comparable to the parental KS483 cells in differentiation characteristics and retained

full capacity to differentiate into osteoblasts and chondrocytes. This clone was used for the generation of isogenic stable cell lines by transient co-transfection of an FRT-targeting vector and an Flp-recombinase expression vector. FRT-targeting vectors were a derivation of the pEF5/FRT/V5-DEST vector (Invitrogen), containing either luciferase cDNA as a control or full length human Nherf1 or human Nherf2 cDNAs, under the control of the EF1α promoter. Due to Flp-mediated recombination at the genomic FRT-site, this targeting vector was incorporated in the genome. Simultaneously, a shift in antibiotic resistancy was introduced allowing positive selection for integrants in the genomic FRT-site only and negative selection for random integrants in one single step. This method allows the generation of isogenic stable cell lines after a single transfection by eliminating variability of multiple integrated copies and results in consistent long-term stable expression of the transgene[13].

Cell culture

For osteoblast differentiation KS483 cells were seeded at a density of 12000 cells/cm^2 as described previously[14]. Medium was changed every 3 to 4 days. At confluence (from day 4 of culture onward), ascorbic acid (50 μg/ml. Merck Inc., NY, USA) and when nodules appeared (from day 11 of culture onward) β-glycerol phosphate (5mM, Sigma Chemical Co., St Louis, MO, USA) were added. From day 11 the cells were cultured in the absence or presence of 10^{-7} M PTHrP (1-34) (Bachem, PA, USA), stained at day 18 for matrix mineralization with alizarin and alizarin was subsequently measured as described before[14].

For chondrocyte differentiation KS483 cells were cultured as pellets. For this, 300000 cells were seeded in a 96 wells plate (Greiner, Alphen a/d Rijn, The Netherlands) in high glucose DMEM (Invitrogen, Breda, The Netherlands), containing 40 μg/ml proline (Sigma, Zwijndrecht, The Netherlands), 100 μg/ml pyruvate (Sigma), 50 mg/ml ITS + premix (BD Biosciences, San Jose, USA), and 50 μg/ml ascorbic acid and centrifuged for 5 min at 1238 rpm. Medium was changed every 3 to 4 days. From day 1 to 14 medium was supplemented with 10^{-7} M dexamethasone (Sigma), and 10 ng/ml TGFβ3 (R&D systems, Minneapolis, USA). From day 14 to 28 medium was supplemented with 5mM β-glycerol phosphate and 500 ng/ml BMP6 (kindly provided by dr. S. Vukicevic, University of Zagreb, Croatia). At day 28 pellets were used for glycosaminoglycan (GAG) analysis or used for histology.

Glycosaminoglycan assay

After 28 days 3 to 4 pellets were washed with PBS and incubated overnight in 0.1 mM EDTA, containing 40 μg/ml proteinase K (Invitrogen) at 56°C. From each sample 50 μl was used for GAG analysis and 10 μl was used for DNA measurements. For GAG analysis, 50 μl sample was calibrated against a GAG standard (0,1,2,3, and 5 μg) using the Blyscan sulphated GAG assay (Biocolor, Krimpen a/d IJssel, The Netherlands) according to the manufacturers protocol, with small modifications. In short, each sample was diluted 1:1 in 0.1 mM EDTA/40 μg/ml proteinase K and 500 μl Blyscan dye reagent was added. Next, the samples were mixed for 30 minutes and centrifuged for 10 minutes at 13000 rpm. Then, pellets were dissolved in 200 μl dye dissociation reagent and mixed for 15 minutes. Absorbance was measured at 650 nm. For DNA measurements, 10 μl sample was calibrated against a DNA standard (0.5 – 10 μg) as described before[14].

Von Kossa staining

At day 28, two pellets were fixed in 10% formalin for 1 hour and embedded in paraffin. Section of 5 µm were deparaffined and hydrated through graded ethanol steps and rinsed with bidest. Subsequently, they were incubated in 1% $AgNO_3$ for 30 seconds and rinsed with bidest. Next, sections were treated for 3 minutes in 0.01% pyrogallol and rinsed with bidest. Then, they were incubated in 5% Nathiosulfate and rinsed with bidest. Sections were counterstained with light green and afterwards rinsed three times with bidest. Finally, the sections were air dried and embedded in histomount-diluted 1:1 in xylene.

RNA isolation

Cells were cultured under osteoblastic conditions as described before[14], for 4, 7, 11, 14, 18 days and RNA was isolated using Trizol LS reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

Quantitative PCR

Quantitative PCR (qPCR) was performed using the BioRad iCycler (Biorad, Veenendaal, The Netherlands). For each gene a set of primers was designed (table 1), which spanned at least 1 intron-exon boundary and had an optimal annealing temperature of 60˚C, using Beacon designer (United Bioinformatica Inc., Calgary, Canada). cDNA (5 ng) was amplified in triplicate using the qPCR core kit for SYBR green 1 (Eurogentec, Maastricht, The Netherlands), under the following conditions: cDNA was denatured for 10 minutes at 95˚C, followed by 40 cycles, consisting of 15 s at 95˚C, 20 s at 60˚C, and 40 s at 72˚C. From each sample a melting curve was generated to test for the absence of primer dimer formation and DNA contamination. Each reaction contained 5 µl cDNA (1 ng/µl), 10x reaction buffer, 3 or 4 mM $MgCl_2$ (table 1), 40 µM dNTP's, 300 nM primer, 0,75 µl SYBR green, and 0,1 µl HotGoldStar polymerase in a total volume of 25 µl. Fold changes, adjusted for the expression of β2m, were calculated and log transformed using the comparative method[15]. Significant changes were calculated using the Double Delta Model (DDM) (J. Hoogendam et al., Chapter 6).

Name	F/R	Primer	MgCl
β2µ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
mouse nherf1	F	CCAGCGATACCAGTGAGGAGC	3 mM
	R	GAGGAGGAGGAGGAGGTAGATGAG	
mouse nherf2	F	GCTCTCCTGCTTCTCACTCTGG	3 mM
	R	TCGTCCTCCTGTGCCTTGATTC	
human nherf1	F	TACAGAAGGAGAACAGTCGTGAAGC	4 mM
	R	GCCAGGGAGATGTTGAAGTCTAGG	
human nherf2	F	CCGACAAGGACACTGAGGATGG	4 mM
	R	CGCTTGTTGACTCGCATGGC	

Table 1: quantitative PCR primer sets.
F: forward primer, R: reverse primer.

Statistics

Values represent mean ± SEM. Differences were examined by analysis of variance (ANOVA) followed by the post-hoc least significant difference test (LSD). Results were considered significant at $p<0.05$.

Results

Nherf1 and Nherf2 mRNA expression during endochondral bone formation in vivo

In order to establish whether Nherf1 and Nherf2 mRNAs are expressed by chondrocytes and osteoblasts during endochondral bone formation, in situ hybridizations were performed on 18-days old mouse embryos and on hind paws of 17-days old mouse embryos. Nherf1 (fig. 1 A-D) and Nherf2 (fig. 1 E-H) showed a similar expression pattern in kidney, vertebrae, as well as in metatarsals. The negative controls are shown in figure 1A (Nherf1) and 1E (Nherf2). In kidney Nherf1 and Nherf2 mRNAs were expressed in the tubuli, whereas no expression was found in the glomeruli (fig. 1B and 1F). Nherf1 and Nherf2 mRNAs were expressed by chondrocytes throughout the growth plate and by the osteoblasts in the bone collar (fig. 1C and 1G). In addition, the chondrocytes in vertebrae showed Nherf1 and Nherf2 mRNA expression as well (fig. 1D and 1H). Remarkably, the pre-chondrocytes in the intervertebrae region and the pre-osteoblasts in the perichondrium showed no Nherf1 and Nherf2 mRNA expression. Taken together, Nherf1 and Nherf2 mRNAs are co-expressed by chondrocytes and osteoblasts during endochondral bone formation.

The effect of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation in vitro

We subsequently studied Nherf1 and Nherf2 mRNA expression during osteoblast differentiation *in vitro* using KS483 cells. In agreement with the results of the in situ hybridization, we found stable Nherf1 (fig. 2A) and Nherf2 (fig. 2B) mRNA expression during KS483 osteoblast differentiation. In order to investigate the role of Nherf1 and Nherf2 in osteoblast and chondrocyte differentiation, we generated human Nherf1 and Nherf2 overexpressing KS483 cell lines using the Flp-In system. As a control cell line, cells were stably transfected with the luciferase gene.

The Nherf1 and Nherf2 overexpressing cell lines showed stable overexpression of about 10-fold of human Nherf1 and human Nherf2 mRNA compared to endogenous (mouse) Nherf1 and endogenous (mouse) Nherf2 mRNA (fig. 3). In addition, the expression of endogenous Nherf1 and Nherf2 mRNA at the end of culture (day 18) was not different from the expression at the beginning of culture (day 4) in both overexpressing cell lines, which is comparable to Nherf1 and Nherf2 mRNA expression in the control cell line.

To establish whether Nherf1 and Nherf2 overexpression influences osteoblast differentiation of KS483 cells, the cells were cultured in monolayer for 18 days under osteoblastic conditions. At the end of culture the mineralized osteoblastic matrix was stained with alizarin red (fig. 4A) and alizarin red was quantified (fig. 4B). Nherf2 overexpression stimulated the production of a mineralized osteoblastic matrix. This result was in contrast to the production

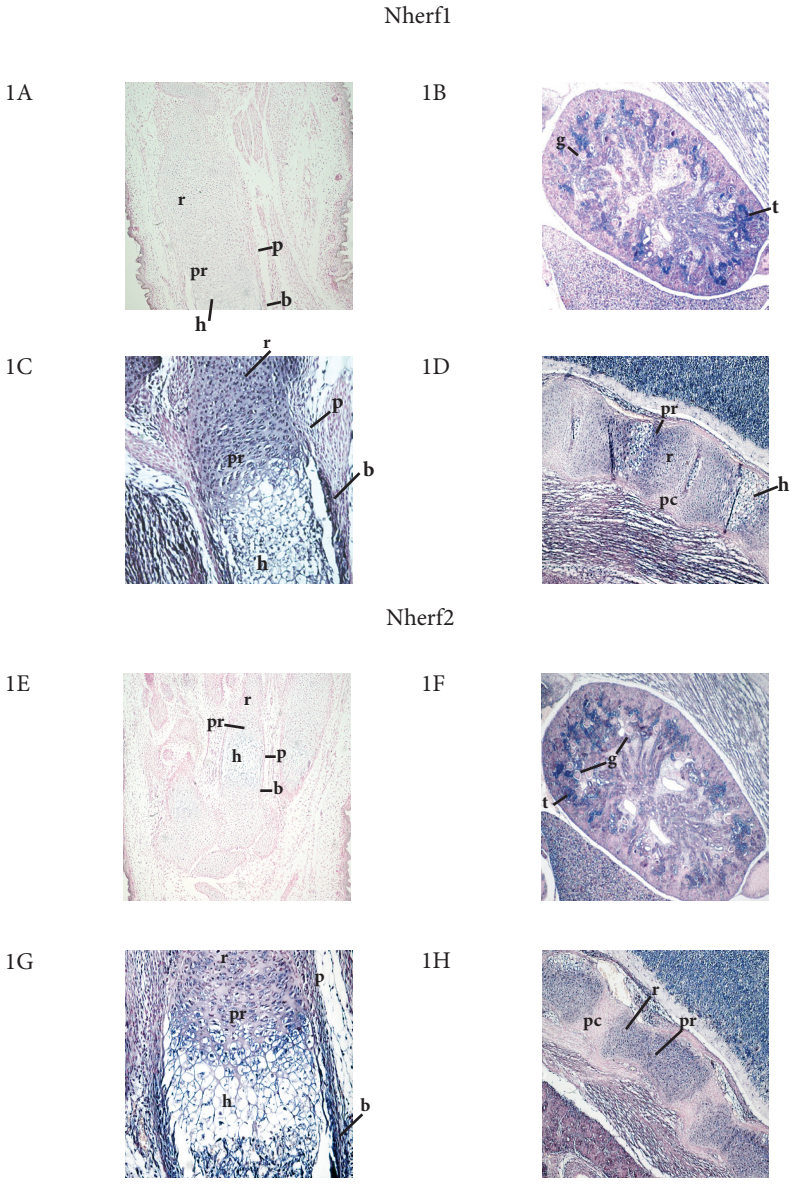

Figure 1: Nherf 1 and Nherf2 mRNA expression in mouse embryo
Nherf1 and Nherf2 mRNA expression were established by performing in situ hybridizations on parasagital sections of 18-days old mouse embryos and on hind paws of 17-days old mouse embryos. A) The negative control (sense probe) for Nherf1 and E) the control for Nherf2 were not stained. In the kidney B) Nherf1 and F) Nherf2 mRNA were expressed in tubuli (t), but not in the glomeruli (g). In the growth plate C) Nherf1 and G) Nherf2 mRNA was expressed throughout the growth plate by resting (r), proliferating (pr), and hypertrophic (h) chondrocytes. Both mRNAs were also expressed by osteoblasts in the bone collar (b) but not by pre-osteoblasts in the perichondrium (p). In vertebrae D) Nherf1 and H) Nherf2 were expressed throughout the growth plate as well. Pre-chondrocytes (pc) in the intervertebrae did not express both Nherf mRNAs. r = Resting, pr = proliferating, and h = hypertrophic chondrocytes.

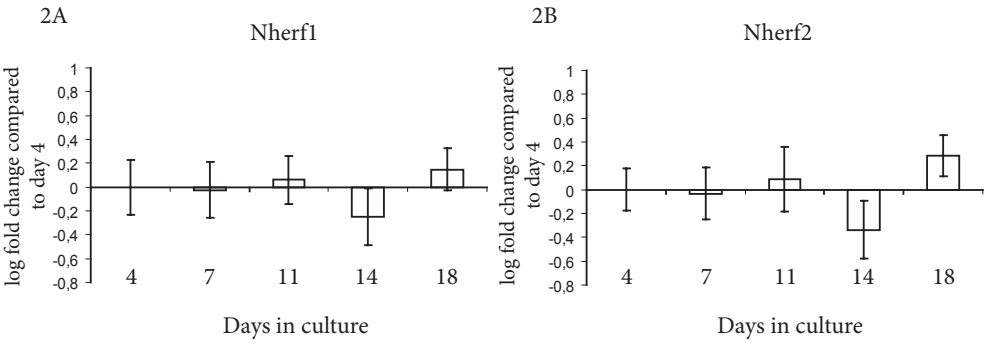

Figure 2: Expression Nherf1 and Nherf2 during KS483 osteoblast differentiation
The mRNA expression of Nherf1 and Nherf2 during KS483 osteoblast differentiation was established using qPCR analysis. Both mRNAs were highly and continuously expressed during osteoblast differentiation. Ct-value β2μ: 29.4; Ct-value Nherf1: 28.5; Ct-value Nherf2: 30.9.

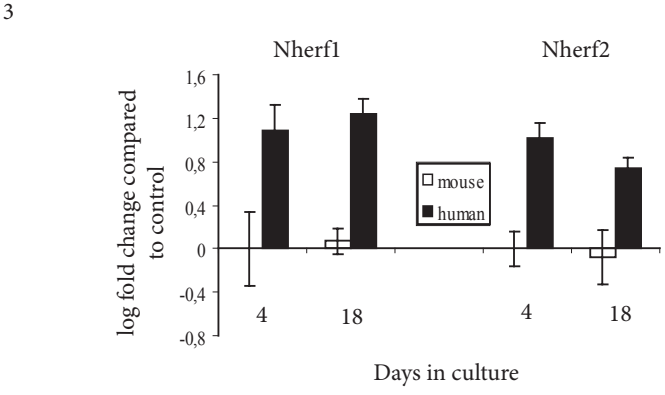

Figure 3: Overexpression of Nherf1 and Nherf2 in KS483 cells
Full length human Nherf1 and human Nherf2 cDNAs were stably transfected in the KS483 cell line. Using qPCR, the mRNA expressions of mouse and human were measured at day 4 and day 18 of osteoblast culture. Mouse mRNAs were continuously expressed during culture. Both human mRNAs showed a stable overexpression of about 10 fold compared to the mouse mRNA expression during differentiation.

of the mineralized matrix by Nherf1 overexpressing cell line, in which the mineralization was decreased, compared to the control cell line.
To examine whether Nherf1 or Nherf2 overexpression affected chondrocyte differentiation as well, cells were cultured as pellets for 4 weeks under chondrogenic conditions. At the end of culture the GAG production was measured (fig. 5) and the mineralized cartilage matrix was stained with von Kossa (fig. 6). While the production of GAGs in the Nherf1 and Nherf2 overexpressing cell lines was comparable to the GAG production in the control cell line (fig. 5),

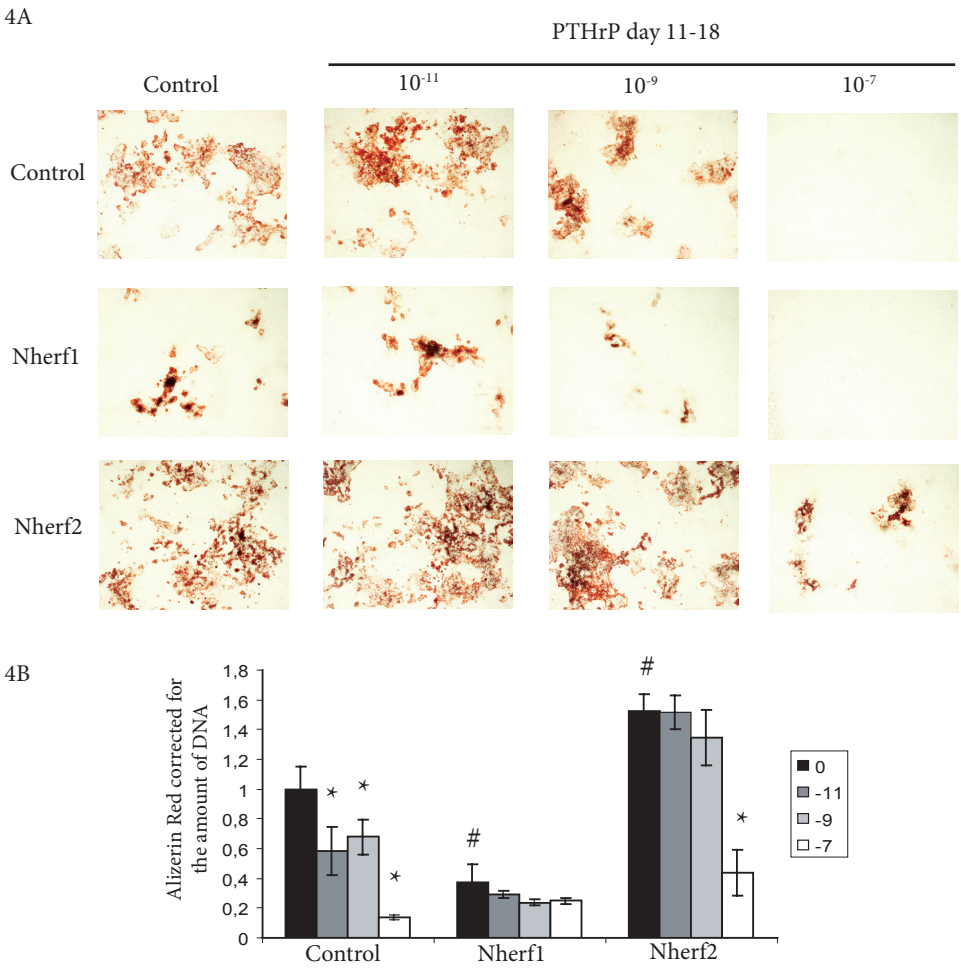

Figure 4: The effects of Nherf overexpression and PTHrP treatment on osteoblastic matrix mineralization
Cells were cultured under osteoblastic conditions for 18 days. From day 11-18, cells were treated with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 18 days the mineralized osteoblastic matrix was stained with alizarin Red (A) and the amount of alizarin Red was measured (B). Nherf1 overexpression inhibited, while Nherf2 overexpression stimulated matrix mineralization. In the control cell line, PTHrP dose dependently inhibited matrix mineralization. The highest dose completely inhibited mineralization. In addition, the highest dose of PTHrP also completely inhibited mineralization of the Nherf1 overexpressing cell line, this was, however, not significant. Matrix mineralization of the Nherf2 cell line was inhibited, although not completely by 10-7 M PTHrP. *Significant $p<0.05$ compared to untreated cultures.

mineralization of the cartilage matrix was not similar (fig. 6). Nherf2 induced mineralization of the cartilage matrix, whereas the Nherf1 overexpressing cell line, like the control cell line, had not produced a mineralized cartilage matrix.
Thus, Nherf1 and Nherf2 overexpression have distinct effects on terminal chondrocyte and osteoblast differentiation of KS483 cells.

The effect of PTHrP treatment in Nherf1 and Nherf2 overexpressing cell lines

In order to investigate whether Nherf1 and Nherf2 overexpression changed the biological response to PTHrP treatment in osteoblast differentiation, monolayer cultures were treated with a dose range of PTHrP from day 11-18. The osteoblastic matrix was stained with alizarin red (fig. 4A) and afterwards alizarin red was measured (fig. 4B). PTHrP inhibited the production of a mineralized matrix in the control cell line dose dependently. Nherf2 overexpression partly reversed this inhibition. Only the highest concentration (10^{-7} M) inhibited matrix mineralization in the Nherf2 overexpressing cell line, whereas all doses of PTHrP inhibited in the control cell line. Treatment with the highest dose of PTHrP also inhibited mineralization of the Nherf1 cell line (fig. 4A), this was however not significant (fig. 4B).

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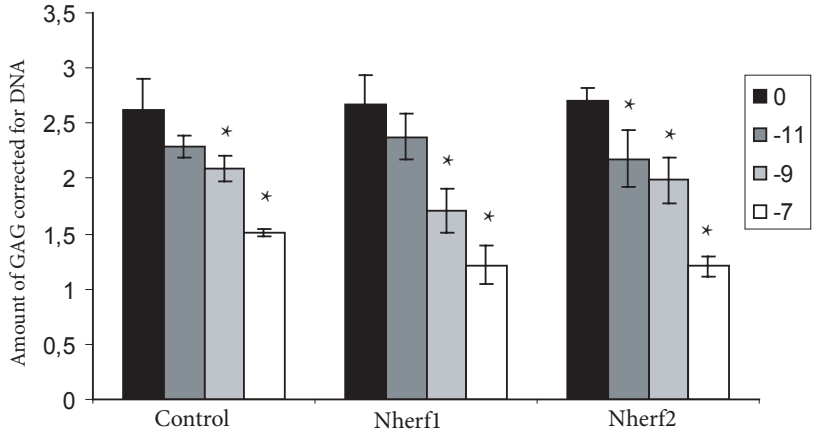

Figure 5: The effects of Nherf overexpression and PTHrP treatment on GAG production
Cells were cultured as pellets, to stimulate chondrocyte differentiation. Pellets were treated from week 2 to 4 with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 4 weeks the GAG production corrected for DNA was determined. The GAG production was not affected by Nherf overexpression. In all cell lines PTHrP inhibited dose dependently the GAG production. *Significant $p<0.05$ compared to untreated pellets.

To examine whether Nherf1 and Nherf2 overexpression altered the biological response to PTHrP treatment in chondrocyte differentiation, pellets were treated with a dose range of PTHrP from week 2-4. After 4 weeks, the GAG production was measured and the mineralized cartilage matrix was stained with von Kossa. In all cell lines, PTHrP treatment resulted in a dose dependent decrease of GAG production (fig. 5). In the control and Nherf1 overexpressing cell lines, a dose of 10^{-9} M PTHrP inhibited GAG production, while in the Nherf2 overexpressing cell line a dose of 10^{-11} M PTHrP already inhibited GAG production. Only the Nherf2 overexpressing cell line had produced a mineralized cartilage matrix and the highest dose of PTHrP (10^{-7} M) inhibited the production of mineral (fig. 6).
Thus, overexpression of Nherf1 or Nherf2 had only minor consequences for the biological actions of PTHrP.

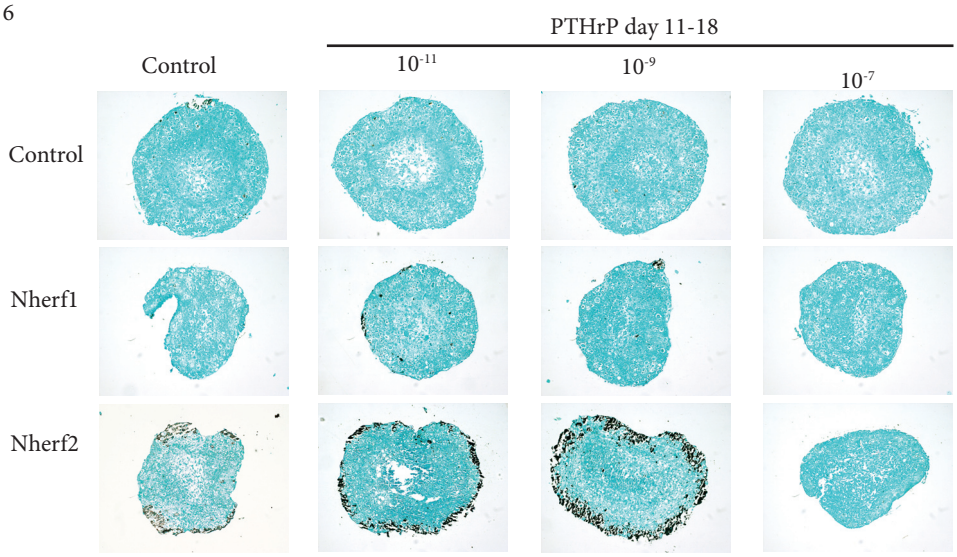

Figure 6: The effects of Nherf overexpression and PTHrP treatment on cartilage matrix mineralization
Cells were cultured as pellets, to stimulate chondrocyte differentiation. Pellets were treated from week 2 to 4 with a dose range of PTHrP (10-11, 10-9, 10-7 M). After 4 weeks the matrix was coloured with von Kossa to stain the mineralized matrix. The control and Nherf1 overexpressing cell line had not produced a mineralized cartilage matrix after 4 weeks. Nherf2 overexpression induced mineralization of the cartilage matrix. The highest dose of PTHrP inhibited the mineralization.

Discussion

Nherf has first been found in renal tubular cells as a regulator of the Na+/H+ exchanger[1]. The two isoforms of Nherf, Nherf1 and Nherf2, have not only been associated with the regulation of ion channels, but they also bind to other proteins, like transcription factors, signalling molecules and receptors, including the PTHR1[4;5]. The interactions of the Nherf proteins and PTHR1 are best studied in renal tubuli, where they redirect signalling from the AC/PKA pathway to the PLCβ/PKC pathway[4]. In this study we examined whether Nherf1 and Nherf2 mRNAs are also expressed by chondrocytes and osteoblasts *in vivo*. In addition, we investigated

the effects of Nherf1 and Nherf2 overexpression on chondrocyte and osteoblast differentiation *in vitro*. Furthermore, we examined whether Nherf1 and Nherf2 overexpression alters the biological response to PTHR1 signalling in chondrocyte and osteoblast differentiation.

To address the first question, whether Nherf1 and Nherf2 are expressed during endochondral bone formation, we performed in situ hybridizations for Nherf1 and Nherf2 on 18-days old mouse embryos and hind paws of 17-days old mouse embryos. We found a widespread expression of Nherf mRNAs, including expression in renal tubuli, which is line with other studies[16;17]. During endochondral bone formation, both Nherf1 and Nherf2 mRNAs were expressed by chondrocytes and by osteoblasts in the bone collar. However, no expression was found in pre-chondrocytes and pre-osteoblasts, suggesting that Nherf1 and Nherf2 exert their actions during later stages of chondrocyte and osteoblast differentiation and have no effect on progenitor cells. In line with the *in vivo* data, we detected Nherf1 and Nherf2 mRNA expression during osteoblast differentiation *in vitro* using KS483 progenitor cells.

The second question we addressed was whether Nherf1 and Nherf2 overexpression altered chondrocyte and osteoblast differentiation of KS483 progenitor cells. Generation of Nherf1 and Nherf2 overexpressing cell lines resulted in stable overexpression of Nherf1 and Nherf2 mRNA of about 10 fold during culture. No data about overexpression of Nherf1 and Nherf2 protein have been collected. Since overexpression of Nherf1 and Nherf2 resulted in biological responses, it is likely that Nherf1 and Nherf2 protein are overexpressed as well. This needs to be confirmed.

Overexpression of Nherf1 and Nherf2 had distinct effects on osteoblast differentiation. Overexpression of Nherf2 resulted in an increase in matrix mineralization. Probably, Nherf2 overexpression results in the recruitment of signalling mediators, as receptors and signalling molecules, involved in mineralization and thereby inducing the mineralization of the osteoblastic matrix. Since Nherf2 overexpression induced cartilage matrix mineralization as well, this mechanism is perhaps also operational in chondrocyte differentiation. In contrast to overexpression of Nherf2, overexpression of Nherf1 inhibited osteoblastic matrix mineralization. The negative effect of Nherf1 in bone formation has been demonstrated before in Nherf1 knockout mice[17]. Some, but not all female Nherf1 knockout mice, showed dwarfism and displayed severe osteoporosis and bone fractures. This is presumably a secondary effect of the mild hypophosphatemia due to ablation of Nherf1, since Nherf1 regulates the phosphate transport by binding to the sodium/phosphate co-transporter type IIa (Npt2a). However, our data suggests that the bone phenotype may also be explained partly by direct effects on osteoblastic matrix mineralization. The effect of Nherf1 overexpression on cartilage matrix mineralization was less clear. However, the control cell line had not yet produced a mineralized cartilage matrix after a culture period of 4 weeks as well.

Distinct functions for Nherf1 and Nherf2 have been reported in the kidney, in the regulation of ion channels[17-19]. Our results also suggest diverse functions in osteoblast and chondrocyte differentiation for Nherf1 and Nherf2. This might be explained by their distinct construction, which consists of several functional domains. Therefore, Nherf1 and Nherf2 may have a preference for binding to different sets of other PDZ proteins, like receptors, ion channels, signalling proteins, transcription factors and cellular structural proteins[2;3;16;20].

The third aim was whether Nherf overexpression alters the biological effect of PTHR1 signalling on osteoblast and chondrocyte differentiation. We have shown earlier that the PTHR1 is

expressed during KS483 osteoblastic differentiation and that PTHR1 signalling inhibits the mineralization of the osteoblastic matrix via various mechanisms[13]. Despite overexpression of Nherf1 and Nherf2, the highest dose of PTHrP inhibited matrix mineralization. Thus, Nherf1 and Nherf2 had only minor effects on the biological effects of PTHrP in this cell model.
Remarkably, while the lowest dose of PTHrP already inhibited osteoblastic matrix mineralization in the control cell line, only a high dose of PTHrP inhibited osteoblastic mineralization in Nherf2 overexpressing cell line. This discrepancy could be a direct effect of Nherf2, but we hypothesize that it is an indirect effect, through the presumably larger amount of mineralization at the time point of PTHrP treatment compared to the control cell line. For complete inhibition of the mineralization of the osteoblastic matrix, PTHrP treatment should start earlier during differentiation. Comparable to the control cell line, PTHrP treatment completely inhibited osteoblastic matrix mineralization in the Nherf1 overexpressing cell line. This inhibition was, however, not significant, because of the already low amount of mineralization in the untreated cells.
The effects of PTHrP on chondrocyte proliferation and differentiation in the growth plate have been studied extensively[8;21;22]. In agreement with these studies, we found an inhibition of PTHrP treatment on cartilage matrix mineralization in the Nherf2 overexpressing cell line. The effects of PTHrP treatment on cartilage matrix mineralization in the control and Nherf1 cell lines were less clear, due to the lack of mineralization in these pellets. Nherf1 and Nherf2 overexpression did not alter the effect of PTHrP treatment on GAG production. PTHrP dose dependently inhibited GAG production with only small differences, irrespective of Nherf overexpression.
Despite overexpression of Nherf1 or Nherf2 mRNA, overexpression did not result in major changes on biological actions of PTHrP. This may be explained by a lack of translation from Nherf mRNA into protein. However, it is likely that Nherf2 protein is overexpressed, since the Nherf2 overexpressing cell line induced biological responses in osteoblast and chondrocyte differentiation. In addition, the Nherf1 overexpressing cell line altered the biological response in osteoblast differentiation, indicating that Nherf1 protein is overexpressed as well. Another explanation could be the inefficient coupling of Nherf1 and Nherf2 to the PTHR1 in osteoblasts and chondrocytes, in contrast to renal kidney cells. In addition, levels of PLCβ could be too low to affect the dominant AC/PKA pathway. The measurements of the activation of the AC/PKA pathway and the PLCβ/PKC pathway are currently underway.
In summary, we showed that Nherf1 and Nherf2 are expressed during endochondral bone formation by chondrocytes and osteoblasts. In addition, our data suggest that Nherf predominantly alters the matrix mineralization of KS483 osteoblasts and chondrocytes. Finally, in our study Nherf overexpressing did not alter the biological response to PTHR1 signalling.

Acknowledgements

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Novel early target genes of PTHrP in chondrocytes

4

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Abstract

We have performed microarray analysis to identify PTHrP target genes in chondrocytes. ATDC5 cells were cultured as micromasses to induce chondrocyte differentiation. At day 8 of culture the cells had a pre-hypertrophic appearance. This time point was chosen for isolation of RNA at 0h, 1h, 2h, and 4h after a challenge with 10^{-7} M PTHrP. Samples were subjected to a cDNA microarray using competition hybridization.

A list of 12 genes ($p< 10^{-3}$), of which the expression regulation by PTHrP was confirmed by qPCR analysis, was generated. This included 7 upregulated and 5 downregulated genes. Three genes were known to be involved in PTHrP regulation and 6 were previously found in growth plate chondrocytes. Most of the genes (10 out of 12) were implicated in signal transduction and regulation. PTHrP also induced the expression of the upregulated genes in KS483 osteoblasts, suggesting involvement in a more generalized response to PTHrP. The vast majority of the upregulated genes (6 out of 7) contained CREB and/or AP-1 transcription factor binding sites in their promoter regions. Remarkably, a number of PTHrP regulated genes contained Stat transcription factor binding sites in their promoters. In transient transfection assays we show that PTHrP is able to positively regulate the activity of Stat3- and negatively regulate the activity of Stat5-specific promoter-reporter constructs in ATDC5 and UMR106 cells. In combination with the expression regulation of genes involved in Jak/Stat signalling, this data is suggestive for a previously not recognized interaction between PTHrP and Jak/Stat signalling.

Introduction

Longitudinal growth results from chondrocyte proliferation and subsequent differentiation in the epiphyseal growth plate, by a process called endochondral ossification. During this process resting chondrocytes in the stem cell zone enter the proliferative zone, start dividing and arrange in typical columns. These cells are characterized by secreting high amounts of collagen II. Subsequently, cells stop proliferating and start to differentiate into pre-hypertrophic chondrocytes, secreting collagen IX. Pre-hypertrophic cells further increase in size and become hypertrophic chondrocytes, which secrete high amounts of collagen X. The extracellular matrix becomes calcified and finally, mature chondrocytes undergo apoptosis, leaving a scaffold for bone formation.

Systemic hormones regulate longitudinal growth, partly via direct actions on growth plate chondrocytes through their receptors[1-3]. The direct and indirect actions of systemic hormones on growth plate chondrocytes were recently reviewed by Van der Eerden et al.[4]. The mechanism of action of the various factors involved in regulating the proliferation and differentiation of chondrocytes within the growth plate is still largely unknown. Systemic hormones probably interact with locally acting growth factors present in the growth plate, such as the Fibroblast Growth Factors (FGFs) or the members of the Indian Hedgehog (IHh)/ Parathyroid Hormone related Peptide (PTHrP) negative feedback loop, which is described to control the pace of chondrocyte differentiation[5;6].

In this study we have focused on the actions of PTHrP on growth plate chondrocytes. PTHrP plays a crucial role in controlling the pace of chondrocyte proliferation and differentiation in the growth plate, which is recognized by a number of studies. Knockout mice for PTHrP

show accelerated chondrocyte differentiation leading to dwarfism[7], while ectopic expression of PTHrP in chondrocytes inhibited their differentiation leading to a smaller cartilaginous skeleton[8]. In addition, PTHrP, the expression of which is tightly controlled by IHh[5], regulates both the rate and extent of chondrocyte proliferation by directly regulating the cell cycle machinery, partly by down regulation of the cyclin-dependent kinase inhibitor $p57^{Kip2}$[9].
PTHrP signals through the PTH/PTHrP Receptor 1 (PTHR1), which is predominantly expressed in pre-hypertrophic chondrocytes in the transition zone of the growth plate[10]. Binding of PTHrP to its receptor activates various signal transduction pathways. The dominant pathways result in activation of adenylate cyclase/protein kinase A (AC/PKA) and phospholipase C/protein kinase C (PLC/PKC)[11]. Downstream targets of PTH-signalling include the transcription factors cAMP response element-binding protein (CREB) and members of the AP-1 family, which are responsible, at least partly, for the genomic response. Indeed various PTH response genes have binding sites for these transcription factors in their promoters[12;13]. Recently, early response genes of Parathyroid Hormone (PTH) in osteoblasts were identified by microarray analysis[14].
In this study the chondrogenic ATDC5 cell line was used to identify PTHrP target genes in pre hypertrophic-like chondrocytes[15]. ATDC5 cells reproducibly differentiate into chondrocytes in four weeks in a monolayer culture[16]. This cell line is a representative model for studying the actions of PTHrP on chondrogenesis. During this process cells become responsive to PTHrP and in agreement with in vivo studies PTHrP inhibits hypertrophic chondrocyte differentiation[17;18].
We selected this cell line for the identification of early response genes of PTHrP. Using cDNA microarray analysis we identified 12 early response genes and confirmed their regulation by PTHrP using quantitative PCR in different cell culture models. Bio-informatic and functional analysis of a subset of these response genes, using transient transfection assays, revealed a previously not recognized level of interaction between PTHrP and Jak/Stat signalling.

Materials and Methods

Cell culture

ATDC5 cells were grown in Dulbecco's modified Eagle's medium /F-12 (DMEM/F12) (Invitrogen, Breda, The Netherlands) containing 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 10% charcoal stripped foetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 10 µg/ml insulin (Sigma Chemical Co., St Louis, MO, USA), 10 µg/ml bovine transferrin (Roche, Almere, The Netherlands), and 3 x 10^{-8} M sodium selenite (Roche), in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The micromass culture technique was modified from Ahrens et al.[19]. Trypsinized cells were resuspended in medium at a concentration of 2 x 10^7 cells/ml, and 3 drops of 10 µl of this cell suspension were placed in a well of a standard 12 wells culture plate. The cells were allowed to adhere for 2 hours at 37°C and 5% CO_2, and then 1 ml medium was added to each well. The medium was replaced every other day. KS483 mesenchymal progenitor cells were differentiated into osteoblasts as described previously[20]. UMR106 cells were cultured in DMEM (Invitrogen) containing 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 10% FCS (Integro BV).

cAMP enzymimmunoassay

To establish the responsiveness of ATDC5 cells to PTHrP, intracellular cAMP accumulation was measured as previously described[21], using an enzymimmunoassay (Amersham, Freiburg, Germany), according to the manufacturers protocol. For this purpose, ATDC5 micromasses were challenged with a dose range of PTHrP(1-34) at day 7 and 14 of culture.

RNA isolation and amplification

Medium was refreshed after 7 days and the ATDC5 micromasses were challenged with 10^{-7} M PTHrP(1-34) at day 8 of culture (time point 0h) and total RNA was extracted at different time points in triplicate by using Trizol LS Reagent (Invitrogen), followed by RNA cleanup with RNeasy mini kit (Qiagen, Maryland, USA). RNA concentrations were determined by measuring the absorbance at 260 nm. Next, RNA samples were pooled and time point 0h was chosen as reference sample. Total RNA (3 µg/reaction) was amplified as described before[22], with slight modifications. In short, first strand cDNA was synthesized by adding 500 ng T7-oligodT primer (5'-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGC $G(T)_{21}$-3') to 10 µl RNA sample. Samples were incubated for 10 minutes at 70°C, followed by 60 minutes at 42°C in a total volume of 20 µl, containing 5x first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 2U Rnasin (Promega, Leiden, The Netherlands), and 200U Superscript II RT (Invitrogen). Next, second strand cDNA was synthesized for 2 hours at 16°C in a total volume of 150 µl, containing 5x second strand buffer, 0.2 mM dNTP's, 10U DNA Ligase (Invitrogen), 40U DNA polymerase I (Invitrogen), and 2U Rnase H (Invitrogen). This was followed by addition 2 µl T4 polymerase (5 U/µl) (Invitrogen) and incubation for 5 minutes at 16°C. The double-stranded cDNA reaction was stopped and the remaining RNA in the mixture was degraded by addition of 7.5 µl of 1 M NaOH, 2 mM EDTA by incubation at 65°C for 10 minutes. Samples were purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction. The volume of the aqueous phase was increased to 450 µl using H_2O. For further purification, samples were transferred to a Centricon-100 microconcentrator column (Millipore, Amsterdam, The Netherlands) (pre-spinned with 450 µl H_2O) and centrifuged for 12 minutes at 2500 rpm. After 3 wash steps with 450 µl H_2O, cDNA was collected in a total volume of 7 µl by inverting the column and by centrifuging for 30 s at 13000 rpm. Subsequently, cDNA was transcribed into cRNA using the T7 high yield transcription kit (Epicentre, Madison, USA). The cDNA solution was incubated at 42°C for 3 hours in a total volume of 20 µl, containing 10x T7 reaction buffer, 7.5 mM ATP, CTP, GTP, and UTP, 10 mM DTT, 2U Rnasin, and 2 µl Ampliscribe T7 enzyme solution, followed by sample concentration using centricon-100 microconcentrator columns. This method was based on the original protocol of Van Gelder et al.[23]. Finally, cRNA concentration was determined by measuring the absorbance at 260 nm.

Probe labelling

cRNA (1.2 µg) was reverse transcribed with random hexamer primers, and labelled by incorporation of cyanine 5-dUTP (Cy5) or cyanine 3-dUTP (Cy3) (NEN, Boston, USA) according to the protocols of Ross et al.[24], with slight modifications. In short, cRNA and 8 µg random primers (Roche) in a total volume of 15 µl was incubated for 10 minutes at 70°C. Subsequently, 6 µl 5x first strand buffer, 3 µl 0.1 M DTT, 0.6 µl low-T dNTPs, 3 µl Cy3

dUTP (time point 0h) or Cy5-dUTP (other time points), and 1 µl Superscript II RT (200 U/µl) were added, incubated for 10 minutes at room temperature, followed by incubation at 42°C for 90 minutes. After 60 minutes fresh Superscript II RT (1 µl) was added. Next, RNA was degraded, by addition of 15 µl 0.1 M NaOH and incubation for 10 minutes at 70°C, after which the solution was neutralized by addition of 15 µl 0.1 M HCl. The labelled samples, supplemented with 180 µl 10 mM Tris, 1mM EDTA, pH 8 (TE) and 10 µl mouse Cot-1 DNA (10 mg/ml) (Invitrogen), were pooled and purified using a centricon-30 microconcentrator column (Millipore) (pre-spinned with 450 µl TE for 8 minutes at 13.000 rpm). PolyA RNA (20 µg) (Amersham) and yeast tRNA (20 µg) (Invitrogen) were added to 450 µl TE during the second wash step. The purified product was collected, by inverting the column and by centrifuging for 1 minutes at 13000 rpm, and finally, resuspended in a total volume of 45 µl hybridization solution, containing 7.65 µl 20x SSC and 1.35 µl 10% SDS.

(Pre-) hybridization

For the hybridization experiments microarrays, on which the NIA 15k mouse cDNA clone set[25] was spotted, were purchased from the Leiden Genome Technology Center (LGTC). DNA was crosslinked by UV irradiation at 65 mJ/cm^2 (Stratalinker mode 1800 UV Illuminator, Stratagene). To prevent non-specific hybridization, the slides were incubated in 45 µl hybridization solution (400 ng/µl yeast tRNA, 400 ng/µl poly(A) RNA, 400 ng/µl herring sperm DNA (Invitrogen), 100 ng/µl mouse Cot1 DNA, 5x Denhardt's solution, 3.2x SSC and 0.4% SDS) at 65°C for 30 minutes. Prior to hybridization, the slides containing the pre-hybridization mixture were incubated for 2 minutes at 80°C to denature the spotted DNA. After pre-hybridization, the slides were washed twice in 2x SSC for 5 minutes at room temperature and dehydrated with subsequent steps of 5 x 5 minutes 70%, 5 minutes 90% and 5 minutes 100% ethanol. For hybridization, the probes were denatured by heating for 2 minutes at 100°C, left at room temperature for 15 minutes, centrifuged for 10 minutes, and placed under a 24 mm x 60 mm glass coverslip. The slides were incubated overnight at 65°C in a hybridization chamber (Corning, Amsterdam, The Netherlands) and washed the next day in 2x SSC for 5 minutes at room temperature and dehydrated using graded ethanols.

Microarray design and statistical analysis

The reference array experiments, 0h vs 0h, were hybridized in duplicate, 1h vs 0h and 2h vs 0h in triplicate, and 4h vs 0h in quadruplicate. Following hybridization, slides were scanned in the Agilent DNA Microarray scanner (Agilent Technologies, Amstelveen, The Netherlands). Genepix 3.0 software (Axon Instruments Inc.) was used to quantify the resulting images. Subsequently, normalization and gene expression analysis were performed with Rosetta Resolver (Rosetta Biosoftware). Due to the overall poor quality of the cDNA spots on the microarray, stringent selection criteria were used for inclusion of spots, to minimize the risk of false positive signals. A spot was only included if the spot settles the selection criteria in all time points. Spots showing an absolute fold change of 2 or spots showing significantly regulation ($p<0.01$) in the reference array, and flagged spots in all arrays were excluded from analysis. For further analysis, spots were selected from the remaining list if the signal intensity of Cy3 or Cy5 was above a cut off level (0.05), and the absolute fold change was under 50.

Analysis of variance (ANOVA) was performed between the remaining spots of the 4 different hybridizations. Spots showing significant ($p<0.001$) differential expression regulation, with intensity value above background value, and with a fold change >1.8, for upregulated genes, and <0.55, for downregulated genes, were selected for further analysis. To identify the selected spots, the PCR-amplified cDNA of each spot (500 ng) was sequenced by the LGTC using 12 pmol M13 primers in a total volume of 24 µl.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from ATDC5 micromasses, cultured for 7 and 14 days, using Trizol LS Reagent (Invitrogen), and reverse transcribed into cDNA, using random hexamer primers (Amersham). Semiquantitative PCR was performed for beta-2 microglobulin (β2m), collagen II, collagen IX, collagen X, and PTHR1 under the following conditions: cDNA was denatured at 94°C for 5 minutes, followed by cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, and final extension at 72°C for 10 minutes (table 1).

Name	F/R	Primer	cycli
β2µ	F	TGACCGGCTTGTATGCTATC	25
	R	CAGTGTGAGCCAGGATATAG	
PTHR1	F	TGCTTGCCACTAAGCTTCG	30
	R	TCCTAATCTCTGCCTGCACC	
Collagen II	F	GCCAAGACCTGAAACTCTGG	27
	R	GCGATGCTGTTCTTACAGTGG	
Collagen IX	F	CTGTGTGTGCAGTTGTCTGG	27
	R	CCAGTGCTTTTCAAGTGTGC	
Collagen X	F	GCCTCGAGCCCAAAAGGCGTCTCATATTT	35
	R	GGCTCGAGCCCAGGAGCAGTGGATAATG	

Table 1 : RT-PCR primer sets
F: forward primer, R: reverse primer

RNA isolation from KS483 osteoblasts

After 11 day of culture, KS483 cells have formed bone nodules and the cells within the nodules start to mineralize the extracellular matrix. This time point was chosen to stimulate the cells with 10^{-7} M PTHrP(1-34). RNA was isolated after 1, 3, and 6 hours using Trizol LS Reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

Quantitative PCR (qPCR)

To validate the expression patterns of PTHrP target genes, quantitative PCR was performed using the BioRad iCycler (Biorad, Veenendaal, The Netherlands). For each gene a set of primers was designed (table 2), which spanned at least 1 intron-exon boundary and had an optimal annealing temperature of 60°C, using the Applied Biosystems software program Primer Express. cDNA (5 ng) was amplified in triplicate using the qPCR core kit for SYBR

green 1 (Eurogentec, Maastricht, The Netherlands), under the following conditions: cDNA was denatured for 10 minutes at 95˚C, followed by 40 cycles, consisting of 30 s at 95˚C, 20 s at 60˚C, and 40 s at 72˚C. From each sample a melting curve was generated to test for the absence of primer dimer formation and DNA contamination. Each reaction contained 5 µl cDNA (1 ng/µl), 10x reaction buffer, 3 or 4 mM $MgCl_2$ (table 2), 40 µM dNTP's, 300 nM primer, 0.75 µl SYBR green, and 0.1 µl HotGoldStar polymerase in a total volume of 25 µl. Fold changes, adjusted for the expression of β2m, were calculated and log transformed using the comparative method[26].

Name	F/R	Forward primer	MgCl
β2µ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
RGS2	F	GGAAGCCCAAAACTGGCAA	3 mM
	R	CTGCCCAGAGCTGCGCT	
SGK	F	CTCCTCCGCCAAGTCCCT	3 mM
	R	TGCCTAGCCAGAAGAACCTTTC	
Ptp4a1	F	CCTTGGCAGAGCTCCGGT	3 mM
	R	GGAGCCGCATTTTCGGAC	
Upar	F	CAATGGTGGCCCAGTTCTG	3 mM
	R	TCCCAGCACATCTAAGCCTGT	
IER3	F	CTCTACCCTCGAGTGGTCCG	4 mM
	R	CTCAGGTGTCACGGCGC	
Stat3	F	AGCCCCGGAGACAGTCGA	3 mM
	R	AAACACCAACGTGGCATGTG	
Csrp2	F	CCACTCGGAATGCCTGTCTG	3 mM
	R	GCCACTGTTGTGCTGTCTAAATTTT	
Sf3a2	F	CATCAGACTAACTTGGCCCGG	3 mM
	R	TCTCAGGGTAGTCAATCTGGAACAG	
Acvr2b	F	GACGGGCCTGTCGATGAGTA	3 mM
	R	TCATGGTCCCAGCACTCCTC	
Gab1	F	GACAGAAAAGTCAAGCCGGC	3 mM
	R	GGACATGGGAAACCTAGAGGAGT	
LamRI	F	CAACAACAAGGGAGCTCACTCA	4 mM
	R	TCTCAATCTCCTCTGGGTCTCTG	
Dym	F	AAAGTCCCCCGGCAGCTA	3 mM
	R	TGGGCCTTGCATCAGGTACT	
Arpc4	F	CGCGATGACTGCCACTCTC	3 mM
	R	CAATTTCATCGGCCTGCTTC	
Aurkb	F	GGACTGCCACGATCATGGA	4 mM
	R	CGCACATGGTCTTCCTCCTC	
Uchl5	F	TTTTTGCCAAGCAGGTAATTAATAATG	4 mM
	R	GCCCCTTCATAGCTGCATCA	
Akr1b8	F	CACCTGGAAGTCTCCCCCA	3 mM
	R	AATGAAGAGGTCCTCCCGCT	

Table 2 : qPCR primer sets.
F: forward primer, R: reverse primer

Identification of transcription factor binding sites

To find evolutionary conserved transcription factor binding sites in promoters of target genes, sequences surrounding the transcription start site, from –1000 bp to +100 bp, were extracted from the NCBI database and were aligned to either the human or rat genome using the evolutionary conserved regions (ECR) browser[27]. rVista 2.0 was used to predict conserved regulatory elements and define transcription factor binding sites, with similarity predefined as 0.85 (complete match has a value of 1)[28]. Consensus binding sequences, which were predicted in 4 or more promoters, were selected.

Transient transfection

ATDC5 cells were seeded at a density of 10000 cells/cm^2 and UMR106 cells were seeded at a density of 30000 cells/cm^2 in a 24-wells plate. The cells were kept in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The second day the cells were transiently co-transfected with 1 μg of the reporter construct and with either 100 ng HA-Stat3 expression vector (kindly provided by T. Hirano, Department of Molecular Oncology, Osaka university, Japan[29]), 100 ng Stat5a, 100 ng Stat5b expression vector (kindly provided by Warren J. Leonard, National Institutes of Health, Bethesda, Maryland, USA[30]) or 100 ng pcDNA3.1 expression construct using Fugene 6 transfection reagent (Roche, Basel, Switzerland), according to the manufacturers protocol. The following reporter constructs were used: a Stat3 specific reporter promoter construct (kindly provided by Dr. I. Touw, Erasmus Medical Center, Rotterdam, The Netherlands[31]), and a Stat5 specific reporter promoter construct (kindly provided by Peter Storz, University of Stuttgart, Stuttgart, Germany[32]). To correct for transfection efficiency, 25 ng CMV renilla was included in all transfection experiments. The next morning the medium was changed and at the end of the day cells were treated with a dose range of PTHrP(1-34) (10^{-9}, 10^{-8} and 10^{-7} M). After 20 hours luciferase assays were performed using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturers protocol. Luciferase activity was measured using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin-Elmer, Boston, MA, USA). Firefly luciferase activity was corrected for renilla luciferase activity.

Statistics

Values represent mean ± SEM. Differences were examined by analysis of variance (ANOVA) followed by the post-hoc least significant difference test (LSD). Results were considered significant at $p<0.05$.

Results

Characterization of ATDC5 micromass cultures

To induce chondrocyte differentiation, ATDC5 cells were cultured as micromasses. Already after 7 days a homogeneous cell pellet was formed, containing an alcian blue positive cartilage matrix. Both the size of the micromasses and the intensity of the alcian blue staining increased further after 14 days of culture (fig. 1A). Histological analysis at 7 days showed rounded chondrocytes embedded in an alcian blue positive extracellular matrix (fig.1B). RNA was isolated and used for PCR analysis to study the expression of typical cartilage markers (fig. 1C). Collagen II was expressed at 7 days and tended to decline at 14 days. Markers for pre-

hypertrophic (collagen IX) and for hypertrophic chondrocytes (collagen X) were present at 7 days and were increased at 14 days. PTHR1 mRNA did not change in the differentiation process (fig. 1C), but the responsiveness of the ATDC5 cells to PTHrP increased with differentiation (fig. 2).
Taken together, culturing the ATDC5 cells as micromasses efficiently induced chondrocyte differentiation. After 1 week of culture, the cells responded to PTHrP and expressed markers of pre-hypertrophic chondrocytes, which are the main target cells for PTHrP in the growth plate. For this reason, this time point was chosen for identification of PTHrP target genes.

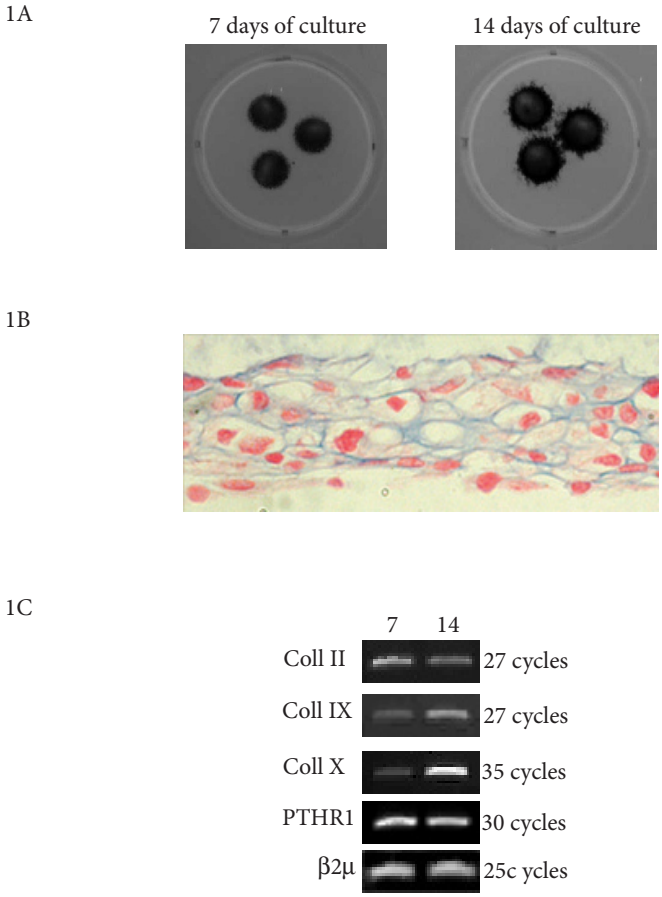


Figure 1: Phenotypic characterization of ATDC5 micromass cultures.
(A) ATDC5 micromass cultures were cultured for 7 and 14 days and stained for alcian blue. (B) Section of an ATDC5 micromass, cultured for 9 days, and stained for alcian blue (C) Expression pattern of coll II, coll IX, coll X, PTHR1, and β2μ mRNAs at day 7 and 14 of ATDC5 micromass differentiation.

2

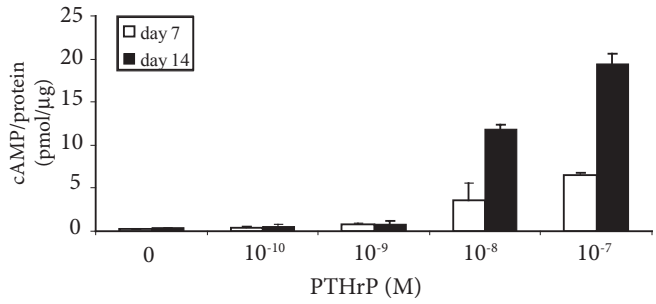


Figure 2: Responsiveness of ATDC5 cells to PTHrP during differentiation.
Intracellular cAMP concentration corrected for protein was measured at day 7 and 14 of culture, following stimulation with a dose range of PTHrP.

Identification and selection of PTHrP target genes

ATDC5 cells were cultured as micromasses and stimulated at day 8 with 10^{-7} M PTHrP for 1, 2, and 4 hours and cDNA microarray analysis was performed. After applying stringent selection criteria, 7843 out of the 15442 spots were taken in the analysis. A list of 31 spots, which exhibited significant ($p<0.001$) differential expression after 1, 2 or 4 hours following PTHrP stimulation, was generated. Sequence analysis revealed that 3 genes were present in two different spots and 1 gene in 3 spots. The expression patterns for the duplicate and triplicate spots were identical. This reduces the number of response genes to 26 (table 3). From this list 16 genes were chosen for validation experiments in ATDC5 cells. Exclusion criteria were insufficient information on gene identity, for example ESTs, and insufficient information for the design of qPCR primer sets, which span intron-exon boundaries.

The expression patterns of the selected genes were first validated by studying expression profiles using qPCR on the same RNA samples subjected to microarray analysis (fig. 3B, D, and F). As internal standard β2µ, which was not regulated by PTHrP in microarray analysis (data not shown), was used. Four out of 16 genes could not be validated and were discarded from further study, leaving 12 genes for more detailed analysis (table 4). In qPCR experiments RGS2, Stat3, Csrp2, Upar, and IER3 showed the same expression profile compared to the data from microarray analysis (fig. 3B and D). The expression pattern of early upregulated target genes SGK and Ptp4a1 were slightly different. SGK was only upregulated after 1 hour and Ptp4a1 after 2 and 4 hours, instead of continuously up regulation of both target genes revealed by microarray analysis. The downregulated target genes, Sf3a2, Acvr2b, Gab1, LamRI, and DYM showed in the qPCR data down regulation at all time points, instead of transient down-regulation at 1 hour after PTHrP stimulation found by microarray analysis (fig. 3F). The expression patterns were confirmed in RNA samples from ATDC5 cells isolated in a new independent experiment (data not shown).

Name	Acc. Nr.	up/down	Ratio 0h[2]	Ratio 1h[2]	Ratio 2h[2]	Ratio 4h[2]	selected
Regulator of G-protein signaling 2 (RGS2)	NM_053453	up	1,00	8,53	22,18	2,85	X
Serum glucocortioid regulated kinase (SGK)	NM_011361	up	0,90	2,63	1,88	2,82	X
Protein tyrosine phosphatase 4a1 (Ptp4a1)	NM_011200	up	1,01	1,78	2,63	4,13	X
Urokinase plasminogen activator receptor (Upar)	NM_011113	up	0,90	1,84	3,74	1,16	X
Immediate early response 3 (Ier3)	NM_13366	up	0,86	2,75	3,54	2,93	X
Signal transducer and activator of transcription 3 (Stat3)	NM_011486	up	0,86	0,74	1,17	2,72	X
Cysteine and glycine-rich protein 2 (Csrp2)	NM_007792	up	1,12	1,15	1,78	2,26	X
Splicing factor 3a, subunit 2 (Sf3a2)	NM_013651	down	1,04	0,34	0,74	0,85	X
Activin receptor IIb (Acvr2b)	NM_007397	down	1,13	0,47	0,93	0,955	X
Growth factor receptor bound protein 2–associated protein 1 (Gab1)	NM_021356	down	1,38	0,42	1,01	0,92	X
Laminin receptor I (LamRI)	NM_011029	down	1,62	0,22	0,96	1,34	X
Dymeclin (Dym)	NM_027727	down	0,87	0,17	0,59	0,71	X
GDP-mannose pyrophosphorylase B (gmppb)	NM_177910	down	1,06	0,12	0,58	0,99	
actin related protein 2/3 complex, subunit 4 (Arpc4)[1]	NM_026552	down	0,85	0,55	1,01	0,83	X
RNA binding motif protein 16 (Rbm16)	BC075621	down	1,02	0,27	0,88	0,89	
aldolase 1 (Aldoa)	NM_007438	down	0,88	0,53	0,89	0,94	
Aurkb: aurora kinase B[1]	NM_011496	down	0,78	0,38	0,60	0,67	X
heparan sulfate (glucosamine) 3-O-sulfotransferase 1 (Hs3st1)	NM_010474	down	0,79	0,30	0,60	0,71	
ubiquitin carboxyl-terminal esterase L5 (Uchl5)[1]	NM_019562	down	1,03	0,04	0,65	0,86	X
aldo-keto reductase family 1, member B8/7 (Akr1b8)[1]	NM_008012	down	0,94	0,49	0,85	0,89	X
phosphatidylinositol glycan, class T (Pigt)	NM_133779	down	1,20	1,89	0,75	0,84	
KCNQ1 overlapping transcript 1 (Kcnq1ot1)	AF119385	down	1,03	0,07	0,57	0,64	
betaine-homocysteine methyltransferase (Bhmt)	NM_016668	down	1,18	0,24	0,79	0,84	
eukaryotic translation elongation factor 1 alpha 1 (Eef1a1)	NM_010106	down	0,76	0,48	0,75	0,81	
EST	NM_133349	down	0,83	0,84	0,53	1,09	
EST	AC155250	down	1,11	0,21	0,71	0,87	

Table 3: Genes regulated by PTHrP in chondrocytes revealed by statistical analysis of the microarray data.

[1]) These genes could not be validated in qPCR analysis

[2]) Significant ($p<0.001$) differential expression regulation.

Reg.[1]	Abbr.[2]	Name	Function	rp[3]	gp[4]
up	RGS2	Regulator of G-protein signaling 2	Inhibitor of G-protein coupled receptor protein activation.	yes	no
up	SGK	Serum glucocortioid regulated kinase	involved in signal transduction pathways.	yes	no
up	Ptp4a1	Protein tyrosine phosphatase 4a1	Plays a regulatory role in a variety of cellular processes.	no	yes
up	Upar	Urokinase plasminogen activator receptor	Regulator of cell-surface plasminogen activation.	yes	no
up	IER3	Immediate early response 3	Anti apoptotic characteristics.	no	no
up	Stat3	Signal transducer and activator of transcription 3	Transcription factor involved in GH signalling.	no	yes
up	Csrp2	Cysteine and glycine-rich protein 2	partner of protein inhibitor of activated STAT1 (PIAS1).	no	yes
down	Sf3a2	Splicing factor 3a, subunit 2	Binds microtubules and can bundle microtubules in vitro.	no	no
down	Acvr2b	Activin receptor IIb	Member of TGFb-R superfamily, expressed during chondrogenesis.	no	yes
down	Gab1	Growth factor receptor bound protein 2–associated protein 1	Involved in signalling from various receptors (GH/IL6).	no	no
down	LamRI	Laminin receptor I	Involved in interactions between cells and ECM.	no	yes
down	Dym	Dymeclin	Participates in proteoglycan metabolism.	no	yes

Table 4: List of validated PTHrP target genes.

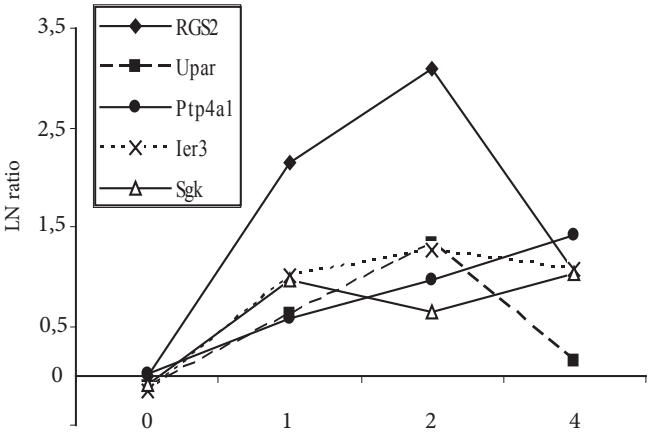
[1]) Reg. = regulation

[2]) Abbr. = abbreviation

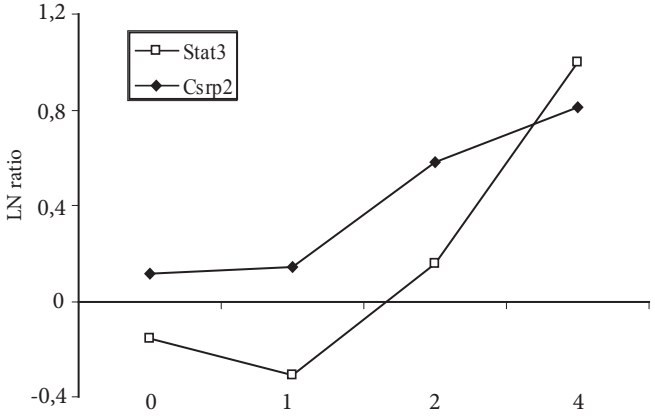
[3]) rp = previously been implicated in PTHrP signaling

[4]) gp = previously been found in growth plate chondrocytes

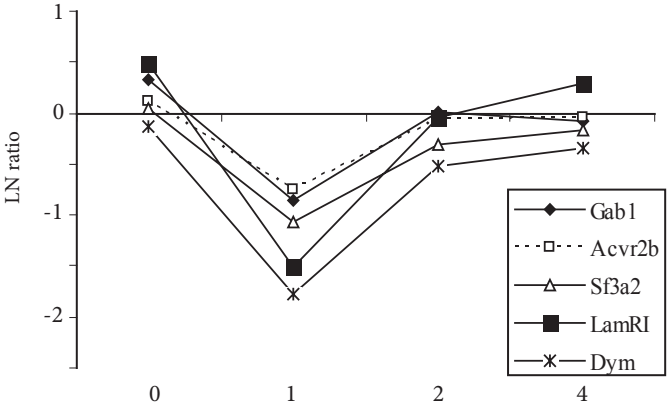
3A


RGS2
Upar
Ptp4a1
Ier3
Sgk
LN ratio
3,5
2,5
1,5
0,5
0
0
1
2
4

3C


Stat3
Csrp2
LN ratio
1,2
0,8
0,4
0
-0,4
0
1
2
4

3E


Gab1
Acvr2b
Sf3a2
LamRI
Dym
LN ratio
1
0
-1
-2
0
1
2
4

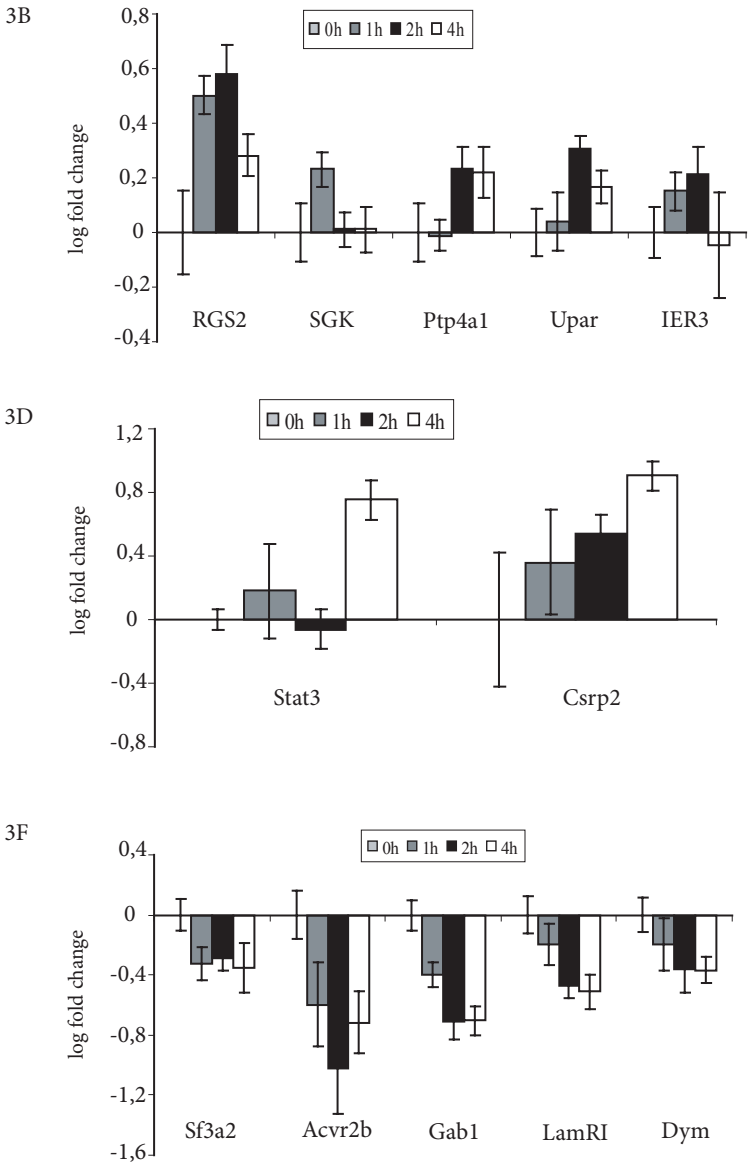


Figure 3: expression profiles of PTHrP target genes in ATDC5 chondrocytes revealed by microarray analysis and qPCR analysis.

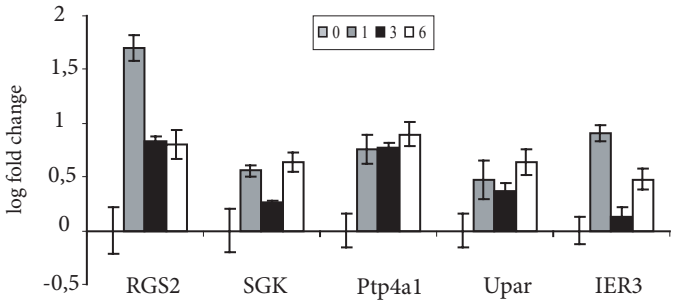
RNA was isolated from ATDC5 micromass cultures at 0h, 1h, 2h and 4h after PTHrP stimulation, amplified and labelled as described. Samples were hybridized against 0h. Based on the expression profiles revealed by microarray analysis, the genes were divided over 3 clusters. (A) Cluster 1 contained 5 immediate early upregulated genes, i.e. RGS2, SGK, Ptp4a1, UPAR, and IER3. The expression pattern revealed by qPCR is shown in (B). (C) Cluster 2 contained 2 immediate early upregulated genes, i.e. STAT3 and Csrp2. The expression pattern revealed by qPCR is shown in (D). (E) Cluster 3 contained 5 immediate downregulated genes, i.e. Sf3a2, Acvr2b, Gab1, LamRI, and DYM. The expression pattern revealed by qPCR is shown in (F).

Validation of PTHrP target genes by qPCR

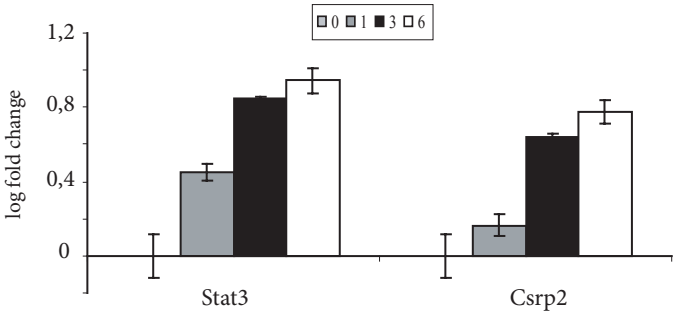
Among these 12 identified PTHrP target genes (table 3) 7 genes were upregulated and 5 genes were downregulated. Most of the genes were implicated in signal transduction and regulation and were intracellular mediators or receptors. Three genes were already known target genes of PTHrP in osteoblasts and 6 genes have previously been found in growth plate chondrocytes. Based on the expression patterns, the genes were divided into 3 groups, 2 groups for upregulated genes and 1 group for downregulated genes (fig. 3A, C, and E). Genes in expression pattern 1 were upregulated after 1 hour and genes in expression pattern 2 were upregulated after 2 and 4 hours. The genes in expression pattern 3 were downregulated after 1 hour. Expression pattern 1 contained RGS2, SGK, Upar, IER3, and Ptp4a1; Stat3 and Csrp2 formed expression pattern 2; and expression pattern 3 contained Sf3a2, Gab1, DYM, LamRI and Acvr2b.

To test whether the response of these target genes was restricted to chondrocytes or whether it was part of a more generalised response to PTHrP, the expression patterns were analysed in differentiated KS483 osteoblasts using qPCR (fig. 4). For this purpose, RNA was isolated after 0, 1, 3, and 6 hours after a challenge with PTHrP. The expression patterns of all upregulated genes were comparable with the expression patterns found in chondrocytes. The expression patterns of the downregulated genes in KS483 osteoblasts were less clear. All genes responded to PTHrP. Only Gab1 was downregulated, although this regulation was transiently instead of continuously as in ATDC5 cells. Remarkably, Sf3a2 and Dym were upregulated instead of downregulated. No consistent pattern of regulation was found for Acvr2b and LamRI.

4A



4B



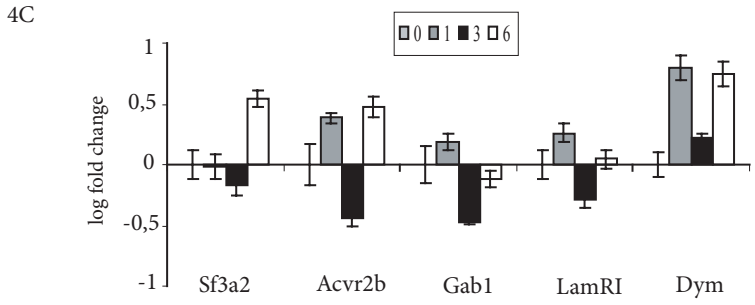


Figure 4: Expression patterns of PTHrP target genes in KS483 osteoblasts.
The genes were grouped according to expression profiles revealed by microarray analysis in chondrocytes as described in fig. 3. RNA was isolated from KS483 osteoblasts, stimulated with PTHrP at day 11, after 0h, 1h, 3h and 6h and qPCR was performed. The expression of the genes in expression pattern 1 in chondrocytes (A), in expression pattern 2 in chondrocytes (B), and in expression pattern 3 in chondrocytes (C) were determined by qPCR.

Transcription factor binding sites

We subsequently analyzed promoter regions (sequences from -1000 bp to + 100 bp from the transcription start site) of target genes to identify common regulatory elements involved in their expression regulation. To decrease the number of false positive results, we searched for transcription factor binding sites that were conserved in human and rat genomes. The promoter regions of RGS2, SGK, Ptp4a1, Stat3, Acvr2b, Gab1, and LamRI were aligned to the human genome and the promoter regions of IER3, Upar and Csrp2 to the rat genome. Insufficient information was available in the database to align the promoter regions of Sf3a2 and Dym with the human and the rat genomes, preventing the selection of evolutionary conserved binding elements in these genes. Table 5 shows transcription factors of which binding sequences have been found in 4 or more promoters of the identified PTHrP target genes.

ATF/CREB binding sites were predicted in the promoter regions of 6 out of 7 upregulated genes, 4 times in combination with an AP-1 binding site. ATF/CREB binding sites were also predicted in 2 downregulated genes, but never in combination with an AP-1 binding site. Remarkably, 1 of these genes was Dym, which was upregulated in KS483 osteoblasts. Another interesting finding was the identification of Stat transcription factor binding sites, predominantly in the promoter regions of upregulated PTHrP target genes. Binding sites for the transcription factor ELK1, which is, like ATF/CREB, AP-1, and Stat, involved in immediate early responses, were predicted in 7 promoter regions. Binding sites for transcription factor E2F and SP1, both of which are facilitating factors involved in gene transcription, were distributed over up and downregulated target genes and were predicted in 10 out of 12 and 6 out of 12 promoter regions, respectively.

Reg.[1]	Name	ATF/ CREB[2]	AP1	E2F[2]	SP1[2]	ELK1[2]	Stat1[2]	Stat3[2]	Stat6[2]
up	RGS2	X	X				X		X
up	SGK	X	X	X		X	X	X	X
up	Ptp4a1	X(3)	X	X(2)					
up	Upar	X	X		X		X		
up	IER3			X(2)	X(2)	X(2)	X	X	X(2)
up	Stat3	X(2)		X	X(2)	X(2)	X	X	
up	Csrp2	X		X(4)	X	X(2)		X(3)	X(2)
down	Sf3a2			X(3)	X	X(2)	X(2)	X	
down	Acvr2b			X(2)	X(4)				
down	Gab1			X					
down	LamRI	X		X		X			
down	Dym	X(2)		X(4)		X(2)	X(2)	X	

Table 5: Transcription factor binding sites.
Conserved transcription factor binding sites predicted 4 times or more in the promoter regions of the PTHrP target genes. X: transcription factor binding site is present in promoter region. [1]) Reg.: Regulation [2]) In case more than 1 site is present in the promoter regions the number of hits is indicated between brackets.

Stat regulation by PTHrP

In order to investigate the biological significance of the interactions between PTHrP and Stat signalling, transient transfection experiments were performed. In contrast to undifferentiated cells, differentiated ATDC5 (and KS483) cells could not be transfected. Since undifferentiated ATDC5 cells express very low amounts of the PTHR1, experiments were also performed in UMR106 cells, which can easily be tranfected and have higher PTHR1 expression levels.
In line with the upregulation of Stat3 mRNA revealed by microarray analysis, PTHrP induced activity of a Stat3 reporter in both cell types dose dependently with a maximal fold induction of 1.4 in ATDC5 cells (fig. 5A) and 2.5 in UMR106 cells (fig. 5B). In the presence of Stat3 expression vector, luciferase activity of the Stat3 reporter was enhanced in ATDC5 cell, but not in UMR106 cells (fig. 5A and B). PTHrP also further increased Stat3 reporter activity in the presence of Stat3 expression vector in both cell lines.
To investigate whether the regulation of Stat proteins by PTHrP is part of a more generalized interaction between PTHrP and Jak/Stat signalling, we subsequently studied the regulation of Stat5a and Stat5b by PTHrP. Comparable to Stat3, the mRNA expression of Stat5a (fig. 6A) and Stat5b (fig. 6B) was upregulated after 4 hours of PTHrP treatment in ATDC5 cells by qPCR. Transient transfection experiments were performed in ATDC5 cells and in UMR106 cells, using a Stat5 promoter-reporter construct in the absence of Stat5 expression vector. PTHrP inhibited luciferase activity of the Stat5 reporter, with a maximal fold change of 0.5, in UMR106 cells (fig. 6D), but had no effect in ATDC5 cells (fig. 6C). This observation is in contrast to the increase in mRNA expression of Stat5a and Stat5b. Addition of Stat5a or Stat5b enhanced the reporter activity in ATDC5 and in UMR106 cells. In both cell lines, PTHrP inhibited Stat5 reporter activity in the presence of Stat5b expression vector, whereas in the presence of Stat5a expression vector reporter activity was inhibited in UMR106 cells only.

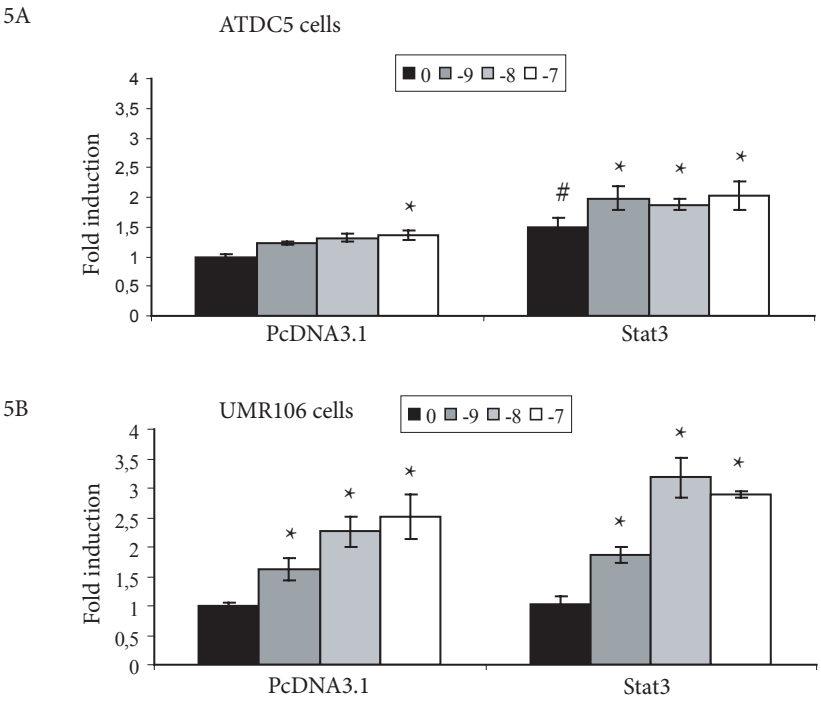
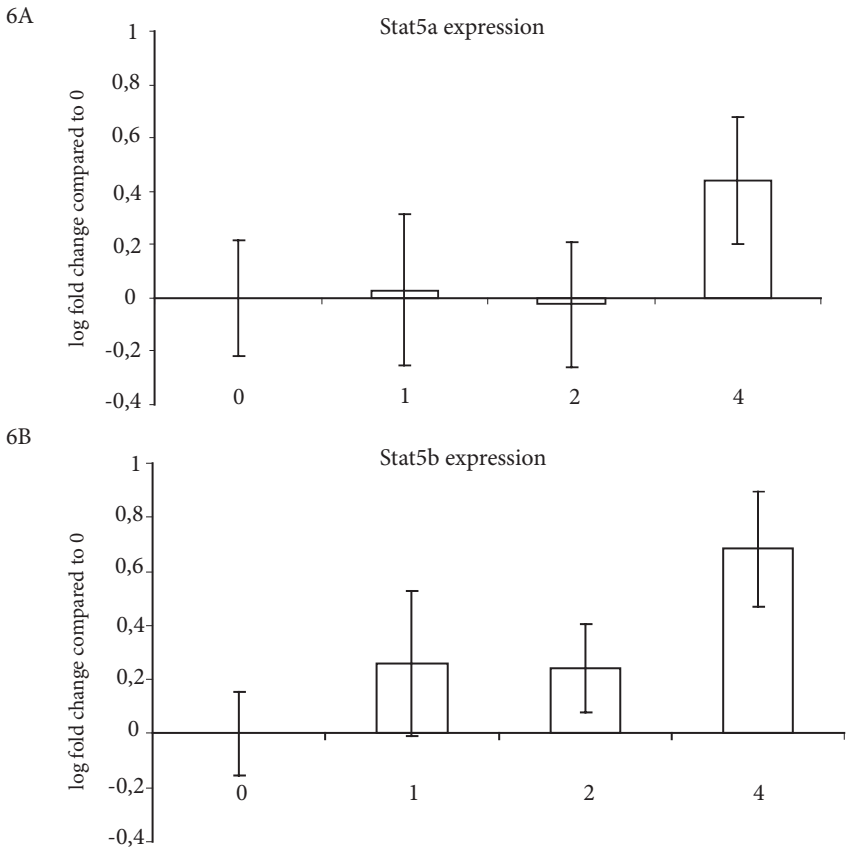


Figure 5: Regulation of the activity of Stat3 protein by PTHrP.
A) ATDC5 cells and B) UMR106 cells were transiently transfected with the stat3 specific promoter-reporter construct and co-transfected with 100 ng pcDNA3.1 or 100 ng stat3 expression vectors and treated with a dose range of PTHrP (0, 10-9, 10-8, 10-7 M). Data are expressed as fold induction compared to control after correction for transfection efficiency. Experiments were performed in quadruplicate and repeated at least twice. *Significant $p<0.05$ compared to vehicle stimulation. #Significant $p<0.05$ compared to vehicle stimulation in pcDNA3.1 co-transfections.

Discussion

In the present study, we have identified early response genes of PTHrP in chondrocytes using the chondrogenic ATDC5 cell line. Disadvantages of the ATDC5 cell line differentiated under standard conditions in monolayer are the formation of a heterogeneous culture and the necessity for prolonged culture periods of at least 28 days[15]. In monolayer cultures chondrogenesis is initiated when multi cell layers appear, suggesting that cell-cell contacts are crucial for chondrocyte differentiation. These cell-cell contacts are the initial trigger for mesenchymal stem cells (MSCs) to form chondrocytes in embryogenesis. To accelerate the formation of cell-cell contacts, we used the micromass culture technique, resembling the condensation of MSCs *in vivo* by formation of a three-dimensional spheroid structure[19;33]. Aggregation of ATDC5 cells resulted in more homogeneous cultures. Already after 7 days type II collagen was abundantly expressed compared to expression after 14 days in monolayer culture[16]. Type IX and type X collagen were expressed at low levels after 7 days compared to expression after 14 and 21 days, respectively, in monolayer culture[16]. In both cultures the responsiveness of the ATDC5 cells to PTHrP increased during differentiation[18]. We decided to stimulate the



micromasses at day 8 with PTHrP. At this time point, the chondrocytes respond to PTHrP and have pre-hypertrophic characteristics as demonstrated by the expression of collagen type IX. Late-proliferating and pre-hypertrophic cells are the main target cells of PTHrP in the growth plate[34]. The higher levels of collagen X expression at day 14 of culture suggested that more cells have already undergone chondrocyte hypertrophy at this time point.

To identify early response genes of PTHrP the NIA 15k mouse cDNA bank was used. This bank was amplified and spotted by the Leiden Genome Technology Center. The quality control of custom-made cDNA microarray, compared to commercially available microarrays, is a well-known problem. The quality of the spots of the microarray used in this study was low, due to heterogeneous spot morphologies ("doughnuts"), deposition inconsistencies, and oversized spots[35]. In addition, identification of the spots was only possible by direct sequencing the cDNAs used in the spotting process, because of contamination. This contamination is most likely introduced during multiple rounds of replication of the bank by PCR, as previously suggested[36]. Because of the uncertainty of the identity of the spots, our results could not be used for pathway screening or genome wide analysis. By applying very stringent selection criteria, the microarray could still be used to identify a subset of PTHrP target genes. The

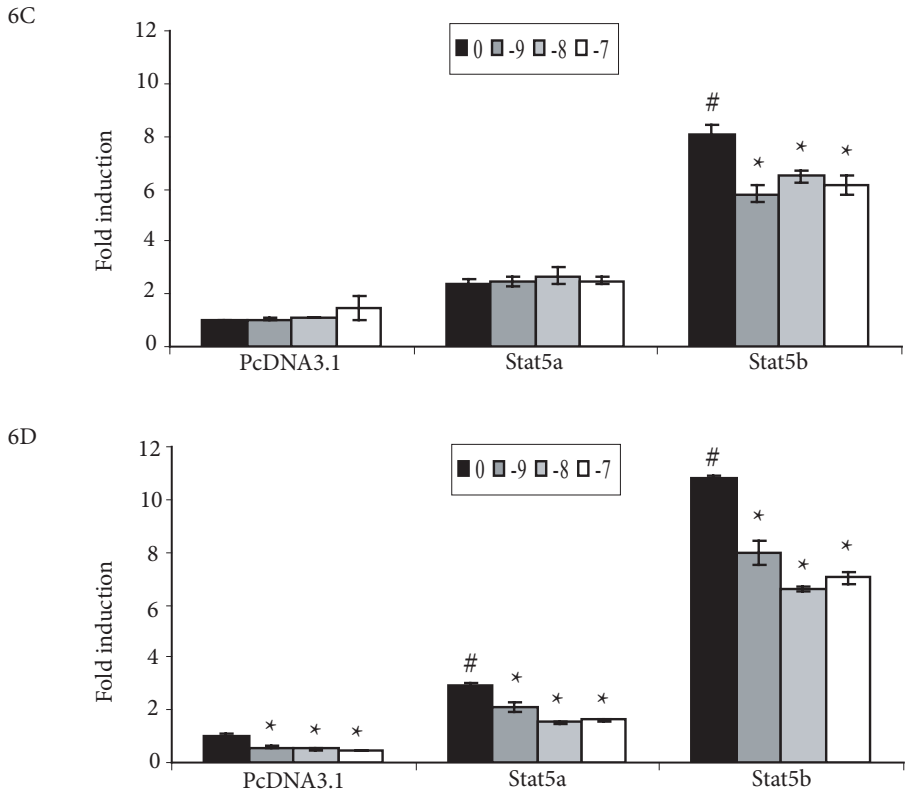


Figure 6: Regulation of Stat5 mRNA expression and Stat5 protein activity by PTHrP.
RNA was isolated at day 11 from ATDC5 micromass cultures at 0h, 1h, 2h and 4h after PTHrP stimulation and qPCR was performed for A) Stat5a and B) Stat5b. C) ATDC5 cells and D) UMR106 cells were transiently transfected with the stat5 specific promoter-reporter construct and co-transfected with 100 ng pcDNA3.1, 100 ng Stat5a or 100 ng Stat5b expression vectors and treated with a dose range of PTHrP (0, 10-9, 10-8, 10-7 M). Data are expressed as fold induction compared to control after correction for transfection efficiency. Experiments were performed in quadruplicate and repeated at least twice. *Significant $p<0.05$ compared to vehicle stimulation. #Significant $p<0.05$ compared to vehicle stimulation in pcDNA3.1 co-transfections.

validity of this approach was subsequently shown by qPCR in different cell models and by bio-informatics analysis. We were able to classify 12 out of 16 genes as bonafide target genes of PTHrP. qPCR analysis is an established method for validation of microarray data. However, due to the distinct methodologies and in our case also the relative poor quality of the custom made arrays, the overlap was not 100%. Others have also reported this, particularly with respect to the fold changes[37-39].

Bio-informatic analysis revealed that RGS2, Upar, and SGK were already identified as PTH target genes in osteoblasts[14;40;41]. Indeed, in this study RGS2, Upar and SGK were also induced in osteoblasts as well as in chondrocytes by PTHrP. Six out of 12 target genes have not been demonstrated in growth plate chondrocytes before. The majority of the identified PTHrP target genes (10 out of 12) were involved in signal transduction pathways and modulation

of these pathways. These include RGS2, SGK, Ptp4a1, Ier3, Stat3, Csrp2, and Gab1. These factors are involved in various signal transduction pathways, like AC/PKA, ERK, Jak/Stat and PI3-kinase/AKT pathways, suggesting that PTHrP signalling could influence these signalling cascades[41-46]. In addition, PTHrP could also influence other pathways via regulation of expression of receptors, like Upar, Acvr2b, and LamRI.

Quantitative PCR resulted in validation of the upregulated PTHrP target genes identified by microarray analysis, in chondrocytes as well in osteoblasts. These data suggest that the upregulated genes are part of a more generalized response to PTHrP, which is not restricted to chondrocytes. Verification of the downregulated target genes revealed a more cell type dependent picture. For instance, two genes, Dym and Sf3a2, were downregulated in chondrocytes, but upregulated in osteoblasts.

The dominant pathway activated by PTHrP is the AC/PKA pathway, which results in activation of the transcription factors CREB and/or AP-1[47]. Indeed, in various early response genes of PTH or PTHrP functional CREB and AP-1 response elements have been identified[48]. In line with this, promoter analysis revealed CREB transcription factor binding sites predominantly in the upregulated genes (6 out of 7), 4 times in combination with an AP-1 site. A less consistent picture was found for the downregulated genes. In the promoter regions of 2 downregulated genes (LamRI and Dym) CREB transcription factor binding sites were predicted. Remarkably, Dym was induced by PTHrP in KS483 osteoblasts. In addition, the presence of SP1, E2F and ELK1 were predicted in both up- and downregulated genes. Recently, Qin et al used a statistical approach to identify transcription factor binding sites used by PTH-signalling in osteoblasts[14] instead of enrichment for evolutionary conserved binding sites applied in this paper. Comparable to our study were the predictions of CREB and AP-1 transcription factor binding sites predominantly in upregulated genes. Also the presence of Sp1 sites in up- and downregulated genes were predicted by both methods. The validity of our approach was furthermore underscored by previous data showing the presence of a CREB transcription factor binding site in the Stat3 promoter[49]. Furthermore, RGS2 is induced by cAMP, suggesting a CREB transcription factor binding site in its promoter[50]. In addition, SP1 binding sites have been described before in the promoter regions of IER3 and Csrp2[51;52].

A remarkable finding in our study was the expression regulation of proteins involved in the Jak/Stat signalling cascade, like Stat3 and Csrp2. Csrp2 is a binding partner of Pias1, which is an inhibitor of Stat1[42]. In addition, a novel observation was the prediction of several Stat transcription factor binding sites in the promoter regions of genes induced by PTHrP (6 out of 7), often in combination with CREB and AP1 sites. These observations were of biological significance, since we also observed that PTHrP induced the activity of a Stat3 reporter construct, either in the presence or absence of extra Stat3. This result is comparable with another study, in which Stat3 reporter activity was induced by activation of the AC/PKA pathway, by increasing the posttranslational activation of Stat3 proteins in rat thyroid cells[53]. In addition, we showed that the regulation of Stat proteins by PTHrP was not only restricted to Stat3, but also included other members of the Jak/Stat family, like Stat5a and Stat5b. Despite the induction of Stat5a and Stat5b mRNA, PTHrP inhibited Stat5 reporter activity in the absence and presence of exogenous Stat5a and Stat5b. This observation is most likely explained by an effect of PTHrP on the posttranslational actions of Stat5a and Stat5b, mediated by the

AC/PKA pathway. Indeed, the inhibiting effect on Stat5 activity after activation of the AC/PKA pathway has been described before in T lymphocytes[54]. It was shown that AC/PKA signalling inhibited tyrosine phosphorylation of Stat5a and Stat5b, thereby preventing their activation. Our results suggest that this mechanism may also be operation in chondrocytes and osteoblasts after activation of PTHR1 signalling.

The effects of PTHrP on Stat3 and Stat5 reporters were observed in ATDC5 and UMR106 cells with only slight differences. Generally, the responses in ATDC5 cells were lower than observed in UMR cells. This is most likely explained by low PTHR1 responses of the undifferentiated ATDC5 cells. Due to the excessive formation of cartilage matrix, which prevented efficient transfections, the transfection experiment could not be repeated in differentiated ATDC5 cells and KS483 cells, which express higher levels of PTHR1. The data suggest, however, that a crosstalk between PTHR1 and Jak/Stat signalling is a more generalized mechanism. Taken together, we provide evidence for interactions between PTHrP and Jak/Stat signalling, not only at the level of mRNA expression regulation, but also at the level of posttranslational modification, resulting in either activation of Stat3 or repression of Stat5a and Stat5b mediated gene transcription.

The involvement of Jak/Stat proteins in signalling cascades of other growth factors in chondrocyte differentiation has been described before. Stat1 and Stat3 are involved in the effects of fibroblast growth factor (FGF) on chondrocyte proliferation within the growth plate[55]. In addition, the Jak/Stat cascade is involved in growth hormone (GH) signalling. Stat5b is the most important Stat protein with respect to the actions of GH on growth and is responsible for the induction of insulin-like growth factor-1 (IGF-1)[56]. Since Jak/Stat signalling plays an important role in chondrocyte proliferation, modulation of this pathway by PTHrP might be an essential mechanism involved in the actions of PTHrP in keeping the growth plate chondrocytes in a proliferation competent state.

In summary, we have applied new culture conditions to induce chondrogenic differentiation of ATDC5 cells. In addition, we have identified 12 PTHrP target genes. Among them were several genes involved in distinct signalling pathways operational within the growth plate, suggesting the presence of a crosstalk with PTHrP signalling. In addition, we report for the first time the presence of a previously not recognized interaction between PTHrP and Jak/Stat signalling.

Acknowledgements

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Novel late target genes of PTHrP in chondrocytes

5

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Abstract

To gain more insight into the downstream effectors of PTHrP signalling in chondrocytes, we performed microarray analysis to identify late PTHrP response genes using the chondrogenic ATDC5 cell line. At day 8 of micromass culture ATDC5 cells have pre-hypertrophic-like characteristics and at this time point the cells were stimulated with 10^{-7} M PTHrP for 24h and 72h and RNA was isolated. PTHrP treatment inhibited the outgrowth of cartilage matrix and decreased the expression of Col10a1 mRNA, which is in line with the inhibitory effects of PTHrP on chondrocyte differentiation.

Using cDNA microarray analysis, a list of 9 genes ($p< 10^{-3}$) was generated, including 3 upregulated (IGFBP4, Csrp2, and Ecm1) and 6 downregulated (Col9a1, Col2a1, Agc, Hmgn2, Calm1, and Mxd4) response genes. Four out of 9 genes (Ecm1, Calm1, Hmgn2, and Mxd4) are novel PTHrP response genes and 2 out of 9 (Calm1 and Hmgn2) have not yet been identified in cartilage. Four out of 9 (Ecm1, Col2a1, Col9a1, and Agc) genes are components of the extra cellular matrix and the remaining genes are involved in signal transduction and transcription regulation.

The response to PTHrP was validated by quantitative PCR, using the same RNA samples as labelled in the microarray experiments and RNA samples isolated from a new experiment. In addition, we examined whether these genes also reacted to PTHrP in other PTHrP responsive models, like KS483 osteoblasts and explanted metatarsals. The expression of late PTHrP response genes varied between ATDC5 chondrocytes, KS483 osteoblasts and metatarsals, suggesting that the expression of late response genes is dependent on the cellular context of the PTHrP responsive cells.

Introduction

Parathyroid hormone (PTH) related peptide (PTHrP) regulates the pace of chondrocyte differentiation during endochondral bone formation[1;2]. In the growth plate the main target cells for PTHrP are late-proliferating and pre-hypertrophic chondrocytes, which express the PTH/PTHrP receptor (PTHR1)[1;3]. Since both decreased and increased PTHrP signalling lead to severe growth plate abnormalities, the expression of PTHrP must be tightly controlled[4-7]. The protein controlling PTHrP expression in the growth plate is the growth factor Indian Hegdehog (IHh), which is expressed by pre-hypertrophic chondrocytes. Vice versa, PTHrP regulates the expression of IHh by inhibiting the transition from proliferating chondrocytes into hypertrophic chondrocytes, thereby delaying IHh production, which completes the negative feedback loop.

PTHrP not only controls the pace of chondrocyte differentiation in the growth plate, but is required for keeping the chondrocytes in the proliferative competent stage as well[8]. The effect of PTHrP on proliferation is mediated by controlling the expression of cell cycle regulators, like cyclin dependent kinase inhibitor p57kip2, as described recently[9].

Two major pathways are involved in PTHR1 transduction, namely the adenylate cyclase (AC)/ protein kinase A (PKA) and the phopholipase C (PLC)/ protein kinase C (PKC) pathway. Recently it was shown that the AC/PKA pathway is sufficient for the effects of PTHrP on chondrocyte differentiation, suggesting only a minor role for the PLC/PKC pathway[10]. Signalling via AC/PKA leads to the activation of the transcription factors, cAMP

response-element binding protein (CREB) and members of the AP-1 family. These two classes of transcription factors are largely responsible for the biological response to PTHrP. In concordance with this, a majority of the early target genes of PTHrP contain binding sites for CREB and/or AP-1 in their promoters[11-13]. Furthermore, phosphorylation by PKA regulates the activity of one of the master transcription factors in cartilage formation, Sox9[14]. This is another illustration that PTHrP can influence chondrocyte differentiation via the AC/PKA pathway.

Despite the critical role of PTHrP in endochondral bone formation, downstream targets of PTHrP, which transduce its effect on chondrocyte differentiation, are largely unknown. For this reason, we have started microarray experiments with the chondrogenic ATDC5 cell line[15]. This cell line is a representative model for studying the actions of PTHrP on chondrogenesis. The cells reproducibly differentiate into chondrocytes in 7 days in micromass culture. In addition, during this process cells become responsive to PTHrP and in agreement with in vivo studies PTHrP inhibits hypertrophic chondrocyte differentiation[16;17]. Previously, we have identified novel early response genes of PTHrP[18]. In this study we focus on the identification of late PTHrP response genes, regulated 24 or 72 hours after PTHrP treatment. Comparison of the expression patterns of these response genes in osteoblasts and explanted metatarsals with ATDC5 cells, demonstrates that the regulation of these genes by PTHrP is cell type specific and dependent on the cellular context of the PTHrP responsive cells.

Materials and Methods

Cell culture

ATDC5 cells were grown in Dulbecco's modified Eagle's medium /F-12 (DMEM/F12) (Invitrogen, Breda, The Netherlands) containing 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), 10% charcoal stripped foetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 10 µg/ml insulin (Sigma Chemical Co., St Louis, MO, USA), 10 µg/ml bovine transferrin (Roche, Almere, The Netherlands), and 3 x 10^{-8} M sodium selenite (Roche), in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. The micromass culture technique was modified from Ahrens et al.[19]. Trypsinized cells were resuspended in medium at a concentration of 2 x 10^7 cells/ml, and 3 drops of 10 µl of this cell suspension were placed in a well of a standard 12 wells culture plate. The cells were allowed to adhere for 2 hours at 37°C and 5% CO_2, and then 1 ml medium was added to each well. The medium was replaced every other day.

RNA isolation and amplification

Medium was refreshed after 7 days and the ATDC5 micromasses were challenged with 10^{-7} M PTHrP or vehicle at day 8 of culture (time point 0h) and total RNA was extracted at different time points in triplicate using Trizol LS Reagent (Invitrogen), followed by RNA cleanup with RNeasy mini kit (Qiagen, Maryland, USA). RNA concentrations were determined by measuring the absorbance at 260 nm. Next, RNA samples were pooled and time point 0h was chosen as reference sample. Total RNA (3 µg/reaction) was amplified as described before[20], with slight modifications. In short, first strand cDNA was synthesized by adding 500 ng T7-oligodT primer (5'-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGC

$G(T)_{21}$-3') to 10 µl RNA sample. Samples were incubated for 10 minutes at 70°C, followed by 60 minutes at 42°C in a total volume of 20 µl, containing 5x first strand buffer, 10 mM DTT, 0.5 mM dNTPs, 2U Rnasin (Promega, Leiden, The Netherlands), and 200U Superscript II RT (Invitrogen). Next, second strand cDNA was synthesized for 2 hours at 16°C in a total volume of 150 µl, containing 5x second strand buffer, 0.2 mM dNTPs, 10U DNA Ligase (Invitrogen), 40U DNA polymerase I (Invitrogen), and 2U Rnase H (Invitrogen). This was followed by addition of 2 µl T4 polymerase (5 U/µl) (Invitrogen) and incubation for 5 minutes at 16°C. The double-stranded cDNA reaction was stopped and the remaining RNA in the mixture was degraded by addition of 7.5 µl of 1 M NaOH, 2 mM EDTA followed by incubation at 65°C for 10 minutes. Samples were purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction. The volume of the aqueous phase was increased to 450 µl using H_2O. For further purification, samples were transferred to a Centricon-100 microconcentrator column (Millipore, Amsterdam, The Netherlands) (pre-spinned with 450 µl H_2O) and centrifuged for 12 minutes at 2500 rpm. After 3 wash steps with 450 µl H_2O, cDNA was collected in a total volume of 7 µl by inverting the column and by centrifuging for 30 s at 13000 rpm. Subsequently, cDNA was transcribed into cRNA using the T7 high yield transcription kit (Epicentre, Madison, USA). The cDNA solution was incubated at 42°C for 3 hours in a total volume of 20 µl, containing 10x T7 reaction buffer, 7.5 mM ATP, CTP, GTP, and UTP, 10 mM DTT, 2 U Rnasin, and 2 µl Ampliscribe T7 enzyme solution, followed by sample concentration using centricon-100 microconcentrator columns. This method was based on the original protocol of Van Gelder et al.[21]. Finally, cRNA concentration was determined by measuring the absorbance at 260 nm.

Probe labelling

cRNA (1.2 µg) was reverse transcribed with random hexamer primers, and labelled by incorporation of cyanine 5-dUTP (Cy5) or cyanine 3-dUTP (Cy3) (NEN, Boston, USA) according to the protocols of Ross et al.[22] with slight modifications. In short, cRNA and 8 µg random primers (Roche) in a total volume of 15 µl was incubated for 10 minutes at 70°C. Subsequently, 6 µl 5x first strand buffer, 3 µl 0.1 M DTT, 0.6 µl low-T dNTPs, 3 µl Cy3 dUTP (time point 0h) or Cy5-dUTP (other time points), and 1 µl Superscript II RT (200 U/µl) were added, incubated for 10 minutes at room temperature, followed by incubation at 42°C for 90 minutes. After 60 minutes fresh Superscript II RT (1 µl) was added. Next, RNA was degraded, by addition of 15 µl 0.1 M NaOH and incubation for 10 minutes at 70°C, after which the solution was neutralized by addition of 15 µl 0.1 M HCl. The labelled samples, supplemented with 180 µl 10 mM Tris, 1mM EDTA, pH 8 (TE) and 10 µl mouse Cot-1 DNA (10 mg/ml) (Invitrogen), were pooled and purified using a centricon-30 microconcentrator column (Millipore) (pre-spinned with 450 µl TE for 8 minutes at 13000 rpm). PolyA RNA (20 µg) (Amersham) and yeast tRNA (20 µg) (Invitrogen) were added to 450 µl TE during the second wash step. The purified product was collected, by inverting the column and by centrifuging for 1 minutes at 13000 rpm, and finally, resuspended in a total volume of 45 µl hybridization solution, containing 7.65 µl 20x SSC and 1.35 µl 10% SDS.

(Pre-) hybridization

For the hybridization experiments microarrays, on which the NIA 15k mouse cDNA clone

set[23] was spotted, were purchased from the Leiden Genome Technology Center (LGTC). DNA was crosslinked by UV irradiation at 65 mJ/cm^2 (Stratalinker mode 1800 UV Illuminator, Stratagene). To prevent non-specific hybridization, the slides were incubated in 45 µl hybridization solution (400 ng/µl yeast tRNA, 400 ng/µl poly(A) RNA, 400 ng/µl herring sperm DNA (Invitrogen), 100 ng/µl mouse Cot1 DNA, 5x Denhardt's solution, 3.2x SSC and 0.4% SDS) at 65°C for 30 minutes. Prior to hybridization, the slides containing the pre-hybridization mixture were incubated for 2 minutes at 80°C to denature the spotted DNA. After pre-hybridization, the slides were washed twice in 2x SSC for 5 minutes at room temperature and dehydrated with subsequent steps of 5 x 5 minutes 70%, 5 minutes 90% and 5 minutes 100% ethanol. For hybridization, the probes were denatured by heating for 2 minutes at 100°C, left at room temperature for 15 minutes, centrifuged for 10 minutes, and placed under a 24 mm x 60 mm glass coverslip. The slides were incubated overnight at 65°C in a hybridization chamber (Corning, Amsterdam, The Netherlands) and washed the next day in 2x SSC for 5 minutes at room temperature and dehydrated using graded ethanols.

Microarray design and statistical analysis

The reference array experiment, 0h vs 0h, was hybridized in duplicate, 24h vs 0h and 72h vs 0h for PTHrP and vehicle treated samples were hybridized in triplicate. Following hybridization, slides were scanned in the Agilent DNA Microarray scanner (Agilent Technologies, Amstelveen, The Netherlands). Genepix 3.0 software (Axon Instruments Inc.) was used to quantify the resulting images. Subsequently, normalization and gene expression analysis were performed with Rosetta Resolver (Rosetta Biosoftware, seattle, USA). Due to the overall poor quality of the cDNA spots on the microarray, stringent selection criteria were used for inclusion of spots, to minimize the risk of false positive signals. A spot was only included in the analysis if it passed all of the following selection criteria. 1) Spots should have an absolute fold change of less than 2 in the reference array. 2) Spots should show no significantly regulation ($p<0.01$) in the reference array. 3) Only non-flagged spots in any of the arrays were included. 4) The signal intensity of Cy3 or Cy5 of the spots should be above a cut off level (0.05). 5) Spots should show an absolute fold change less than 50. Next, analysis of variance (ANOVA) was performed between the remaining spots of the reference and PTHrP treated hybridizations. 6) Spots should show significant ($p<0.001$) differential expression regulation, with intensity value above background value. 7) Spots should not be differentially expressed ($p<0.01$) during time in the vehicle treated cultures. To identify the selected spots, the PCR-amplified cDNA of each spot (500 ng) was sequenced by the LGTC using 12 pmol M13 primers in a total volume of 24 µl.

Histology

Micromasses were fixed for 10 minutes in 10% formalin, subsequently dehydrated using graded ethanols, and embedded in paraffin. Sections of 5 micron were deparaffined with Paraclear (EarthSafe Technologies Inc, Belle Mead, NJ, USA) and hydrated using graded ethanols. Subsequently, the sections were treated for 3 minutes with 3% acetic acid, stained with 1% Alcian Blue for 30 minutes, rinsed first with 3% acetic acid and then with bidest. Hereafter, the sections were immersed in Nuclear Fast Red for 5 minutes and then rinsed with

bidest. Next, the sections were dehydrated using graded ethanols. Finally, the sections were embedded in histomount-diluted 1:1 xylene.

RNA isolation from mouse bone explants

Metatarsalsia were isolated from 15-days old Swiss Albino mouse embryos. The mice were kept in a light and temperature controlled room (12 hours light, 20-22°C) with food and water available *ad libitum*. Experiments were approved by the local ethical committee for animal experiments. The bone explants were cultured in αMEM with 10% FCS and the next day metatarsals were challenged with 10^{-7} M PTHrP or vehicle in αMEM containing 1% BSA. RNA was isolated after 24 and 72 hours from metatarsals, using Trizol LS Reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

RNA isolation from KS483 osteoblasts

KS483 mesenchymal progenitor cells were differentiated into osteoblasts as described previously[24]. At day 11, KS483 cells have formed bone nodules and the cells within the nodules start to mineralize the extracellular matrix. This time point was chosen to treat the cells with 10^{-7} M PTHrP. RNA was isolated after 24 and 72 hours using Trizol LS Reagent (Invitrogen) and reverse transcribed into cDNA using random hexamer primers (Amersham).

Quantitative PCR (qPCR)

To validate the expression patterns of PTHrP response genes, quantitative PCR was performed using the BioRad iCycler (Biorad, Veenendaal, The Netherlands). For each gene a set of primers was designed (table 1), which spanned at least 1 intron-exon boundary and had an optimal annealing temperature of 60˚C, using Beacon designer (United Bioinformatica Inc., Calgary, Canada). cDNA (5 ng) was amplified in triplicate using the qPCR core kit for SYBR green 1 (Eurogentec, Maastricht, The Netherlands), under the following conditions: cDNA was denatured for 10 minutes at 95˚C, followed by 40 cycles, consisting of 15 s at 95˚C, 20 s at 60˚C, and 40 s at 72˚C. From each sample a melting curve was generated to test for the absence of primer dimer formation and DNA contamination. Each reaction contained 5 µl cDNA (1 ng/µl), 10x reaction buffer, 3 or 4 mM $MgCl_2$ (table 1), 40 µM dNTP's, 300 nM primer, 0.75 µl SYBR green, and 0.1 µl HotGoldStar polymerase in a total volume of 25 µl. Fold changes, adjusted for the expression of β2m, were calculated and log transformed using the comparative method[25]. Significant changes were calculated using the Double Delta Model (DDM) (Chapter 6).

Name	F/R	Forward primer	MgCl
β2μ	F	CACTGACCGGCCTGTATGC	3 mM
	R	GAATTCAGTGTGAGCCAGGATATAGA	
IGFBP4	F	AACTGTGACCGCAACGGC	4 mM
	R	ACGCTGTCCGTCCAGGG	
Csrp2	F	CCACTCGGAATGCCTGTCTG	3 mM
	R	GCCACTGTTGTGCTGTCTAAATTTT	
Ecm1	F	TCGCCCACCTAGCTCCCTATC	4 mM
	R	AGCAGCGGACGGTCATATTCTG	
Col2a1	F	CTTCGGAGAGTGCTGTCCCATC	4 mM
	R	CCCTTGTCACCACGATCACCTC	
Col9a1	F	CACTGGGCTGCCTGGTATCC	4 mM
	R	GGCTCCTGTGTCTGGTCTCTTG	
Agc	F	CGAATGGAACGACGTCCC	3 mM
	R	CTGATCTCGTAGCGATCTTTCTTCT	
Calm1	F	GTGTCCGTGGTGCCGTTACTC	3 mM
	R	TGCGAGCGAAGGAAGGAAGAAC	
Hmgn2	F	TCCAAAGCCAGAGCCCAAACC	4 mM
	R	GGCATCTCCAGCACCTTCAGC	
Mxd4	F	CCCGAACAACAGGTCTTCACAC	4 mM
	R	GCACGCTTCAGAAGGCTCAG	
Ap3d	F	AATGCGGAGGCGGTGAAGTC	4 mM
	R	GGGTGGTGGACAGCCAGAAGC	
Brd4	F	GGCTCGAAAACTCCAGGATGTG	4 mM
	R	TGTCGCTGCTGCTGTCACTAG	
Nme1	F	CAGGCAGGGGACCAGCAAC	4 mM
	R	GCCTGAAGGAGGTAAGGAATGC	
Nsd1	F	CACACAGAACAAGGGCTTCAGG	3 mM
	R	CCACAGGACAGCGGACACAG	
Tgfβ1i4	F	CTCCCTCCCGCCCTCTCTC	4 mM
	R	GGTTCCCAGCAAAGACGACAAG	

Table 1 : qPCR primer sets

Results

Effect of PTHrP treatment on ATDC5 micromass cultures

ATDC5 cells were cultured as micromasses for 8 days to stimulate chondrocyte differentiation. Previously we have shown that the cells have a pre-hypertrophic phenotype at this stage of culture[18]. PTHrP treatment at day 8 resulted in inhibition of the outgrowth of cartilage matrix 72 hours later (fig. 1A). Histological analysis of the micromasses showed rounded chondrocytes embedded in an alcian blue positive extracellular matrix (fig.1B). Treatment with PTHrP for 24 and 72 hours did not change the cell structure or the intensity of the alcian blue staining in the micromasses. RNA was isolated 24 and 72 hours after PTHrP treatment and used for qPCR analysis to study the expression of collagen 10 (Col10a1), a marker for hypertrophic chondrocytes. PTHrP decreased Col10a1 mRNA expression after 72 hours of treatment (fig. 1C).

Taken together PTHrP treatment at day 8 of culture inhibited new formation of cartilage and inhibited the differentiation into hypertrophic Col10a1 expressing chondrocytes. For this reason, this time point was chosen for identification of PTHrP response genes.

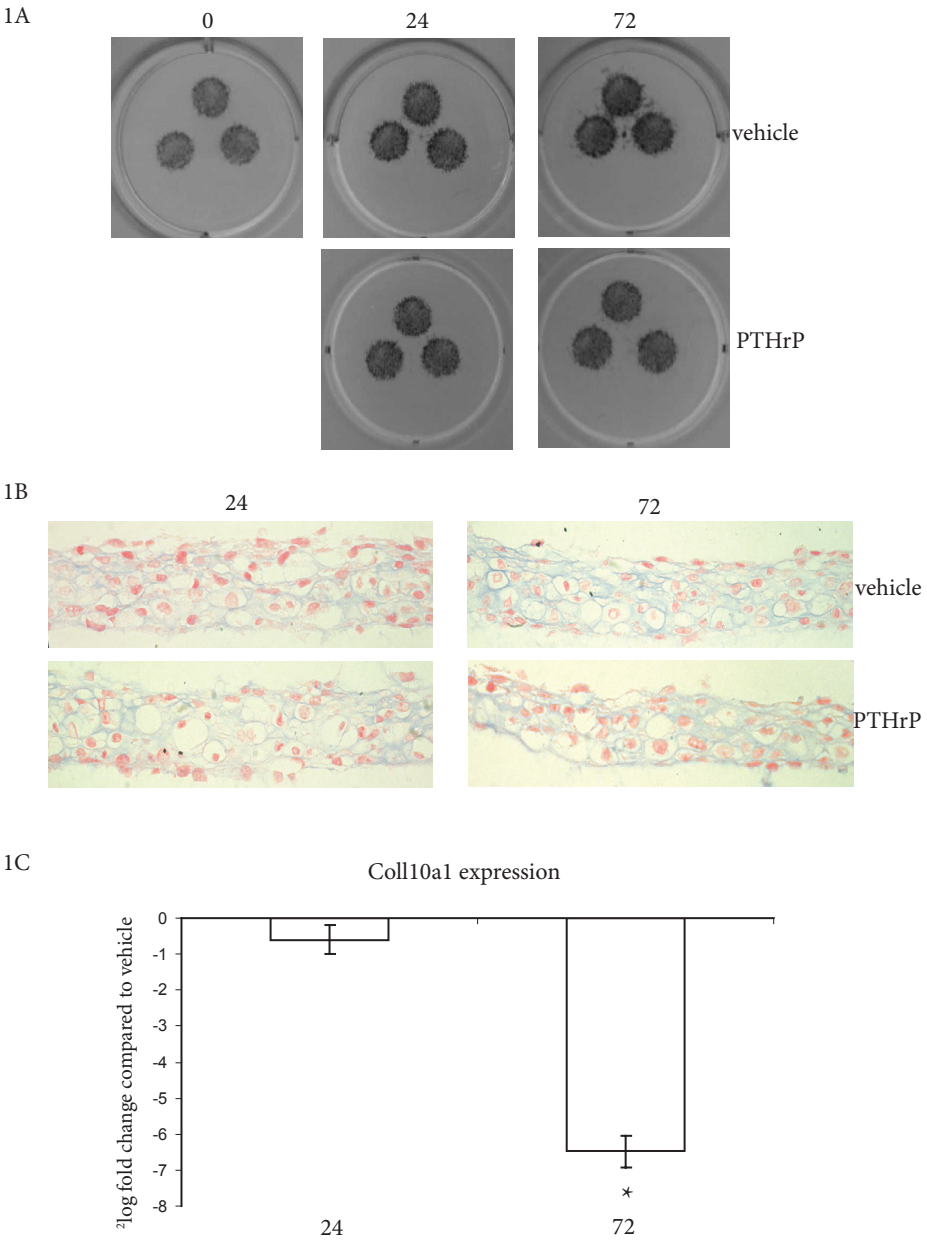


Figure 1: characterization of ATDC5 micromass cultures.
(A) ATDC5 micromass cultures were cultured for 8 days and then treated with vehicle or 10-7 M PTHrP for 0, 24 and 72 hours. Cultures were stained with alcian blue. (B) Alcian blue stained sections of ATDC5 micromasses, cultured for 8 days and then treated with vehicle or 10-7 M PTHrP for 24 and 72 hours. (C) qPCR analysis of col10a1 mRNA in micromasses cultured for 8 days and then treated with 10-7 M PTHrP for 24 and 72 hours. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. * Significant vs vehicle treated samples ($p<0.05$).

Name	Accession number	Up/down	Ratio 0h[2]	Ratio 24h[2]	Ratio 72h[2]	selected
Extracellular matrix protein 1 (Ecm1)	NM_007899	up	0.75	1.56	1.82	X
Transforming growth factor beta 1 induced transcript 4 (TGFβ1i4)1	NM_009366	up	1.01	1.71	2.36	X
Cysteine and glycine-rich protein 2 (Csrp2)	NM_007792	up	1.12	2.71	1.92	X
CD2 antigen (cytoplasmic tail) binding protein 2 (Cd2bp2)	NM_027353	up	1.11	4.94	3.26	
Insulin-like growth factor binding protein 4 (IGFBP4)	NM_010517	up	0.81	1.84	1.42	X
Interferon induced transmembrane protein 3 (Ifitm3)	NM_025378	up	0.85	1.85	2.14	
Carbonyl reductase 2 (Cbr2)	NM_07621	up	0.86	2.42	2.12	
Nuclear receptor-binding SET-domain protein 1 (NSD1)1	AF419220	up	1.12	1.71	2.07	X
NADH dehydrogenase (ubiquinone) Fe-S protein 5 (Ndufs5)	NM_134104	up	0.76	1.60	3.17	
Guanosine monophosphate reductase (Gmpr)	NM_025508	up	0.91	1.25	2.56	
EST	AC122296	up	1.11	3.82	2.60	
Phosphatidylinositol glycan, class T (Pigt)	NM_133799	down	1.31	0.38	0.99	
Adaptor-related protein complex 3, delta 1 subunit (Ap3d)1	NM_007460	down	0.86	0.45	0.52	X
Aggrecan (Agc)	NM_007424	down	0.86	0.25	0.14	X
Procollagen, type IX, alpha 1 (Col9a1)	NM_007740	down	0.91	1.03	0.20	X
Stearoyl-Coenzyme A desaturase 2 (Scd2)	NM_009128	down	1.00	0.69	0.40	
Max dimerization protein 4 (Mxd4)	NM_010753	down	0.79	0.55	0.40	X
Expressed in non-metastatic cells 1 (Nme1)1	NM_008704	down	1.26	0.61	0.51	X
Splicing factor, arginine/serine-rich 1 (ASF/SF2)	NM_173374	down	0.92	0.60	0.39	
Hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1)	NM_013556	down	0.96	0.64	0.49	
Procollagen, type II, alpha 1 (Col2a1)	NM_031163	down	1.08	0.81	0.28	X
SID1 transmembrane family, member 2	NM_172257	down	0.90	0.71	0.56	
Ubiquitin specific protease 34 (usp34)	AK033182	down	0.96	0.66	0.44	
Selenoprotein (Sep15)	NM_053102	down	0.96	0.79	0.52	
Calmodulin 1 (Calm1)	NM_009790	down	0.87	0.63	0.40	X
Bromodomain-containing 4 (Brd4)1	NM_020508	down	0.84	0.53	0.42	X
High mobility group nucleosomal binding protein domain 2 (Hmgn2)	NM_016957	down	0.97	0.64	0.42	X
EST	AC127579	down	0.84	0.71	0.52	
EST	NM_026561	down	0.87	0.38	0.35	
EST	NM_028043	down	0.91	0.66	0.40	
EST	NM_134007	down	1.04	0.72	0.57	

Table 2: Genes regulated by PTHrP in chondrocytes revealed by statistical analysis of the microarray data.

[1]) These genes could not be validated in qPCR analysis

[2]) Significant ($p<0.001$) differential expression regulation.

Selection of PTHrP response genes

ATDC5 cells were cultured as micromasses and treated at day 8 with 10^{-7} M PTHrP for 24 and 72 hours and cDNA microarray analysis was performed. After applying stringent selection criteria, 8319 out of the 15442 spots were taken in the analysis. A list of 31 spots, which exhibited significant ($p<0.001$) differential expression after 24 or 72 hours following PTHrP treatment and did not change in the vehicle treated cultures, was generated (table 2). From this list 14 genes were chosen for validation experiments in ATDC5 cells. Exclusion criteria were insufficient information on gene identity, for example ESTs, or insufficient information for the design of qPCR primer sets, which span intron-exon boundaries.

Validation of PTHrP response genes in ATDC5 cells by qPCR

The expression patterns of the selected genes were first validated by studying expression profiles using qPCR in the same RNA samples subjected to microarray analysis (fig. 2B, D, F and H) and in RNA samples from ATDC5 cells isolated in a new independent experiment (data not shown). Beta-2-microglobulin (β2μ) was used as internal standard, as it was not regulated by PTHrP in microarray analysis (data not shown). Five out of 14 genes could not be validated in the second RNA panel and were discarded from further study, leaving 9 genes for more detailed analysis (table 3). Among these 9 identified PTHrP response genes, 3 genes were upregulated and 6 genes were downregulated. Five genes were already known response genes of PTHrP and 7 genes have previously been found in growth plate chondrocytes (table 3). Four out of 9 response genes were extracellular matrix proteins, namely Ecm1, Col2a1, Col9a1 and Agc. The other response genes were implicated in protein binding and were involved in the regulation of signalling and transcription.

Based on the expression patterns, the genes were divided into 4 groups, 2 groups for upregulated genes and 2 groups for downregulated genes (fig. 2A, C, E and G). The expression of the genes in expression pattern 1 peaked at 24 hours while the gene in expression pattern 2 was continuously upregulated. The gene in expression pattern 3 was only downregulated after 72 hours. In expression pattern 4 the genes were continuously downregulated. Expression

Reg.[1]	Abbr.[2]	Name	Function	rp[3]	gp[4]
up	IGFBP4	Insulin-like growth factor binding protein 4	Negative modulator of IGF-1 activity	yes	yes
up	Csrp2	Cystein and glycine-rich protein 2	Binding partner of protein inhibitor of activated STAT1 (PIAS1)	yes	yes
up	Ecm1	Extracellular matrix protein 1	Negative regulator of endochondral bone formation	no	yes
down	Col9a1	Collagen, type IX, alpha 1	Extracellular matrix protein	yes	yes
down	Col2a1	Collagen, type II, alpha 1	Extracellular matrix protein	yes	yes
down	Agc	Aggrecan	Extracellular matrix protein	yes	yes
down	Calm1	Calmodulin 1	Principal mediator of the calcium signal	no	no
down	Hmgn2	High mobility group nucleosome binding protein 2	Nucleosome binding protein	no	no
down	Mxd4	Max dimerization protein 4	Member of Myc/Max/Mad network	no	yes

Table 3 : List of validated PTHrP target genes.

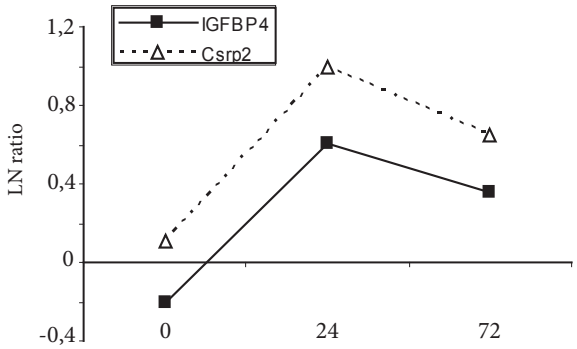
[1]) Reg. = regulation

[2]) Abbr. = abbreviation

[3]) rp = previously been implicated in PTHrP signaling

[4]) gp = previously been found in growth plate chondrocytes

2A


IGFBP4
Csrp2
LN ratio
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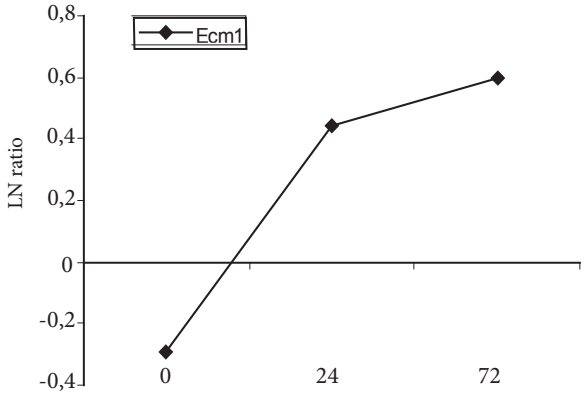
0,8
0,4
0
-0,4
0
24
72

2C


Ecm1
LN ratio
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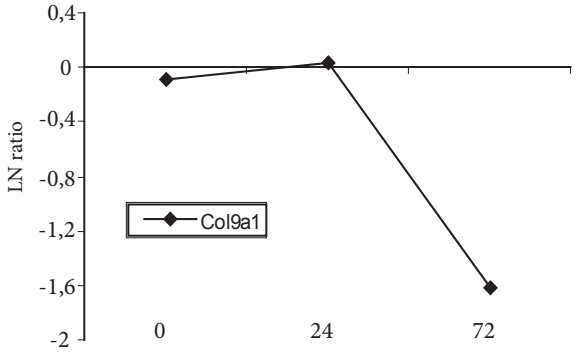
0,6
0,4
0,2
0
-0,2
-0,4
0
24
72

2E


Col9a1
LN ratio
0,4
0
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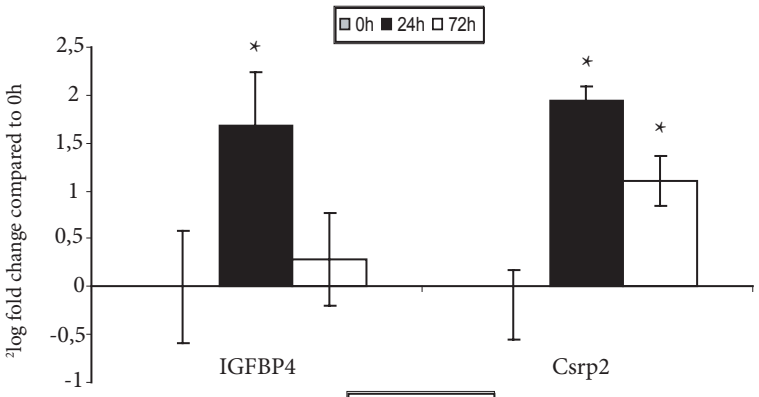
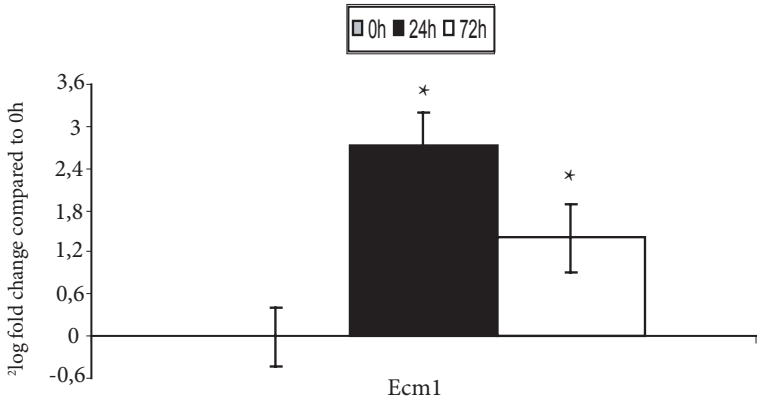
-0,8
-1,2
-1,6
-2
0
24
72

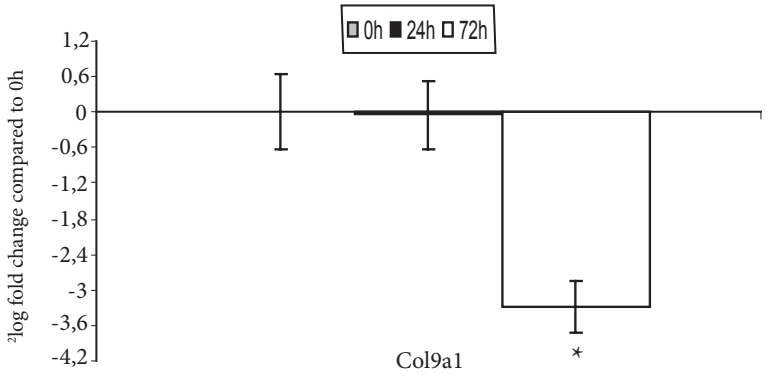
2B

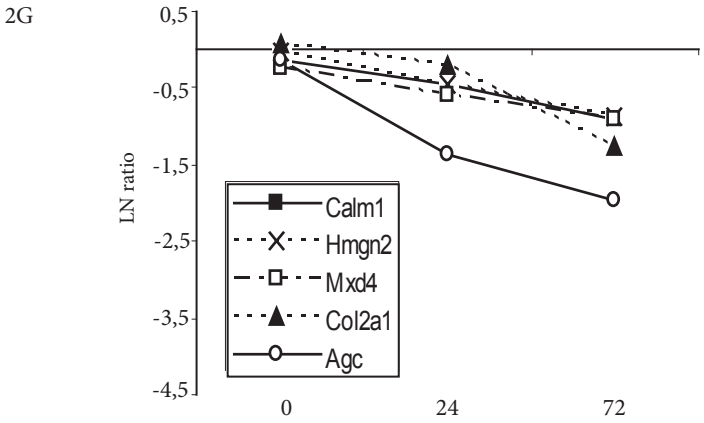

0h 24h 72h
2,5
2
1,5
1
0,5
0
-0,5
-1
2log fold change compared to 0h
IGFBP4
Csrp2

2D


0h 24h 72h
3,6
3
2,4
1,8
1,2
0,6
0
-0,6
2log fold change compared to 0h
Ecm1

2F


0h 24h 72h
1,2
0,6
0
-0,6
-1,2
-1,8
-2,4
-3
-3,6
-4,2
2log fold change compared to 0h
Col9a1



pattern 1 contained Csrp2 and IGFBP4 and expression pattern 2 contained Ecm1. Col9a1 formed expression pattern 3. In expression pattern 4 were Col2a1, Agc, Calm1, Hmgn2, and Mxd4.
In qPCR experiments all genes, except for Ecm1, showed the same expression profile compared to the data from microarray analysis (fig. 2). Ecm1 showed the same expression pattern as the genes in expression profile 1 (fig. 2B and D), upregulation at 24 hours followed by a slight decrease of mRNA expression after 72 hours, instead of continuously upregulation revealed by microarray analysis.

Validation of PTHrP response genes in metatarsals by qPCR

To test whether the identified response genes also respond to PTHrP in chondrocytes *ex vivo*, metatarsals from 15-day old mouse embryos were treated with 10^{-7} M PTHrP for 24 and 72 hours and qPCR analysis was performed. Metatarsals from 15-day old mouse embryos consist of undifferentiated cartilage surrounded by a perichondrium in which osteoblast and osteoclast precursors are present. They differentiate normally during a 1 week culture period into a mineralized bone explant[26]. In response to PTHrP the expression of Col10a1 decreased after 24 hours, indicating a biological response to PTHrP (fig. 3A). All genes except 1, Calm1, responded to PTHrP in the metatarsal model. However, there were remarkable differences, both in the temporal regulation and in the direction of regulation, in the response to PTHrP when compared to the response in ATDC5 cells (compare fig. 3 with fig. 2). For instance, all downregulated genes in the ATDC5 cultures were upregulated in the metatarsal model. In addition, the peak in expression of the upregulated genes, IGFBP4, Csrp2, and Ecm1 differed between the two models. In metatarsals the peak expression of IGFBP4, Csrp2, and Ecm1 was after 72 hours compared to the peak expression in ATDC5 cells after 24 hours.

Validation of PTHrP response genes in KS483 osteoblasts by qPCR

To test whether the response of the response genes were restricted to chondrocytes or whether

2H

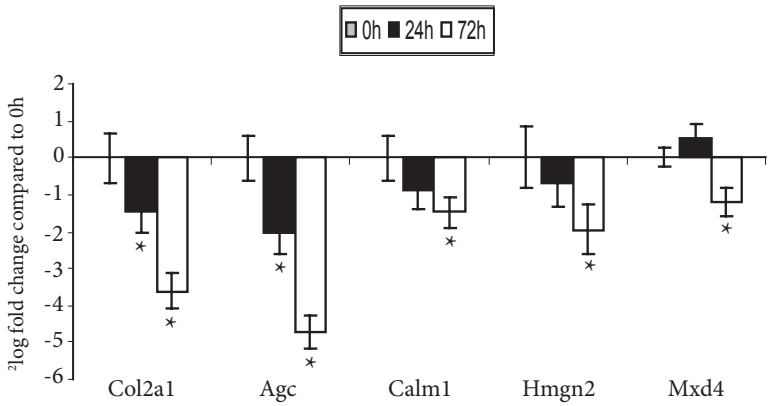


Figure2: expression profiles of PTHrP response genes in ATDC5 chondrocytes revealed by microarray analysis and qPCR analysis.
RNA was isolated from ATDC5 micromass cultures, at 0, 24 and 72 hours after PTHrP (10-7 M) stimulation, amplified and labelled as described. Samples were hybridized against 0 hours in duplicate or triplicate (for details see materials and methods). In the left panel the expression profiles revealed by microarray analysis are shown (A, C, E, and G). In the right panel the corresponding patterns revealed by qPCR are shown (B, D, F, and H). Every sample was run in triplicate.
(A and B) Expression profile 1 contained 2 upregulated genes, i.e. IGFBP4 and Csrp2. (C and D) Expression profile 2 contained 1 upregulated gene, i.e. Ecm1. (E and F) Expression profile 3 contained 1 downregulated gene, i.e. Col9a1. (G and H) Expression profile 4 contained 5 downregulated genes, i.e. Col2a1, Agc, Calm1, Hmgn2, and Mxd4. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. *Significant vs 0h ($p<0.05$).

they were part of a more generalised response to PTHrP, the expression patterns were analysed in differentiated KS483 osteoblasts using qPCR (fig. 4). For this purpose, RNA was isolated after 24 and 72 hours after a challenge with 10^{-7} M PTHrP. The expression patterns of the typical chondrocyte markers Agc, Col2a1 and Col9a1 were not established in this osteoblast culture system. The expression of 3 out of 6 response genes changed after PTHrP treatment in the KS483 culture system, including IGFBP4, Ecm1, and Mxd4. The expression regulation of IGFBP4 and Ecm1 was comparable to the regulation in metatarsals and was different in peak expression compared to the ATDC5 cells. Mxd4 mRNA expression increased in response to PTHrP in osteoblasts, instead of a decrease in expression in ATDC5 chondrocytes, however the expression was comparable to the PTHrP response in metatarsals. Csrp2, Calm1, and Hmgn2 were not significantly regulated by PTHrP in KS483 osteoblasts.

Discussion

In the present study, we have identified late response genes of PTHrP in chondrocytes using the chondrogenic ATDC5 cell line. ATDC5 cells were cultured as micromasses to induce chondrocyte differentiation. After 1 week this resulted in a homogeneously differentiated cell culture, which contained chondrocytes with pre-hypertrophic-like characteristics. Previously, we have shown high expression of PTHR1 in these cells[18]. Because late-proliferating and pre-hypertrophic cells are the main target cells of PTHrP in the growth plate[2], we decided to stimulate the micromasses at day 8 with PTHrP for 24 and 72 hours, to identify late PTHrP

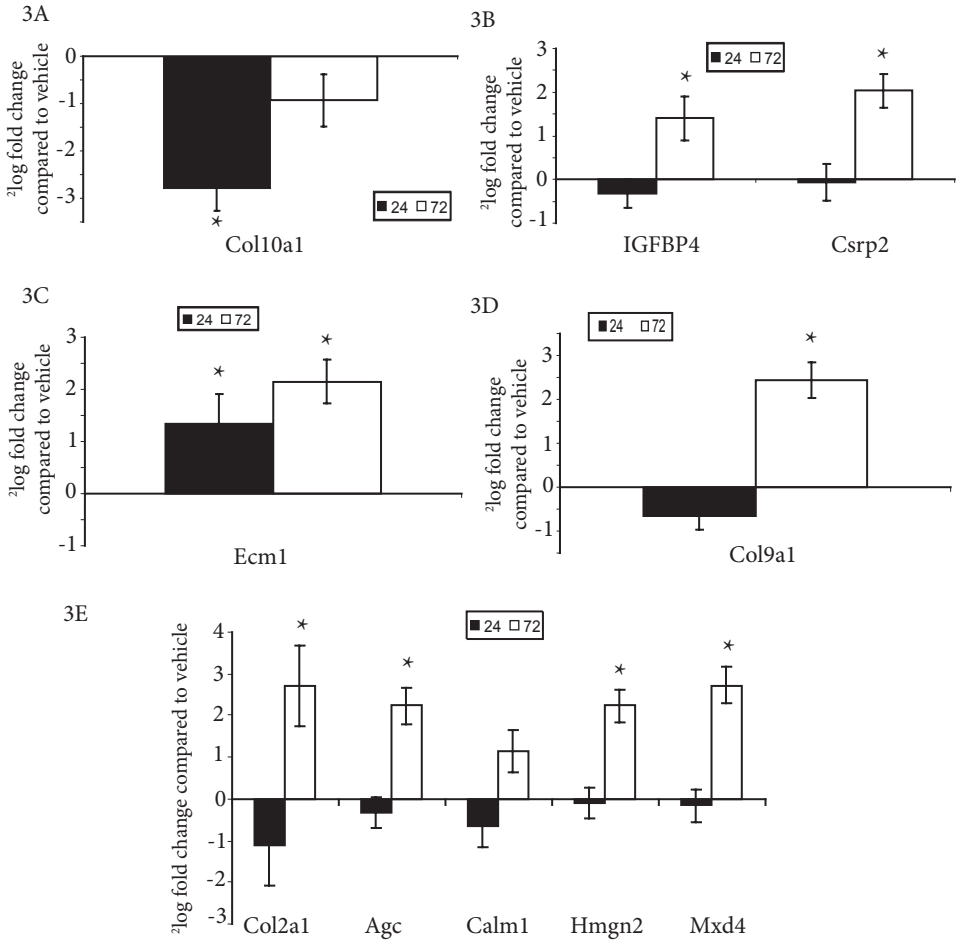


Figure 3: expression patterns of PTHrP response genes in 15-day old metatarsals.
RNA was isolated from 15-day old metatarsals and treated with 10-7 M PTHrP for 24 and 72 hours, and qPCR was performed. Every sample was run in triplicate. (A) PTHrP treatment decreased Col10a1 mRNA expression as expected. (B) Expression regulation of genes from expression pattern 1, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (C) Expression regulation of genes from expression pattern 2, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (D) Expression regulation of genes from expression pattern 3, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. (E) Expression regulation of genes from expression pattern 4, as revealed by microarray analysis in ATDC5 cells, in explanted metatarsals. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. *Significant vs vehicle treated samples ($p<0.05$).

response genes. A challenge with PTHrP resulted in a biological response at 72 hours, as shown by downregulation of the hypertrophic marker Col10a1 and inhibition of the outgrowth of the cartilage matrix, but did not result in a change of cell structure within the micromasses. This is in agreement with current evidence that PTHrP does not inhibit formation of chondrocytes per se, but inhibits their differentiation into hypertrophic cells.
To identify late response genes of PTHrP we used the NIA 15k mouse cDNA bank, which was

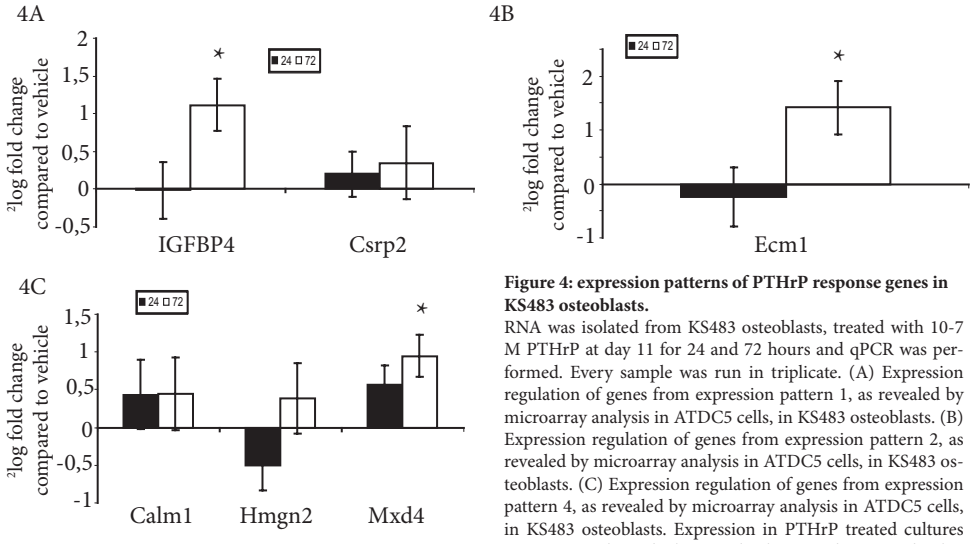


Figure 4: expression patterns of PTHrP response genes in KS483 osteoblasts.
RNA was isolated from KS483 osteoblasts, treated with 10-7 M PTHrP at day 11 for 24 and 72 hours and qPCR was performed. Every sample was run in triplicate. (A) Expression regulation of genes from expression pattern 1, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. (B) Expression regulation of genes from expression pattern 2, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. (C) Expression regulation of genes from expression pattern 4, as revealed by microarray analysis in ATDC5 cells, in KS483 osteoblasts. Expression in PTHrP treated cultures was compared to vehicle treated cultures and expressed as log fold change. Typical cartilage markers were not studied. *Significant vs vehicle treated samples ($p<0.05$).

amplified and spotted by the Leiden Genome Technology Center. Compared to commercially available microarrays, the quality control of custom made cDNA microarray is a well known problem. The quality of the spots of the microarray used in this study was low, due to heterogeneous spot morphologies ("doughnuts"), deposition inconsistencies, and oversized spots as described previously[18;27]. In addition, identification of the spots was only possible by direct sequencing the cDNAs used in the spotting process, because of contamination. This contamination is most likely introduced during multiple rounds of replication of the bank by PCR, as previously suggested[28]. Pathway screening or genome wide analysis could not be used in this study, because of the uncertainty of the identity of the spots. However, the microarray could still be used to identify PTHrP response genes by applying very stringent selection criteria. Using qPCR in different cell models and bio-informatics analysis we showed the validity of this approach. We were able to classify 9 out of 14 genes as bonafide response genes of PTHrP. However, due to the distinct methodologies and in our case also the relative poor quality of the custom made arrays, the overlap was not 100%. Others have also reported this, particularly with respect to the fold changes[29-31].

Bio-informatic analysis revealed that 4 genes, Ecm1, Calm1, Hmgn2 and Mxd4, have not yet been described as response genes of PTHrP. IGFBP4 was already identified as PTHrP response gene in osteoblasts[32] and the typical chondrocyte markers, Agc, Col2a1, and Col9a1, in chondrocytes[17]. In addition, Csrp2 has previously been identified as an early target gene of PTHrP in chondrocytes as well as in osteoblasts in our previous study[18]. Two out of 9 late response genes, Calm1 and Hmgn2, have not been demonstrated in growth plate chondrocytes before. Furthermore, 4 out 9 genes (Ecm1, Col2a1, Col9a1, and Agc) were matrix proteins compared to 0 out of 12 genes among the early response genes. In addition,

4 out of 9 late response genes (Csrp2, Hmgn2, Calm1 and Mxd4) were involved in signal transduction and regulation, compared to 10 out of 12 early response genes[18]. Although we had only a small list of response genes, this is in agreement with the notion that the majority of early target genes are implicated in signal transduction and regulation and that the majority of late response genes code for structural proteins belonging to the extracellular matrix[29;33]. The remaining late response gene, IGFBP4, is involved in protein binding and modulation of signal transduction.

Detailed analysis on the role of some of our targets in chondrocyte differentiation is currently lacking. For example, an interesting feature of the transcription factor Hmgn2 is the presence of a HMG domain. Remarkably, the three major transcription factors involved in chondrocyte differentiation, Sox9, Sox6, and L-Sox5, contain a HMG box. The HMG domain containing protein family can be divided in two subfamilies, the first one containing the subclass of Sox proteins and Hmgn2 belongs to the second subfamily[34]. Together with other as yet unidentified HMG-box containing proteins, the Sox transcription factors form a complex, which binds to several consensus HMG binding sites in the collagen 2 promoter[35;36]. Hmgn2 may be one of these unknown binding proteins. Thus besides regulation of Sox9 activity by phosphorylation, PTHrP may also influence the expression of putative binding partners of Sox9. Remarkably, Hmgn2 is downregulated in ATDC5 and upregulated in metatarsals, but not regulated in osteoblasts, suggesting chondrocyte specific regulation of Hmgn2 expression by PTHrP.

Another response gene of PTHrP involved in protein binding and transcription regulation was Mxd4, a member of the Mad gene family. Mxd4 heterodimerizes with Max forming a transcriptional repression complex. Mxd4 competes for Max binding with Myc, which heterodimerizes with Max forming a transcriptional activation complex. Through regulation of Mxd4 expression, PTHrP could influence the activity of c-Myc, which plays an important role in cell proliferation and prevents the differentiation of cultured chondrocytes into hypertrophic chondrocytes[37;38]. Regulation of c-Myc activity might be one of the mechanisms by which PTHrP keeps the chondrocytes in a proliferative competent stage.

The remaining proteins involved in protein binding and signalling regulation were IGFBP4, Csrp2, and Calm1. PTHrP may play a role in changing intracellular Ca2+ levels by regulating Calm1 expression, which appears to be limited to the ATDC5 cells[39]. The expression regulation of Csrp2, a binding partner of Pias1, which inhibits Stat1, could indicate a possible crosstalk between PTHrP signalling and FGF or Growth Hormone (GH) signalling cascades as mentioned before[18;40]. Both factors use Jak/Stat signalling to exert their effects on chondrocyte proliferation and differentiation. In addition, our data indicates a crosstalk between PTHrP and the IGF signalling cascade as well. IGFBP4 is shown to exert inhibitory effects on IGF1-promoted growth[41;42]. Via upregulation of IGFBP4, PTHrP might be able to counteract IGF1-activity.

A remarkable finding in this study was the decrease of Agc mRNA expression in ATDC5 chondrocytes and an increase of Agc mRNA expression in explanted metatarsals after PTHrP treatment. In contrast to the metatarsals, which contain chondrocytes at various stages of differentiation as well as other cell types like osteoblasts and –clasts, the cultures of the ATDC5 cells are more homogenous. It may well be that regulation of Agc by PTHrP is dependent on the developmental and differentiation stage of the chondrocyte. This is supported by

observations in rat costal chondrocytes in which PTHrP either induced, slightly repressed or had no effect on Agc mRNA expression depending on the gestational age of the cells[43].
Previously we have shown that the majority of early PTHrP effects were part of a more generalised response, not only restricted to chondrocytes[18]. To address whether this was also the case for late PTHrP responses, the expression regulation of these genes by PTHrP were also established in KS483 osteoblasts and in explanted metatarsals, which consist of different cell types, like chondrocytes and osteoblast and osteoclast precursors in the perichondrium. In both systems differences were found in gene expression compared to the expression in ATDC5 chondrocytes in response to PTHrP. Most of the upregulated genes showed a temporal change in expression in metatarsals and osteoblasts. In addition, the direction of the expression of downregulated genes was changed or the expression was not regulated in osteoblasts and metatarsals. This suggest that between early and late effects mechanisms must be operational that translate the PTHrP responses from a more generalised effect, as seen in the immediate early response, into a cell type and cellular context dependent effect, as seen in the late response. Results of other studies also indicate such a mechanism[29;33]. The factors involved in this process are not known.
In summary, we have identified 9 late PTHrP response genes, including 4 novel target genes, Ecm1, Calm1, Hmgn2, and Mxd4. Among them were structural proteins, binding proteins and regulators of signalling. The expression of the late PTHrP response genes varied between cell types, suggesting a functional mechanism, translating the PTHrP responses from a more generalised early response, into a cell type and cellular context dependent late response.

Acknowledgements

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Confidence bands and p-values for qPCR data using the double delta model (DDM)

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Abstract

Background

Several mathematical models exist to calculate data derived from qPCR analysis. However, a model providing computations for expression ratios and p-values in experiments in which all samples are run in triplicate in a single qPCR experiment, well organized in a single spreadsheet, is not available.

Results

We have developed the double delta model (DDM) to calculate p-values and confidence bands in single qPCR experiments in which reference and target samples are run in triplicate. In this model, which is derived from the $2^{-\Delta\Delta Ct}$ method, the variances of the gene of interest as well as the variances of the reference are taken into account.

Conclusions

This model is particularly useful when working with many samples, because the calculations of the ratios and the calculations of the p-values are well organized in a single spreadsheet. Because the DDM does not include corrections for qPCR efficiency, it can only be used when the amplification efficiencies of the target and reference gene are close to 1. The DDM may also be applied to other data sets, in which experimental values are correlated with reference values.

Background

The quantitative Polymerase Chain Reaction (qPCR) is a powerful method to quantify gene expression (for review see[1]). Two different methods to analyze qPCR data are mainly used. The standard curve method is performed in circumstances where it is necessary to determine the absolute transcript copy number[2]. This method is also called absolute quantification and is usually performed by relating the PCR signal to a standard curve. In most situations, however, relative quantification will suffice. This is for example the case in experimental designs in which one would like to examine the effect of a drug or treatment on the expression of a given gene. Many mathematical models exist to calculate the mean normalized gene expression[3-6]. In these methods, the expression of the gene of interest is determined in control and treated samples and related to the expression of a reference gene, which is measured in the same samples. This reference gene is often a house-keeping gene, of which the expression is presumed not to be affected by the treatment.

An appropriate method for calculating expression ratios in data derived from a single qPCR experiment, in which the samples of the gene of interest and the reference gene are run in triplicate, is the $2^{-\Delta\Delta Ct}$ method. The derivations and assumptions have been described elsewhere[5]. Since different efficiencies will generate errors when using this method, the amplification efficiencies of the target and reference gene must be close to 1. This is often the case for amplicons designed to be less than 150 bp and for which the PCR conditions are properly optimized. However, this method does not include calculations of p-values and confidence bands.

A suitable model for calculating expression ratios and p-values in a single qPCR experiment, in which all samples are run in triplicate, is the relative expression software tool (REST). This tool is based on the correction for PCR efficiencies and the mean Ct-value deviation

between sample and control group[6]. In addition, REST also includes statistical analysis by using the pair-wise fixed reallocation randomization test. However, a disadvantage of the REST tool is the requirement of a new spreadsheet for the calculations of each sample in experiments in which the expression of 1 gene is determined in many different samples. This is a lot of work and could be confusing. Examples of such experiments are time series, dose range series or series using different stimuli.
In this letter we propose a novel statistical model, the double delta model (DDM). For calculations of expression ratios, p-values and confidence intervals, using qPCR data derived from a single qPCR experiment in which all samples are run in triplicate. This model is based on the $2^{-\Delta\Delta Ct}$ method[5]. Like the $2^{-\Delta\Delta Ct}$ method, the variances of the Ct-values of the gene of interest as well as the variances of the Ct-values of the reference gene are taken into account. This model does not include corrections for amplification efficiencies, so we want to emphasize that this model can only be used when these values are close to 1. This model is especially useful for calculating differences in gene expression in experiments with many samples. The computations of the ratios and p-values can be calculated in a single spreadsheet, making it well organized and easy to produce graphs.

Implementation

Here, we present a short derivation of the required formulas and show how to implement them as an Excel spreadsheet (table 1). Let *ypcr* be a probe concentration, where *p* indicates the type of probe (reference or not), *c* indicates the condition, and *r* indicates a repetition. Let $X_{pcr} = log_2 Y_{pcr}$. We assume that *x* is approximately normally distributed. This is more realistic than assuming a normal distribution for the concentrations, which are inherently positive and can vary over an enormous range. We also assume that the normal distribution has the same variance for all combinations of *p* and *c*.
The mean of all X_{pcr} for any combination of *p* and *c* will be indicated by

$$mpc = \sum_{r} X_{pcr} / n_{pc},$$

where *npc* is the number of observations. The DDM computes the contrast

$$d = (m_{22} - m_{21}) - (m_{12} - m_{11}) = m_{22} - m_{21} - m_{12} + m_{11} .$$

For explanation of symbols, see result section and table 1. The variance in each group can be estimated as

$$vp = \sum_{r} (X_{pcr} - m_{pc})^2 / n_{pc} - 1 .$$

These can be combined to estimate the pooled variance as

$$v = \sum_{p} \sum_{c} v_{pc} / DF ,$$

where

$$DF = \sum_{p} \sum_{c} n_{pc} - 1$$

Let se_{pc} indicate the standard error of m_{pc}. It is given by sepc = $\sqrt{\text{v/n}_{\text{pc}}}$. In other words: the variance of m_{pc} is v/n_{pc}. From elementary mathematical statistics it shows that the variance of a sum or difference of independent variables is equal to the sum of the variances. We thus find:

$$var(d) = v(1/n_{11} + 1/n_{12} + 1/n_{21} + 1/n_{22}),$$

and

$$se(d) = \sqrt{v(1/n11 + 1/n12 + 1/n21 + 1/n22)}$$

Under the assumption of normality, $d/\sqrt{se(d)}$ has a *t*-distribution with degrees of freedom (DF). This allows the computation of confidence bands and p-values. The confidence limits (CL) are computed as: $d \pm \tilde{t}(0.05;DF)*se(d)$, where $\tilde{t}(0.05;DF)$ is the critical value of the *t*-distribution with *DF* degrees of freedom at the 0.05 confidence level.

	A[1]	B[2]	C[3]	D[4]	E	F	G	H	I
1	Group	Stim.	Type	Symbol	Data1	Data2	Data3	Data4	Formula
2	ref.	-	PCR 1	x111	21,7	21,7	21,7	21,7	
3	ref.	-	PCR 2	x112					
4	ref.	-	PCR 3	x113	22,1	22,1	22,1	22,1	
5	ref.	+	PCR 1	x121	21,7	21,7	20,9	20,9	
6	ref.	+	PCR 2	x122		21,4	21,3	21	
7	ref.	+	PCR 3	x123	22,1	21,9	21,4	21,5	
8	GOI	-	PCR 1	x211	28,6	28,6	28,6	28,6	
9	GOI	-	PCR 2	x212	28	28	28	28	
10	GOI	-	PCR 3	x213	28,8	28,8	28,8	28,8	
11	GOI	+	PCR 1	x221	28,6	26,6	25,5	27,4	
12	GOI	+	PCR 2	x222	28	26,7	26	27,2	
13	GOI	+	PCR 3	x223	28,8	26,4	26	27,2	
14									
15	ref.	-	Mean	m11	21,90	21,90	21,90	21,90	=AVERAGE(E$2:E$4)
16	ref.	+	Mean	m12	21,90	21,67	21,20	21,13	=AVERAGE(E$5:E$7)
17	GOI	-	Mean	m21	28,47	28,47	28,47	28,47	=AVERAGE(E$8:E$10)
18	GOI	+	Mean	m22	28,47	26,57	25,83	27,27	=AVERAGE(E$11:E$13)
19	ref.	-	Var	v11	0,080	0,080	0,080	0,080	=VAR(E$2:E$4)
20	ref.	+	Var	v12	0,080	0,063	0,070	0,103	=VAR(E$5:E$7)
21	GOI	-	Var	v21	0,173	0,173	0,173	0,173	=VAR(E$8:E$10)
22	GOI	+	Var	v22	0,173	0,023	0,083	0,013	=VAR(E$11:E$13)
23	ref.	-	Count	n11	2	2	2	2	=COUNTIF(E$2:E$4, “>-9e99”)
24	ref.	+	Count	n12	3	3	3	3	=COUNTIF(E$5:E$7, “>-9e99”)
25	GOI	-	Count	n21	3	3	3	3	=COUNTIF(E$8:E$10, “>-9e99”)
26	GOI	+	Count	n22	3	3	3	3	=COUNTIF(E$11:E$13, “>-9e99”)
27									
28	Pooled	Pooled	SSQ	s	0,853	0,600	0,733	0,660	=(E23-1)*E19+(E24-1)*E20 +(E25-1)*E21+(E26-1)*E22
29	Pooled	Pooled	DF	DF	6	7	7	7	=SUM(E23:E26)-4
30	Pooled	Pooled	Var	v	0,142	0,086	0,105	0,094	=E28/E29
31									
32	Contrast		Mean	d	0,00	1,76	1,93	0,43	=(E17-E15)-(E18-E16)
33	Contrast		SE	se(d)	0,49	0,36	0,40	0,38	=SQRT(E30*(1/E23+1/E24+1/E25+1/E26))
34	Contrast		t	t	0,00	4,65	4,88	1,15	=E32/E33
35	Contrast		p-value	P	1,00	0,002	0,002	0,287	=TDIST(ABS(E34),E29,2)
36	Contrast		t crit	t(0,05;DF)	2,45	2,36	2,36	2,36	=TINV(0,05,E29)
37	Contrast		CL-		-1,19	0,82	1,00	-0,46	=E32-E36*E33
38	Contrast		CL+		1,19	2,51	2,87	1,32	=E32+E36*E33

Table 1: Example of the double delta model (DDM).

[1]) ref. = reference and GOI = gene of interest. [2]) Stim. = stimulation. [3]) var = variance. SSQ = sum of squares. DF = degrees of freedom. SE = standard error. CL = confidence limit. [4]) Symbols used in the mathematical equations and corresponding with the name in column C. xabc = Ct-value of group (a), stimulation (b), and type (c). mab/vab/nab = mean/variance/counts of group (a) and stimulation (b).

Results

We want to illustrate this model with an example in which the effect of treatment with a drug is examined on the expression of a particular gene at several time points. RNA is isolated from untreated (time point of stimulation) and treated specimens and prepared for qPCR analysis. Samples are run in triplicate in a single qPCR experiment and Ct-values are calculated for the gene of interest and the reference gene (see column E, F, G, and H table 1). Using the DDM we show that the drug has a significant effect on gene expression after 1 ($p = 0.002$) and after 2 hours ($p = 0.002$) (table 1 and fig. 1).

The computations can conveniently be organized as an Excel spreadsheet, as shown in table 1. The numbers in one data set are contained in one column (here columns E, F, G, and H). The upper rows (2– 13) hold the individual measurements (Ct-values). Samples (groups) are indicated in column A. Stimulation, where appropriate, is indicated in column B. Column C gives a name to each number in columns E, F, G, and H, while column D shows the correspondence with symbols in the mathematical equations. Column I shows the Excel formulas. This is done here for illustration purposes; in practical use this column (and perhaps also the fourth, containing the symbols) could probably be deleted. The middle rows (15–26) contain the calculations of mean, variance and sample size for each of the four groups. The lower block (rows 28–38) contains the pooled results and the final statistics, labelled as "contrast". The present spreadsheet assumes a maximum group size of 3. It is not difficult to add extra rows for larger sizes. Smaller group sizes or missing data are no problem: one simply leaves spreadsheet cells empty; the calculations are organized in such a way that they automatically handle missing data. In addition, it is not difficult to add extra columns for additional data sets. Figure 1 shows the graph with the mean expression of a gene of interest (GOI) at 0 (data1), 1 (data2), 2 (data3) and 4 (data4) hours after stimulation, compared to the mean expression at time point 0, described in table 1.

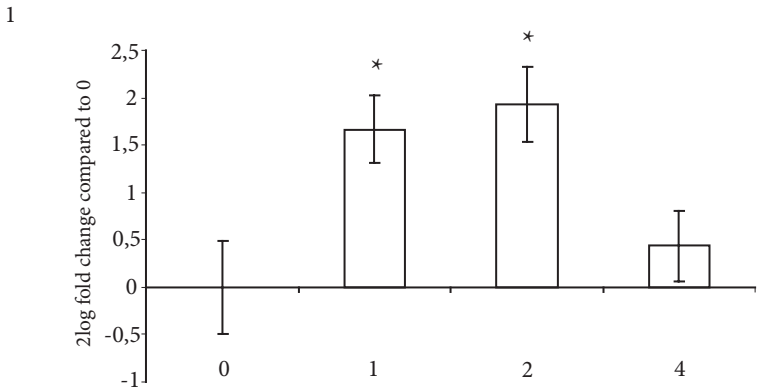

Figure 1. Expression of gene of interest.
The mean expression of the gene of interest (GOI) is shown as 2log fold change compared to 0, time point of stimulation (data1, table 1) at 1 (data2, table 1), 2 (data3, table 1) and 4 (data4, table 1) hours after stimulation. *Significant $p<0.05$.

Conclusions

Several mathematical methods exist to calculate data derived from qPCR analysis[3-6]. However, a model providing calculations for expression ratios and p-values in experiments in which all samples are run in triplicate in a single qPCR experiment, well organized in 1 spreadsheet, is not available. Therefore, we have developed a novel statistical model, the double delta model (DDM) to calculate p-values and confidence bands using data derived from a single qPCR experiment. The DDM is particularly useful when working with many samples. In addition, the DDM is also applicable to other data sets, in which experimental values are correlated with reference values. Examples are experiments, in which enzymatic activity is corrected for cell number or DNA concentration and transient transfection assays, in which luciferase activity is corrected for the transfection efficiency.

Availability

The double delta model is freely available on request from the corresponding author.

Authors' contributions

PE developed the algorithms for the DDM. GH, JW and MK provided biological insight and actively participated in discussion of the project. JH led the project and wrote the paper. All authors read and approved the final manuscript.

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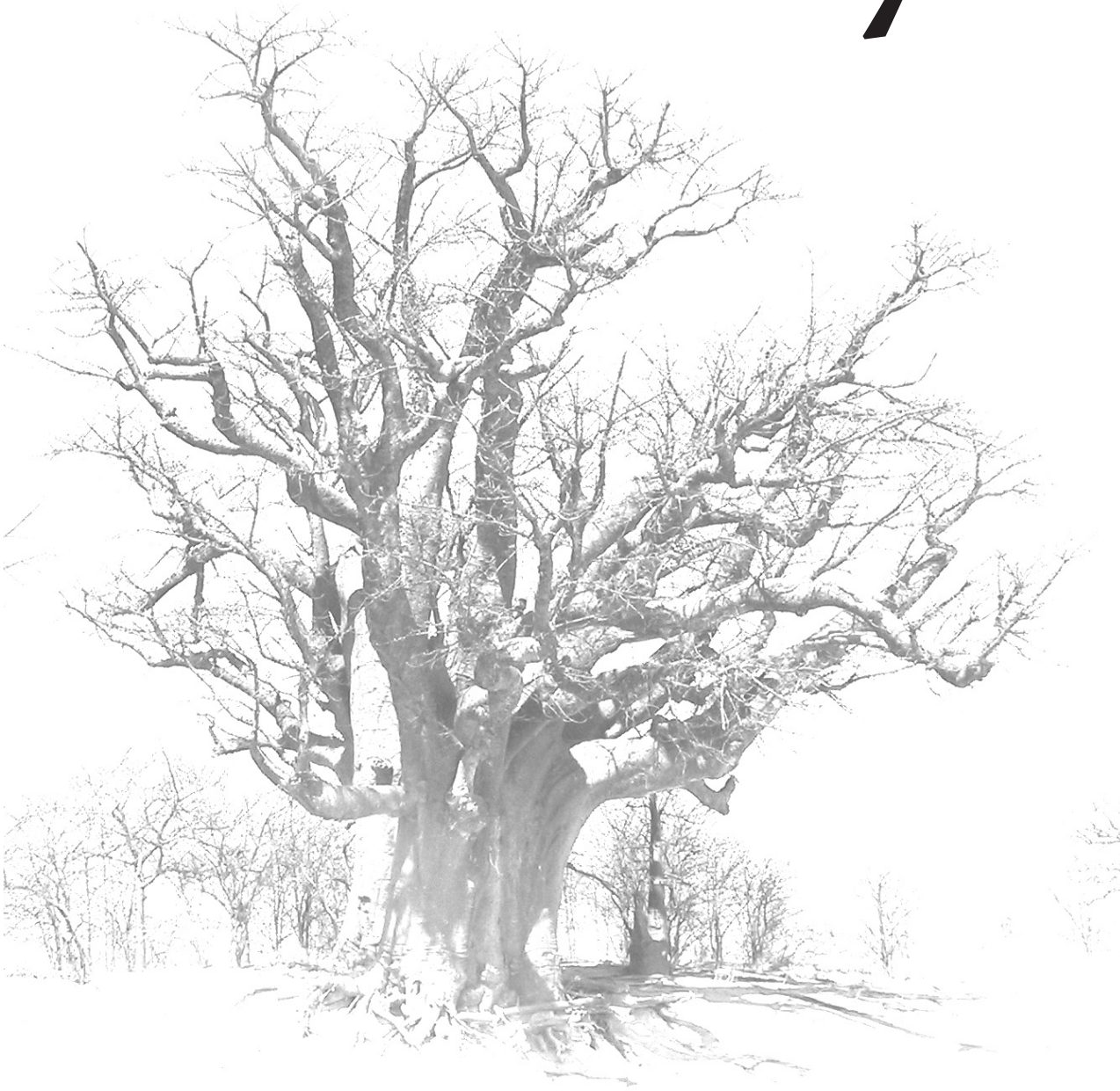
General Discussion

7



Introduction

A network of endocrine and paracrine factors regulates the process of chondrocyte proliferation and differentiation in the growth plate. One of the key regulators involved in this process is Parathyroid Hormone (PTH) related peptide (PTHrP). This has been underlined by several studies, in human and mice, in which type 1 PTH/PTHrP receptor (PTHR1) signalling is interrupted or augmented[1-11]. The exact working mechanism of PTHrP signalling, alone or in combination with other growth factors or systemic hormones, in endochondral bone formation is not completely understood. In this thesis, we further addressed the actions of PTHrP in the complex network of endocrine and paracrine regulation of endochondral bone formation.

The first major result described in this thesis was that the heterogeneity of Blomstrand Osteochondrodysplasia (BOCD) is caused by the impact of a mutation in the PTHR1 gene on the receptor function. In addition, we demonstrated that the PTHR1 adaptor proteins, Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2, are expressed during endochondral bone formation and that they may play a role in matrix mineralization during osteoblast and chondrocyte differentiation. Furthermore, we identified novel early and late PTHrP target genes using cDNA microarray and quantitative PCR (qPCR) analysis. Finally, we showed for the first time interaction between PTHrP and the janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathway.

In this chapter, these major findings will be summarized and critically reviewed.

Genotype-phenotype correlation in Blomstrand Osteochondrodysplasia

BOCD is a lethal osteochondrodysplasia characterized by severe skeletal malformations, due to accelerated endochondral bone formation[12-19]. It is caused by disruption of PTHR1 signalling[2;7-9]. BOCD can be divided into two types, type I and type II, depending on the severity of the skeletal abnormalities, with type I as the most severe phenotype[12]. The molecular basis for this heterogenic clinical presentation is unknown and, therefore, we have addressed the underlying causative factors in chapter 2. For this purpose, we performed mutation analysis in 2 families with type I BOCD and in 3 families with the less severe form, type II BOCD. The latter included 1 case that has not been described before.

Theoretically, there are two possible explanations of the variation in severity of the skeletal abnormalities. First, it is possible that the milder presentation of type II BOCD patients is caused by the genetic background. Support for this explanation comes from two sources. We have identified in two of the type II BOCD families the P132L mutation, which has already been found before in another family with type II BOCD. These families lived in the same region of England and originated from the same ethnic population[7;8]. In addition, it has been shown that the presentation of the phenotype of PTHR1 knockout mice depends on the genetic background[1]. For example, C57BL/6 mice die at mid-gestation, while Black Swiss mice die perinatally.

An alternative explanation is that the clinical severity depends on the degree of inactivation of the PTHR1. We have presented a novel family of different genetic origin, with another PTHR1 mutation causing type II BOCD, providing evidence that type II BOCD does not

depend on the genetic composition. Both mutations resulted in partial inactivation of the receptor with some residual activity. This is in contrast with the mutations identified in type I BOCD. These mutations, including the R104X described in chapter 2, resulted in a complete inactivation of the PTHR1[2;9]. Therefore, we concluded that type I BOCD resulted from a complete inactivation of the PTHR1, whereas a near complete inactivation of the PTHR1, resulting in low levels of residual activity, caused the relatively milder presentation observed in type II BOCD.

This phenomenon is not unique for BOCD. Gradations in the severity of a disease are also found in another syndrome arising from mutations in the PTHR1, namely Jansen's metaphyseal chondrodysplasia (JMC)[20]. This type of osteochondrodysplasia is caused by a constitutively activating mutation in the PTHR1 gene[4;6;11;21]. The T140P mutation caused the classical form of JMC[11]. Another mutation at the same amino acid position, T140R, resulted in ligand independent cAMP formation, which was less pronounced than observed with the T140P mutations[21]. This became clinically evident in a less severe presentation of JMC[21].

Heterogeneity in clinical manifestations due to the nature of the mutation is not only found in diseases arising from the PTHR1 gene. Several types of chondrodysplasia, including hypochondroplasia, achondrodysplasia and thanatophoric dysplasia (from less severe to most severe phenotype) arise from heterozygous mutations in the same gene, namely the Fibroblast Growth Factor (FGF) Receptor 3 (FGFR3)[22]. In these diseases a strong correlation between genotype and phenotype has been found[23].

Genotype-phenotype correlations have also been reported for campomelic dysplasia, a disease characterized by skeletal and also extra-skeletal abnormalities[24]. This disease is caused by mutations in the Sox9 gene, the main transcription factor for chondrocyte development. Both homozygous and heterozygous mutations have been described[25;26]. It has been stated that mutations, resulting in residual transactivation capacity of the transcription factor, may account for a milder phenotype and longer survival of patients[25].

Taken together, these data indicate that the impact of a mutation on the capacity of the receptor to activate downstream signalling pathways, critically determines the clinical presentation.

Regulation of PTHR1 signalling

In chapter 2 we showed that either the type or position of mutations in the PTHR1 resulted in distinct clinical features, due to complete or partial inactivation of the PTHR1. Partial inactivation resulted in residual activity of the PTHR1 and depending on the impact of the mutations, this residual activity could vary. This is also pronounced in patients with an activating mutation in the FGFR3, which can cause hypochondroplasia, achondrodysplasia and thanatophoric dysplasia[11;22].

Two pathways are activated by FGFR signalling in chondrocytes, the Jak/Stat pathway and the extracellular signal regulated kinase (ERK) pathway[27;28]. FGF is a potent inhibitor of chondrocyte proliferation and hypertrophic differentiation[29]. The dominant pathway after FGFR signalling influencing chondrocyte proliferation is the Jak/Stat pathway. FGFR signalling results in the activation of Stat1. Subsequently, Stat1 induces the expression of the cell cycle inhibitor $p21^{waf1/cip1}$, thereby inhibiting chondrocyte proliferation[29-31].

The ERK pathway appears to be more important in the inhibition of hypertrophic

differentiation. Transgenic mice that express a constitutively active mutant of ERK kinase (Mek1) in chondrocytes, displayed incomplete hypertrophy of chondrocytes, but showed no effect on chondrocyte proliferation[32]. Therefore, it has been hypothesized that FGFR signalling inhibits chondrocyte differentiation through the ERK pathway. Thus, the balance between the Jak/Stat pathway and the ERK pathway may adjust the effects of FGFR signalling on chondrocyte proliferation and differentiation[33]. Diverse mutations in the FGFR3 may have distinct impacts on the activity of the two main signalling pathways, which may result in the heterogeneity of the clinical presentation of chondrodysplasia.

Signalling through two pathways is also important in PTHR1 signalling[3;34]. Mouse knockout models have been generated to identify the functions of the two pathways that are activated by PTHR1 signalling in chondrocyte proliferation and differentiation. The two pathways are the adenylate (AC)/protein kinase A (PKA) and the phospholipase C beta (PLCβ)/protein kinase C (PKC) signalling pathway[3;34]. Recently, it has been shown that chondrocyte specific knockout mice, carrying a mutation in the Gαs and thereby disrupting AC/PKA signalling, displayed a phenotype comparable to the PTHrP knockout mice[34;35]. These mice had severe growth plate defects with reduction of the proliferative zone and accelerated chondrocyte differentiation. The opposite was demonstrated in mice carrying a mutant form of the PTHR1 (DSEL mice), which specifically interrupted signalling via the PLCβ/PKC pathway and signalled normally via the AC/PKA pathway[3]. These mice showed an increase in chondrocyte proliferation and a decrease in chondrocyte differentiation. These results indicated that the AC/PKA is the dominant pathway after PTHR1 signalling in stimulating chondrocyte proliferation and inhibiting chondrocyte differentiation. In addition, these results showed that chondrocyte differentiation is stimulated by the activation of the PLCβ/PKC pathway. Thus, the two pathways had opposite effects on chondrocyte proliferation and differentiation. In addition, these results showed that a disbalance between these pathways after PTHR1 activation can result in altered chondrocyte proliferation and differentiation and can cause heterogeneity of diseases, which are caused by mutations in the PTHR1.

The phenotype of the DSEL mice, in which PTHR1 signalling via the PLCβ/PKC pathway was specifically interrupted, was remarkably similar to the phenotype displayed by a consanguineous family with a rare growth disorder, the Eiken syndrome[3;36]. Eiken syndrome is caused by a homozygous mutation in the PTHR1 gene, resulting in a truncated PTHR1[5]. The truncated protein only missed a small part of the C-terminus. Because of the comparable phenotype of the mice model, it is hypothesized that PTHR1 signalling through the PLCβ/PKC pathway is disrupted. Eiken patients were considered normal at birth, but after a few months they developed a skeletal dysplasia, characterized by delayed ossification, principally of the epiphyses, the pelvis, the hands and the feet. Taken together, this indicates that the role of the PLCβ/PKC pathway becomes more pronounced after birth, while the AC/PKA pathway is the dominant pathway during prenatal growth, which has also been suggested previously[3;34].

Normal development of the growth plate requires a balanced signalling between the two main signalling pathways of PTHR1. This balance could be influenced by adapter proteins, like the PDZ domains containing proteins, Nherf1 and Nherf2. Nherf1 and Nherf2 were first found in renal tubuli as regulators of ion channels[37]. Further experiments revealed that the Nherf proteins not only bind to ion channels, but also to transcription factors, signalling molecules,

structural proteins and receptors[38;39]. The binding of Nherf1 and Nherf2 to the PTHR1 and PLCβ is best studied in renal tubuli[38].
In chapter 3 we showed that both Nherf1 and Nherf2 mRNAs were expressed in several organs using in situ hybridizations, which is in line with other studies[40;41]. In addition, we demonstrated Nherf1 and Nherf2 mRNA expression during endochondral bone formation by growth plate chondrocytes and osteoblasts in the bone collar. To establish whether Nherf1 and Nherf2 play a role in osteoblast and chondrocyte differentiation, Nherf1 and Nherf2 overexpressing KS483 cell lines were generated. Nherf1 and Nherf2 overexpression had distinct effects on the differentiation of osteoblasts and chondrocytes. While Nherf1 overexpression inhibited terminal osteoblast differentiation, Nherf2 overexpression stimulated terminal osteoblast and chondrocyte differentiation using matrix mineralization as a read out. The negative actions of Nherf1 in bone formation have been shown before in Nherf1 knockout mice[41]. Some, but not all female Nherf1 knockout mice were dwarfs and they displayed severe osteoporosis and bone fractures. This is most likely a secondary effect. Nherf1 regulates phosphate transport by binding to the sodium/phosphate co-transporter type IIa (Npt2a)[41]. In addition, Nherf1 null mice showed mild hypophosphatemia, which probably affected bone formation. However, our data suggests that the bone phenotype may also be explained partly by direct effects on osteoblastic matrix mineralization. Others have reported distinct functions for Nherf1 and Nherf2 in renal tubuli in the regulation of the Npt2[41-43]. The different functions for Nherf1 and Nherf2 might be explained by the recruitment of different molecules to form a membrane/submembrane bound complex, displaying diverse actions[40;44-46].
In chapter 3 we also showed that Nherf1 and Nherf2 overexpression did not change the actions of PTHR1 signalling on osteoblast en chondrocyte differentiation. We demonstrated, however, overexpression of Nherf1 and Nherf2 mRNA and it is likely that the Nherf1 and Nherf2 proteins are overexpressed as well, because both overexpressing cell lines displayed altered biological responses on osteoblast differentiation. Functional overexpression will probably induce a shift in the balance of PTHR1 signalling, since it has previously been shown that Nherf redirects PTHR1 signalling from the AC/PKA to PLCβ/PKC pathway in renal tubuli[38]. An explanation for the fact that we did not demonstrate an effect of Nherf1 or Nherf2 overexpression on PTHR1 signalling, could be inefficient coupling of Nherf1 and Nherf2 to the PTHR1 in osteoblasts and chondrocytes, in contrast to renal kidney cells. In addition, levels of PLCβ could be too low to affect the dominant AC/PKA pathway. The measurements of the activation of the AC/PKA pathway and the PLCβ/PKC pathway are currently underway.

Microarray and qPCR analysis

In chapter 4 and 5 we identified PTHrP target genes by performing microarray analysis. The microarrays, on which the NIA 15k mouse cDNA clone set[47] was spotted, were custom-made. The quality of the spots of the cDNA microarray was low, due to heterogeneous spot morphologies ("doughnuts"), deposition inconsistencies, and oversized spots. In addition, the quality control of cDNA microarrays is a well-known problem[48;49].
Halgren et al. reported a very high error rate of nearly 38% in the spots on cDNA microarrays[50]. The spots did not contain the cDNAs expected, because of contamination. Also in our study,

all spots containing the regulated cDNAs were sequenced and even a larger error rate was found. Approximately 55% of the cDNAs were not the cDNAs expected (unpublished data). The contamination is most likely introduced during multiple rounds of replication of the bank by PCR, as previously suggested[50;51]. Because of the uncertainty of the identity of the spots cDNA microarray analysis is not the ideal technique for pathway screening or genome wide analysis. For this purpose, commercially available oligonucleotide microarrays are much more reliable. cDNA microarray analysis is, however, a suitable technique for the identification of a subset of target genes, but only by applying very stringent selection criteria, by sequencing the regulated cDNAs, and by validation of regulated genes.

Since the cDNA microarray is used extensively in nearly all areas of biomedical research, investigators should always be aware of any indications that there may be serious unreliability in the microarray data. In addition, a major focus of microarray data appears to be centred on the statistical treatment of microarray data, such as image analysis, normalization, and background subtraction. However, more attention should be given to artefacts generated by amplification of the cDNAs and investigators should be especially cautious when interpreting data obtained from cDNA microarrays.

Microarray data, especially derived from cDNA microarrays, should be validated before jumping to conclusions. Bio-informatic analysis and techniques like Northern blot and qPCR analysis are mainly used for this purpose[52;53]. In our study we used bio-informatic analysis and qPCR analysis in different cell models and we thereby showed the validity of a part of the microarray data. However, due to the distinct methodologies and the relative poor quality of the custom cDNA microarrays, the overlap was not 100%, which is in line with other studies[52-54]. In our study the overlap in expression pattern between microarray and qPCR analysis was 57%. The expression pattern of the remaining genes differed only slightly between microarray analysis and qPCR analysis. In contrast to the expression patterns between microarray and qPCR analysis, which were comparable, the fold changes were dissimilar. A decrease, as well as an increase in fold change were demonstrated, which has also been reported by others[52-54]. cDNA microarray analysis is not a quantitative method in contrast to qPCR and Northern blot. Therefore, direction of changes, but not fold changes, can be determined with cDNA microarray analysis.

While the technical approach of qPCR is straightforward, the statistical analysis of qPCR data is less clear. A model providing computations for expression ratios and p-values in experiments in which all samples are run in triplicate in a single qPCR experiment, well organized in a single spreadsheet, is not available. For this purpose, we generated the double delta model (DDM), which is described in chapter 6. This model is derived from the $2^{-\Delta\Delta Ct}$ method and the variance of the gene of interest as well as the variance of the reference is taken into account[55]. The DDM is particularly useful when working with many samples, because the calculations of the ratios and the calculations of the p-values are well organized in a single spreadsheet. Because the DDM does not include corrections for qPCR efficiency, it can only be used when the amplification efficiencies of the target and reference gene are close to 1. The DDM may also be applied to other data sets, in which experimental values are correlated with reference values.

PTHrP target genes

To unravel how PTHrP exerts its effects on growth plate chondrocytes, we have identified early and late PTHrP target genes, using cDNA microarray analysis. By applying very stringent criteria for data generation and by performing validation studies we have produced a small list of early (12) and late (9) PTHrP response genes. The list of early PTHrP response genes included 7 upregulated (RGS2, SGK, Upar, IER3, Ptp4a1, Stat3, and Csrp2) and 5 downregulated (Sf3a2, Gab1, DYM, LamRI and Acvr2b) genes and the list of late response genes included 3 upregulated (IGFBP4, Csrp2, and Ecm1) and 6 downregulated (Col9a1, Col2a1, Agc, Hmgn2, Calm1, and Mxd4) genes. The majority of the early PTHrP response genes, 83%, were involved in signal transduction and regulation, compared to only 44% of the late PTHrP target genes. In addition, none of the early PTHrP response genes were structural proteins, compared to 44% of the late target genes. This is in agreement with the notion that the majority of early target genes are implicated in signal transduction and regulation and that most of late response genes code for structural proteins belonging to the extracellular matrix[54;56].

In chapter 4 we showed that the majority of early PTHrP effects were part of a more generalized response, not only restricted to chondrocytes, but also present in osteoblasts. In chapter 5 we demonstrated that the expression of most of the late response genes showed temporal and directional changes between the different cell models. This indicates that for studying the actions of PTHrP, the early response genes are most relevant. The late targets of PTHrP are not only manipulated by PTHrP treatment, but have also endured other influences, which are dependent of the cell type and environmental effects. This suggest that between early and late effects mechanisms must be operational that translate the PTHrP responses from a more generalized effect into a cell type and cellular context dependent effect. Results of other studies also indicate such a mechanism[54;56]. The factors involved in this process are not known.

Transcription factor binding sites in promoters of PTHrP target genes

To identify common conserved regulatory elements involved in the expression regulation of early PTHrP target genes, we analyzed their promoter regions. We found several conserved transcription factor binding sites in various early target genes. Among these transcription factor binding sites were cAMP response elements (CRE) and AP-1 response elements. CREs can be bound by CRE binding proteins (CREB). CREB and AP-1 are the main transcription factors activated by the AC/PKA pathway after PTHR1 signalling[3;57]. Remarkably, 6 out 7 of the upregulated genes in chondrocytes contained CREB transcription factor binding sites, 4 times in combination with an AP-1 response element, and only 2 out of 5 of the down regulated genes in chondrocytes contained CREB transcription factor binding sites. In addition, these 2 downregulated target genes were upregulated in osteoblasts. Comparable to our study were the predictions of CREB and AP-1 transcription factor binding sites predominantly in upregulated genes in a study by Qin et al.[58]. They used a statistical approach to identify transcription factor binding sites used by PTH-signalling in osteoblasts instead of enrichment for evolutionary conserved binding sites applied in this paper. This suggests that CREB and AP-1 are predominantly involved in upregulation of transcription of PTHrP target genes.

Among downregulated genes by CREB after PTHR1 signalling is the transcription factor for osteoblast differentiation and hypertrophic chondrocyte differentiation, the runt related transcription factor 2 (Runx2)[59;60]. The suppression of Runx2 transcription is primarily regulated through the AC/PKA signalling pathway[61]. This effect is only partially mediated via CREB, suggesting that other transcription factors are involved in the negative regulation of Runx2 transcription and probably also in the transcription of other downregulated genes[61].

PTHrP and the interactions with other paracrine and endocrine signals

Endochondral bone formation is under the control of endocrine (systemic hormones) and paracrine (growth factors) factors[29;62-64]. From the systemic hormones, the dominant regulator of endochondral bone formation is Growth Hormone (GH)[65]. GH can act directly on the growth plate, via its receptor, which is expressed by growth plate chondrocytes[66]. In addition, GH influences chondrocyte proliferation and differentiation indirectly via the induction of Insulin-like Growth Factor 1 (IGF-1)[67-69]. It is likely that GH also controls the expression of other locally produced growth factors, for instance components of the Indian Hedgehog (IHh)/PTHrP negative feedback loop[62]. In this thesis we provide evidence for the opposite, namely, the regulation of GH actions by PTHrP. Thus, the interaction between GH and PTHrP might be reciprocal.

Besides GH, another important regulator of chondrocyte differentiation in the postnatal growth plate is estrogen. Its main actions are inducing the growth spurt at the beginning of puberty and stimulation of growth plate fusion at the end of puberty[70;71]. While we demonstrated possible interactions between PTHrP and GH, no indications for interactions between PTHrP and estrogens were found in our limited data set.

In chapter 4 we demonstrated for the first time a crosstalk between PTHrP and members of the Jak/Stat family. PTHrP induced the mRNA expression of Stat3, Stat5a, Stat5b, and Csrp2. Csrp2 is a binding partner of PIAS1, which is an inhibitor of Stat1[72]. In addition, several conserved Stat transcription factor binding sites were predicted in the early PTHrP response genes. Furthermore, PTHrP influenced posttranslational activation of Stat3, Stat5a, and Stat5b. Interestingly, activation of Stat3 was increased, while the activation of Stat5a and Stat5b was decreased. Both the increased activity of Stat3 and the decreased activity of Stat5a and Stat5b after AC\PKA signalling have already been demonstrated before in other cell types[73;74].

Stat5b is the most important Stat protein in GH signalling, with respect to longitudinal growth[75;76]. GH induces IGF-1 expression through Stat5b and IGF-1 stimulates chondrocyte proliferation and differentiation[69;77]. By inhibiting Stat5b activity, PTHrP may diminish the positive actions of GH on chondrocyte proliferation and differentiation. Thus, we found that paracrine factors, in our study PTHrP, may influence the actions of endocrine signals, in our study GH. Our experiments were not set up to investigate whether endocrine signals influenced the actions of paracrine factors. This suggests that if indeed endocrine signals exert their effects by modulating paracrine factors, like PTHrP, it is not a one-way direction, but a reciprocal interaction.

Most Stat proteins are also involved in FGFR signalling. The negative regulation of chondrocyte proliferation by FGFR signalling is mediated through Stat1, by inducing the

expression of cell cycle inhibitor p21$^{waf1/cip1}$[30;31]. Stat1 activation is inhibited by PIAS1, which is a binding partner of Csrp2[72]. By inducing the expression of Csrp2 (chapter 4 and 5), PTHrP could alleviate the inhibitory actions of PIAS1 on Stat1 and thereby increasing the negative regulation of chondrocyte proliferation. The inhibitory actions of FGFR signalling on chondrocyte differentiation is mediated through the ERK pathway[32]. No indications for interactions between PTHrP and the ERK pathway were found in our limited data set.
The role of PTHrP in the growth plate is regulating the transition of proliferating into hypertrophic chondrocytes and thereby keeping the chondrocytes in a proliferative competent stage[78]. It has been shown that p57, a member of the CIP/KIP family of inhibitors of cyclin-dependent kinases, is one of the major mechanism used by PTHrP to maintain chondrocyte proliferating and delay their differentiation[79;80]. Studies with transgenic mice demonstrated that the level of PTHrP is very important in normal endochondral bone formation. Both mice with ablation of the PTHrP gene or ectopic expression of PTHrP showed severe dwarfism[35;81]. The expression of PTHrP is tightly controlled. This is accomplished by IHh[62].
In this study we provide evidence that the biological actions of PTHrP might also be controlled by other regulators of endochondral bone formation. PTHrP may influence the signalling pathways of FGF and GH, thereby probably counteracting its positive effect on chondrocyte proliferation, but possibly enhancing its negative effect on chondrocyte differentiation (fig. 1). PTHrP might affect the actions of FGF signalling on chondrocyte proliferation and the actions of GH signalling on both chondrocyte proliferation and differentiation, through influencing Jak/Stat signalling. Through inhibition of the activity of Stat5b by PTHrP, IGF-1 expression may be diminished, presumably resulting in a decrease of chondrocyte proliferation and differentiation. In addition, by inducing Csrp2 expression, PTHrP is potentially able to alleviate the inhibition of PIAS1 on Stat1 activity. This may result in a decrease of chondrocyte proliferation. Experiments to establish whether Stat1 is directly activated by PTHR1 signalling are currently underway. In addition, conflicting data have been reported about Stat3 phosphorylation after FGF activation. Hart et al. found that FGFR signalling could activate Stat3 in the fibroblast cell line NIH3T3, however, another study using rat chondrosarcoma cells (RCS), revealed that FGF treatment had no effect on Stat3 phosphorylation[30;82]. Therefore, the role of Stat3 after FGF activation in this model is unclear.
The transition of proliferating chondrocytes into hypertrophic chondrocytes is under the control of the IHh/PTHrP negative feedback loop[62]. It has been proposed that FGF and BMP signalling affects the negative feedback loop. They show opposite effects on the expression of IHh, on chondrocyte proliferation and on terminal differentiation[29;63]. These actions are believed to be independent of the IHh/PTHrP pathway[29]. By showing that PTHrP influenced the Jak/Stat pathway, which signalling molecules are intracellular mediators of FGF signalling, we provide evidence that a cross talk between PTHrP and FGF signalling may exist.

Final remarks

PTHrP is an essential growth factor in the regulation of growth plate chondrocyte development. It works, however, in a complex environment of other growth factors and endocrine signals, which can influence either their own or each others expression and activity. Thus, the overall effect of the actions of systemic hormones and locally acting growth factors on chondrocyte

1

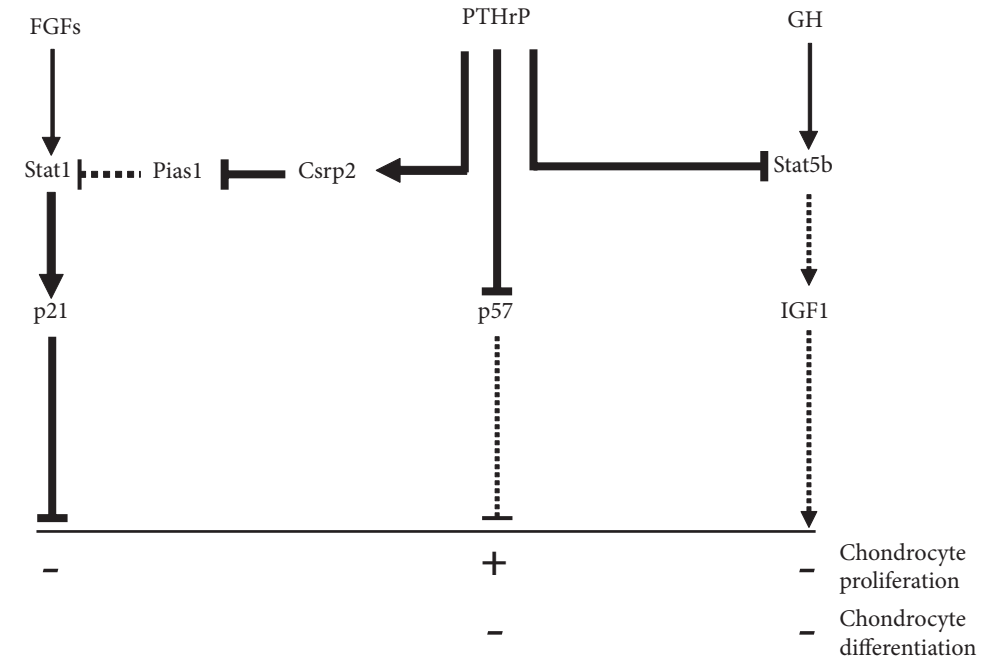


Figure 1: positive and negative actions of PTHrP on chondrocyte proliferation and differentiation.
PTHrP exerts its positive effects on chondrocyte proliferation through the suppression of p57 expression. We hypothesize that PTHrP may also have negative effects on chondrocyte proliferation, through influencing the signalling pathways of FGF and GH and may enhance its negative effect on chondrocyte differentiation, through influencing the GH signalling pathway. PTHrP is potentially able to alleviate the inhibition of PIAS1 on Stat1 activity by inducing the expression of Csrp2. The negative actions of Stat1 on chondrocyte proliferation after FGF activation may be enhanced through inhibition of p21. GH induces IGF-1 expression through Stat5b activation. PTHrP decreases posttranslational activation of Stat5b, thereby probably inhibiting the positive actions of IGF-1 on chondrocyte proliferation and differentiation.

proliferation and differentiation is the result of the activation of signalling pathways and the interactions between them. The occurrence and intensity of specific signalling responses depend upon many factors that may be controlled directly or indirectly by the environment of the chondrocyte. These may include the level of receptor expression, the abundance of adaptor or scaffolding proteins, for instance Nherf, or the expression and activity of downstream kinases and their substrates. In addition, interactions with adjacent cells or with the extracellular matrix could also influence the response to systemic hormones or growth factors.

In the past, most studies have focussed on the actions of a single gene. With the rapid advancements in technology, a growing number of studies are using techniques to determine a wide range of target genes. Among these techniques is genome wide analysis, but another increasing field of interest is the field of bioinformatics and the use of computer models. By using these techniques, the connections between the target genes will lead to new insights.

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Summary/Samenvatting

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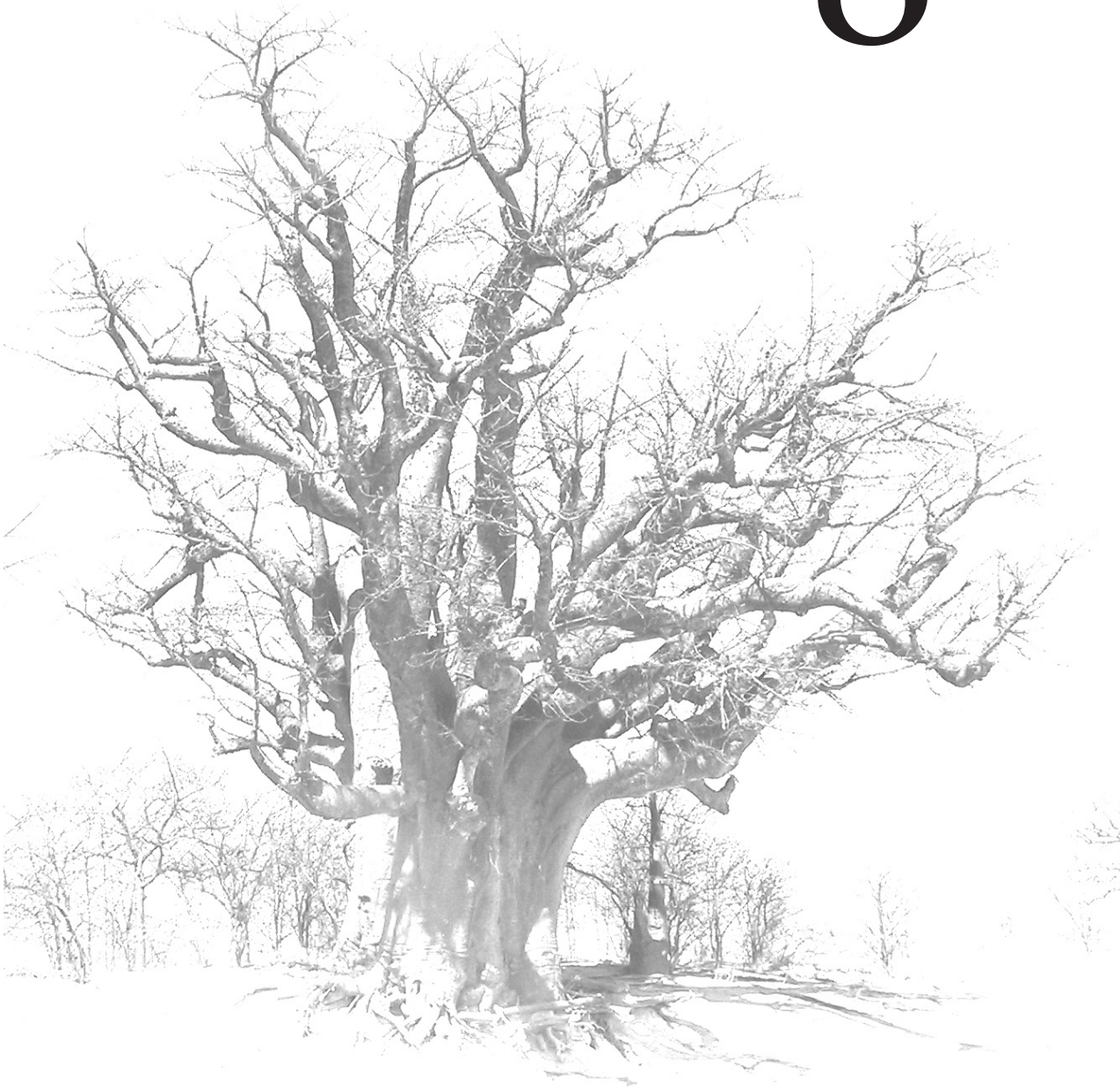

Summary

Longitudinal growth is the key characteristic that distinguishes children from adults. Growth is regulated in the growth plates, which are layers of cartilage located at the ends of the long bones. The cartilage cells are called chondrocytes and go through a coordinated program of proliferation, maturation, hypertrophic differentiation, apoptosis and replacement by bone. This process is called endochondral bone formation. A complex network of hormones (endocrine regulators) is involved in this process. The mechanism of actions of these hormones is not completely understood. A possible mechanism of action is the interaction of systemic hormones with locally produced growth factors (paracrine regulators), like the Parathyroid Hormone (PTH) related Peptide (PTHrP) and its receptor, the type 1 PTH/PTHrP receptor (PTHR1). PTHrP and PTHR1 are among the key regulators in the process of endochondral bone formation. The working mechanism of PTHrP signalling, alone or in combination with other growth factors or systemic hormones, in endochondral bone formation is not completely understood. In this thesis, we have further addressed the actions of PTHrP in the complex network of endocrine and paracrine regulation of endochondral bone formation.

Disruption of PTHR1 signalling, caused by a loss of function mutation in the PTHR1 gene, results in the human in Blomstrand Osteochondrodysplasia (BOCD). BOCD can be divided into two types, depending on the severity of the skeletal abnormalities, with type 1 as the most severe form. The molecular basis for the heterogenic presentation is unknown. In chapter 2 the underlying causative factor of this heterogeneity in clinical presentation of BOCD is addressed. We performed mutation analysis in 2 families with type I BOCD and in 3 families with the less severe form, type II BOCD. The latter group includes 1 case that has not been described before. In one of the type I BOCD cases a nonsense mutation (R104X) was found, resulting in a truncated PTHR1, while in the second type I BOCD case no receptor mutation was identified. A splicing defect (intronM4+27C>T) was demonstrated in one of the type II BOCD cases. In the other 2 families with type II BOCD a previously identified missense P132L mutation was found. Functional analysis demonstrated a near complete inactivation of the PTHR1 with low residual activity in the type II BOCD cases. In combination with published data , we concluded that a complete inactivation of the PTHR1 causes type I BOCD, whereas the relatively milder presentation of type II BOCD is caused by low levels of residual activity due to a near complete inactivation of the PTHR1.

In chapter 3 we investigated the role of the adaptor proteins for the PTHR1, the PDZ domain containing proteins Na+/H+ exchanger regulatory factor 1 (Nherf1) and Nherf2, in endochondral bone formation and PTHR1 signalling. Using in situ hybridization we showed that Nherf1 and Nherf2 mRNAs are expressed during endochondral bone formation by osteoblasts in the bone collar and by chondrocytes in the growth plate. In addition, overexpression of Nherf1 and Nherf2 in KS483 mesenchymal progenitor cells resulted in opposite effects on terminal osteoblast differentiation. Nherf1 overexpression inhibited, while Nherf2 overexpression stimulated matrix mineralization. Cartilage matrix mineralization was increased in the Nherf2, but not in the Nherf1 overexpressing cell line. Overexpression of Nherf1 or Nherf2 had no effect on the production of early chondrocyte markers, the glycosaminoglycans (GAGs). PTHrP inhibited osteoblastic matrix mineralization and GAG production in chondrocytes. Overexpression of Nherf1 or Nherf2 did not change the effect of PTHrP treatment on osteoblast and chondrocyte differentiation. We concluded that the

distinct actions of Nherf1 and Nherf2 are probably due to the recruitment of different target molecules, like transcription factors, receptors and signalling molecules.
We used the chondrogenic ATDC5 cell line and performed cDNA microarray analysis to identify early PTHrP response genes in chondrocytes and to recognize interactions with other regulatory factors in chapter 4. For this purpose, ATDC5 cells were treated for 1, 2, and 4 hours with PTHrP. By applying very stringent criteria for data generation and by performing validation studies we have produced a small list of early PTHrP response genes, including 7 upregulated (RGS2, SGK, Upar, IER3, Ptp4a1, Stat3, and Csrp2) and 5 downregulated (Sf3a2, Gab1, DYM, LamRI and Acvr2b) genes. The majority of these genes were implicated in signal transduction and regulation. The early response genes were part of a generalized response to PTHrP, since they were also identified as PTHrP response genes in osteoblasts. The majority of the upregulated early response genes contained CREB and AP-1 transcription factor binding sites in their promoter regions, suggesting that CREB and AP-1 are predominantly involved in upregulation of transcription of PTHrP target genes. Among the early response genes were mediators of the Janus Kinase (Jak)/Signal transducer and activator of transcription (Stat) pathway. In addition, several Stat transcription factor binding sites were predicted in the promoter regions of the early response genes. Furthermore, in transient transfection assays we showed that PTHrP positively regulated the posttranslational activity of Stat3 and negatively regulated the posttranslational activity of Stat5a and Stat5b. This data is suggestive for a previously not recognized interaction between PTHrP and Jak/Stat signalling.
In chapter 5 the identification of late PTHrP target genes using the ATDC5 cell line and cDNA microarray analysis is described. For the identification of late PTHrP target genes the ATDC5 cells were treated for 24 and 72 hours with PTHrP. By applying very stringent criteria for data generation and by performing validation studies we have produced a small list of late PTHrP target genes. The list of late response genes included 3 upregulated (IGFBP4, Csrp2, and Ecm1) and 6 downregulated (Col9a1, Col2a1, Agc, Hmgn2, Calm1, and Mxd4) genes. The majority of the late response genes code for structural proteins belonging to the extracellular matrix. The expression of late PTHrP response genes varied between different cell types, suggesting that the expression of late response genes is dependent on the cellular context of the PTHrP responsive cells.
The early and late response genes were validated by quantitative PCR (qPCR) analysis. While the technical approach of qPCR is straightforward, the statistical analysis of qPCR data is less clear. We developed the Double Delta Model (DDM) to calculate p-values and confidence bands in data derived from qPCR analysis, which is described in chapter 6.
In chapter 7 the major findings of this thesis are summarized and critically reviewed in relation to current literature. These findings include the discussion of the quality of cDNA microarrays and the interactions of PTHrP with other regulators of endochondral bone formation.

Samenvatting (Summary in Dutch)

Lengtegroei is één van de belangrijkste verschillen tussen kinderen en volwassenen. De groei wordt gereguleerd in de groeischijven. De groeischijven bevinden zich aan de distale uiteinden van de pijpbeenderen en bestaan uit kraakbeen. De cellen in de groeischijf worden kraakbeencellen of chondrocyten genoemd. Deze chondrocyten doorlopen een gecontroleerd

programma van celdeling (proliferatie), maturatie, differentiatie en geprogrammeerde celdood (apoptose). Dit proces eindigt met de vervanging van het kraakbeen door bot en wordt endochondrale botvorming genoemd. Een complex netwerk van hormonen (endocriene regulatoren) is betrokken bij de regulatie van dit proces. Hoe deze regulatie precies verloopt is nog niet helemaal duidelijk. Een mogelijk mechanisme zou kunnen zijn, dat hormonen de activiteit van locaal geproduceerde groeifactoren (paracriene regulatoren) beïnvloeden, bijvoorbeeld door het reguleren van het "Parathyroid Hormone (PTH) related Peptide" (PTHrP) en de bijbehorende type 1 PTH/PTHrP receptor (PTHR1). PTHrP and PTHR1 behoren tot de groep van belangrijkste regulatoren binnen het proces van de endochondrale botvorming. Het mechanisme waarmee PTHrP endochondrale botvorming reguleert, alleen of in combinatie met hormonen, is nog niet helemaal duidelijk. In dit proefschrift hebben we de werking van PTHrP binnen het complexe netwerk van endocriene en paracriene regulatie van de endochondrale botvorming verder uitgediept.

Een homozygote inactiverende mutatie in het PTHR1 gen, waardoor een onderbreking ontstaat in de signaalroute van de PTHR1, leidt in de mens tot Blomstrand Osteochondrodysplasia (BOCD). Afhankelijk van de ernst van de afwijkingen in het skelet wordt deze ziekte in twee types verdeeld, waarbij type I de meest ernstige vorm is. De moleculaire basis voor deze heterogene presentatie is niet bekend. In hoofdstuk 2 hebben we dit onderzocht met behulp van mutatie analyse in 2 families met type I BOCD en in 3 families met de minder ernstige vorm, type II BOCD. Deze laatste groep bevat een familie, die in hoofdstuk 2 voor het eerst wordt beschreven. In één van de type I BOCD families werd een nonsense mutatie (R104X) gevonden, wat resulteerde in een "getrunkeerd" PTHR1 eiwit. In de tweede type I BOCD familie werd geen mutatie in het PTHR1 gen geïdentificeerd. Een splicingsfout (intronM4+27C>T) werd gevonden in één van de type II BOCD families. De andere twee families met type II BOCD hadden de P132L missense mutatie in het PTHR1 gen. Deze mutatie is al eerder beschreven. Functionele analyse liet in de type II BOCD families een bijna complete inactivatie van de PTHR1 met lage restactiviteit zien. In combinatie met reeds gepubliceerde data hebben we geconcludeerd dat een complete inactivatie van de PTHR1 leidt tot type I BOCD en dat type II BOCD veroorzaakt wordt door een lage restactiviteit van de receptor, wat komt door een niet geheel complete inactivatie van de PTHR1.

In hoofdstuk 3 hebben we de rol van de adaptor eiwitten voor de PTHR1, de PDZ domein bevattende eiwitten "Na+/H+ exchanger regulatory factor 1" (Nherf1) en Nherf2, in endochondrale botvorming en in PTHR1 signalering onderzocht. Met behulp van in situ hybridisatie hebben we laten zien dat de Nherf1 en Nherf2 mRNAs tot expressie worden gebracht gedurende endochondrale botvorming, in de bot aanmakende cellen (osteoblasten) in de botkoker en in de chondrocyten in de groeischijf. Daarnaast hebben we laten zien dat overexpressie van Nherf1 en Nherf2 in KS483 mesenchymale voorlopercellen resulteerde in tegenovergestelde effecten in terminale osteoblast differentiatie. Nherf1 overexpressie remde de matrix mineralisatie, terwijl Nherf2 overexpressie deze stimuleerde. De mineralisatie van de kraakbeenmatrix was eveneens toegenomen door overexpressie van Nherf2, maar niet door overexpressie van Nherf1. Overexpressie van Nherf1 of Nherf2 veranderde de productie van de vroege chondrocyte markers, de glycosaminoglycanen (GAGs), niet. PTHrP remde de mineralisatie van de botmatrix en remde de GAG productie door chondrocyten. Overexpressie van Nherf1 of Nherf2 veranderde dit effect van PTHrP op osteoblast en chondrocyte

differentiatie niet. De afwijkende effecten van Nherf1 en Nherf2 worden mogelijk veroorzaakt door de interactie met verschillende target moleculen, zoals transcriptie factoren, receptoren en signaal moleculen.
In hoofdstuk 4 hebben we gebruikt gemaakt van ATDC5 cellen, die te beschouwen zijn als voorlopercellen van chondrocyten, en cDNA microarray analyse om vroege PTHrP respons genen in chondrocyten te identificeren en om interacties met andere regulatiefactoren te vinden. Voor het identificeren van vroege PTHrP respons genen, zijn de ATDC5 cellen voor 1, 2 en 4 uur gestimuleerd met PTHrP. Door gebruik te maken van zeer strenge selectie criteria en door het doen van validatie studies hebben we een kleine lijst met vroege PTHrP respons genen kunnen maken. Deze lijst bestond uit 7 opgereguleerde (RGS2, SGK, Upar, IER3, Ptp4a1, Stat3 en Csrpe) en 5 downgereguleerde (Sf3a2, Gab1, DYM, LamRI en Acvr2b) genen. Het grootste deel van deze genen is betrokken bij de regulatie van signaaltransductie. De vroege respons genen maken deel uit van een meer algemene PTHrP respons, omdat deze genen ook in osteoblasten zijn gevonden als vroege PTHrP respons genen. In de promoter regionen van de meeste opgereguleerde vroege PTHrP respons genen hebben we CREB en AP-1 transcriptiefactor bindingsplaatsen gevonden. Dit suggereert dat CREB en AP-1 bovenal betrokken zijn bij de opregulatie van transcriptie van PTHrP respons genen. De groep van vroege respons genen bevatte eveneens genen die betrokken zijn bij de "Janus Kinase" (Jak)/"Signal transducer and activator of transcription" (Stat) signaalroute. Ook werden verschillende Stat transcriptie factor bindings domeinen voorspeld in de promoter regionen van de vroege respons genen. Daarnaast hebben we laten zien, door gebruik te maken van transiente transfectie analyses, dat PTHrP de posttranslationele activiteit van Stat3 positief beïnvloedde en dat PTHrP de posttranslationele activiteit van Stat5a en Stat5b negatief beïnvloedde. Deze bevindingen zijn suggestief voor een niet eerder gevonden interactie tussen PTHrP en Jak/Stat signalering.
In hoofdstuk 5 hebben we late PTHrP respons genen geïdentificeerd, door gebruik te maken van ATDC5 chondrocyte voorlopercellen en cDNA microarray analyse. Om late PTHrP respons genen te identificeren, zijn de ATDC5 cellen voor 24 en 72 uur gestimuleerd met PTHrP. Door gebruik te maken van zeer strenge selectie criteria en door het doen van validatie studies hebben we een kleine lijst met late PTHrP respons genen kunnen maken. Deze lijst bestond uit 3 opgereguleerde (IGFBP4, Csrp2 en Ecm1) en 6 downgereguleerde (Col9a1, Col2a1, Agc, Hmgn2, Calm1 en Mxd4) genen. Het grootste deel van de late repons genen codeerde voor structurele eiwitten behorende tot de extracellulaire matrix. De expressie van de late PTHrP respons genen verschilde tussen celtypes, wat suggereerde dat de expressie van late response genen afhankelijk is van de cellulaire context van de PTHrP responsieve cellen.
De vroege en late PTHrP repons genen zijn gevalideerd met behulp van kwantitative PCR (qPCR) analyse. De technische procedure van qPCR is duidelijk. De statistische analyse van qPCR data daarentegen is minder duidelijk. Om p-waarden en betrouwbaarheids intervallen te berekenen in data van qPCR analyse, hebben we het "Double Delta Model" (DDM) ontwikkeld. Dit is beschreven in hoofdstuk 6.
Tenslotte zijn in hoofdstuk 7 de belangrijkste bevinden van dit proefschrift samengevat en bediscussieerd in het licht van de bestaande literatuur. Deze bevindingen omvatten onder andere de discussie over de kwaliteit van de cDNA microarrays en de interacties van PTHrP met andere regulatoren van endochondral botvorming.

Curriculum Vitae

Curriculum Vitae

Jakomijn Hoogendam werd geboren op 2 maart 1979 te Delft. Na het behalen van haar Atheneum diploma op het Christelijk Lyceum Delft (CLD) in 1997, begon zij in datzelfde jaar met de studie Biomedische Wetenschappen aan de Universiteit Leiden. In het derde en vierde jaar werd onderzoek verricht op de afdelingen Endocrinologie en Stofwisselingsziekten onder leiding van dr. R van Bezooijen en Infectieziekten onder leiding van Professor dr. JT van Dissel van het Leids Universitair Medisch Centrum. Dit werd gevolgd door haar afstudeerstage aan de afdeling Moleculaire Celbiologie in Leiden onder leiding van dr. K de Rooij.
Na haar afstuderen als doctorandus in 2001, werd zij in september 2001 aangesteld voor 4 jaar als assistent in opleiding bij de afdeling Kindergeneeskunde in het Leids Universitair Medisch Centrum onder leiding van Professor dr. JM Wit en dr. M Karperien. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

List of publications

Deckers MM, Van Bezooijen RL, Van der Horst G, **Hoogendam J**, Van der Bent C, Papapoulos SE, Löwik CW. 2002 Bone morphogenetic proteins stimuate angiogenesis through osteoblast-derived vascular endothelial growth factor A. Endocrinology. 143(4):1545-53

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Van der Horst G, **Hoogendam J**, Farih-Sips H, Feitsma A, Löwik CW, Karperien M. 2006 Fast generation of stable cell lines to study gene function during mesenchymal differentiation, using gene overexpression or RNA silencing. Manuscript in preparation

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Hoogendam J, Parlevliet E, Miclea R, Lowik CW, Wit JM, Karperien M. 2006 Novel early target genes of PTHrP in chondrocytes. Endocrinology. 147(6):3141-52

Hoogendam J, Farih-Sips HCM, Van Beek E, Löwik CW, Wit JM, Karperien M. 2006 Novel late target genes of PTHrP in chondrocytes. Submitted for publication

Hoogendam J, Van der Horst G, Wit JM, Karperien M, Eilers PHC. 2006 Confidence bands and p-values for qPCR data using the double delta model (DDM). Submitted for publication