1 Introduction

1.1 Skeleton of vertebrates

The skeleton plays very important roles for the vertebrates, as it is responsible for the stability and mechanical properties of the body. In addition the skeleton protects the central nervous system and some of the internal organs, as the heart and the lungs. Moreover, the bone marrow of the long bones harbors the stem cells of the hematopoetic system (Seibel et al., 1999). Anatomically, the vertebrate skeleton is divided into three parts: the skull, the axial skeleton (ribs, vertebrae and pelvis) and the appendicular skeleton (bones of the fore- and hindlimbs) (Kaufmann, 1992). Since various mutations in human result in different dwarfism syndromes affecting the long bones of limbs, this study has been concentrated on investigating the bone forming processes in the forelimb bones. Following from proximal to distal the forelimb is subdivided into scapula, humerus, ulna and radius, carpal, metacarpal and phalangeal bones (Fig.1) (Kaufmann, 1992).

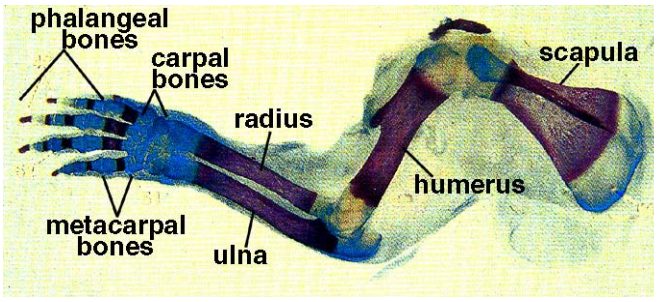

Fig. 1 Skeletal elements of the mouse forelimb

Following from the proximal (right) to the distal (left) direction, the forelimb is divided into scapula, humerus, radius and ulna (shown here as upper bone and lower bone, respectively), the metacarpal and carpal bones, and the phalangeal bones, consisting of the fingers.

Histologically, the vertebrate skeleton consists of various types of skeletal tissues: bone, cartilage, tendons and ligaments. Bone is the main tissue of skeleton. It contains two types of cells: osteoblasts producing a bone matrix, and osteoclasts resorbing the old bone structure (Seibel et al., 1999). During the entire vertebrate life a bone structure is continuously remodeled by osteoblasts and osteoclasts. The bone matrix consists of various glycoproteins, as for example collagen type I, and glycosaminoglycans. Additionally the bone matrix is a heavily mineralized tissue (about 70% of its wet weight), almost due to deposition of calcium phosphates, which provides the mechanical stability of the bones (Cormack, 1987; Seibel et al., 1999). The cartilage is other skeletal tissue, which serves as supporting and connective tissue in the developing skeleton. Chondrocytes, which build the cartilage elements, produce a specific cartilaginous matrix. The cartilaginous matrix is an amorphous structure due to high content of proteoglycans, which helps to hold the interstitial water (Cormack, 1987). In addition, it can be mineralized, but to a less extend than bone matrix (Cormack, 1987); (Olsen

et al., 2000; Seibel et al., 1999). Cartilaginous matrix contains also various glycoproteins, such as collagen type II and type X (Olsen et al., 2000; Seibel et al., 1999) . Much of the cartilage formed in prenatal life is temporal and is later replaced by bone during endochondral bone formation (see next chapter). Other cartilage structures remain throughout life, as for example the periarticular cartilage in the joints, the fibrocartilage in the intervertebral disks, and the elastic cartilage in the external ear (Cormack, 1987). Tendons and ligaments consist of collagen fibers and serve as connections between the skeletal elements and muscles, or between the different bones. Tendons mediate muscular contractions to bones or cartilage structures, and ligaments hold the bones together at the joint region (Cormack, 1987; Seibel et al., 1999).

1.2 Endochondral ossification

Bone formation is a very complex process that starts at early stages of embryonic development and is completed postnatally during puberty. There are two bone-forming processes during embryonic development, intramembranous and endochondral ossification (Erlebacher et al., 1995; Gilbert, 1994; Hinchcliffe and Johnson, 1980; Olsen et al., 2000). The skull and part of the clavicle are formed by the intramembranous ossification, in which bone forms directly from the mesenchymal progenitors. The bones of the axial and appendicular skeleton, as well as some of the facial bones, are formed by endochondral ossification, in which initially a cartilaginous model is formed, which is later replaced by bone (Fig.2). Endochondral ossification starts with mesenchymal cells that condense and differentiate into two types of cells: chondrocytes that form cartilage elements and perichondral cells that surround the cartilage model. Starting from the center of the cartilage elements, chondrocytes undergo several steps of maturation from proliferating chondrocytes to non-proliferating hypertrophic cells. The hypertrophic chondrocytes are bigger in size than proliferating cells and they produce specific proteins of the surrounding matrix. Hypertrophic chondrocytes differentiate into terminally differentiated chondrocytes, which die by apoptosis. Subsequently the blood vessels invade the region of terminal hypertrophic chondrocytes. With vascularization the bone-forming cells, osteoblasts and osteoclasts, arrive and replace terminal hypertrophic chondrocytes by bone marrow and bone. In parallel to the onset of hypertrophic differentiation, the perichondral cells flanking the region of hypertrophic chondrocytes differentiate into an osteoblast-containing layer, the periosteum. The osteoblasts in the periosteum produce primary bone, the bone collar. As a front of differentiation and ossification spreads from the center to the ends of the skeletal elements,

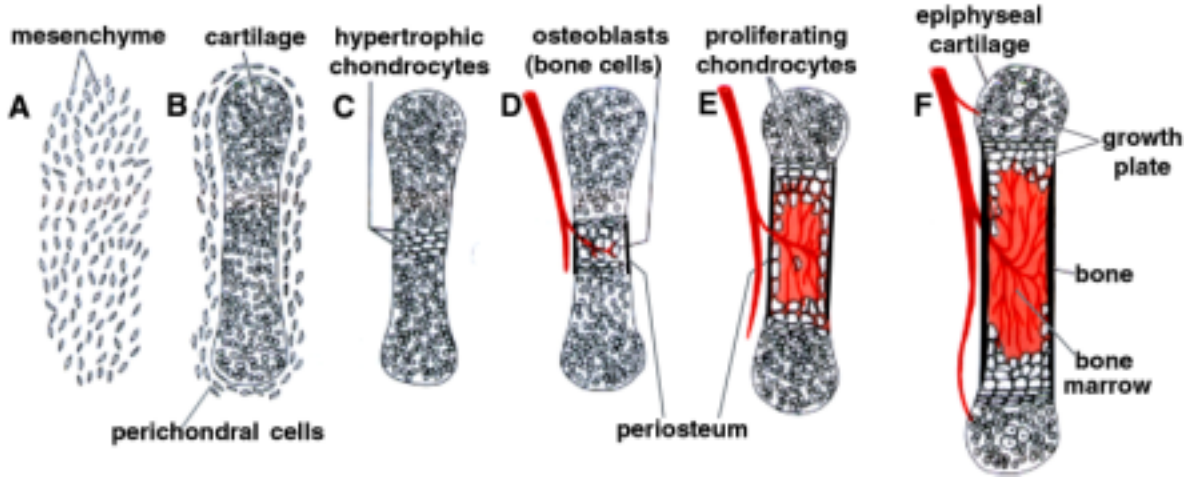

Fig. 2 Schematic diagram of endochondral ossification

Endochondral ossification starts form the mesenchymal cells that condense (A) and differentiate into cartilage cells, chondrocytes, and perichondral cells, surrounding the cartilage model (B). In the center of the skeletal element the chondrocytes undergo hypertrophy (C) and change their extracellular matrix. Terminal hypertrophic chondrocytes die and it allows the blood vessels to invade (D). With the vascularization the osteoblasts and osteoclasts arrive and replace the cartilaginous structure by bone and the bone matrix (E). The cartilage structures, growth plates, stay only in the narrow layers close to the epiphyseal ends (F) (From Gilbert, 1994).

the proliferative and hypertrophic zones are ultimately reduced to narrow bands termed growth plates, located near the ends of the skeletal elements. Longitudinal growth of bones is dependent on proliferation and hypertrophic differentiation of chondrocytes in the growth plate (Erlebacher et al., 1995; Hinchcliffe and Johnson, 1980). As terminal hypertrophic cartilage is continuously replaced by bone, the tight regulation of the various steps of chondrocyte differentiation is critical to balance growth and ossification of the skeletal elements.

In mouse forelimbs the endochondral ossification first starts at embryonic day 10 (E10) and E12.5 the cartilaginous anlagen of almost all skeletal elements are established (Fig. 3A). At E14.5 chondrocytes are differentiated, and bone formation is about to take place. A first sign of initiation of ossification appears in the humerus, where the perichondral cell layer, the perichondrium, differentiates into the periosteum in the middle of the skeletal element (Fig. 3B). At E16.5 bone is present in the middle of the scapula, the humerus, the ulna and the radius (Fig. 3C). The metacarpal and phalangeal elements are ossified at E18.5 (Fig. 3D) (Kaufmann, 1992; St-Jacques et al., 1999).

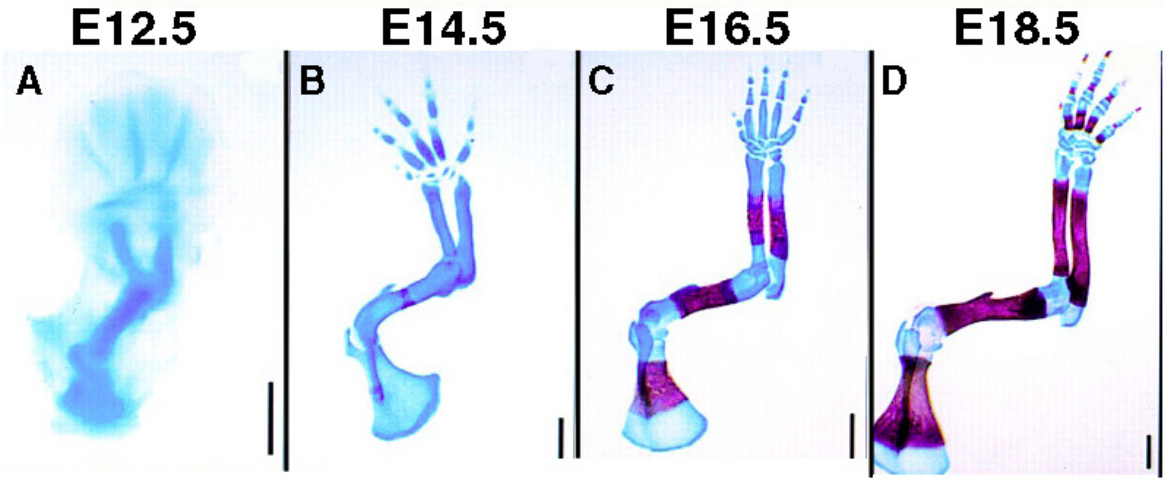

Fig. 3 Endochondral ossification during mouse embryogenesis

(A-D) The skeletal elements of mouse forelimbs at embryonic stages E12.5, E14.5, E16.5 and E18.5 were stained with Alcian blue (for cartilage) and Alizarin Red (for bone). At stage E12.5 the mesenchymal condensations for most skeletal elements have formed (A). At E14.5 the chondrocytes of more proximal elements undergo hypertrophy and in the humerus perichondrium differentiates into periosteum (B). At E16.5 the bone is present in all skeletal elements except of metacarpal and some of the phalangeal bones (C), where the bone fully forms at stage E18.5 (D) (accepted from St-Jaques et al., 1999).

1.3 Structure of developing cartilage

In newborn mice the cartilage of the developing long bones exhibits a highly organized structure that can be divided into several overlapping zones: periarticular, proliferating, prehypertrophic/ hypertrophic and terminal hypertrophic chondrocytes (Fig.4) (Gilbert, 1994). The periarticular chondrocytes consist of small round cells that are present close to the epiphyseal end of the skeletal element. They remain undifferentiated initially, postnatally they develop into the periarticular cartilage of the joints. The proliferating chondrocytes are responsible for the longitudinal growth of the skeletal elements. They produce collagen type II, a protein specific for the cartilage matrix. Proliferating chondrocytes are divided morphologically into two types of cells: small round cells, located close to the epiphyseal ends, and organized in columns of polygonal cells, which are located between resting and differentiating chondrocytes. The cells of the next zone, prehypertrophic/hypertrophic chondrocytes, are the biggest cells in developing skeletal elements. The prehypertrophic chondrocytes express the marker gene Indian hedgehog (Ihh) (Vortkamp et al., 1996). The hypertrophic chondrocytes are characterized by high production of matrix protein collagen type X, ColX (Fig. 4C) (Olsen et al., 2000; Seibel et al., 1999). As the extracellular matrix

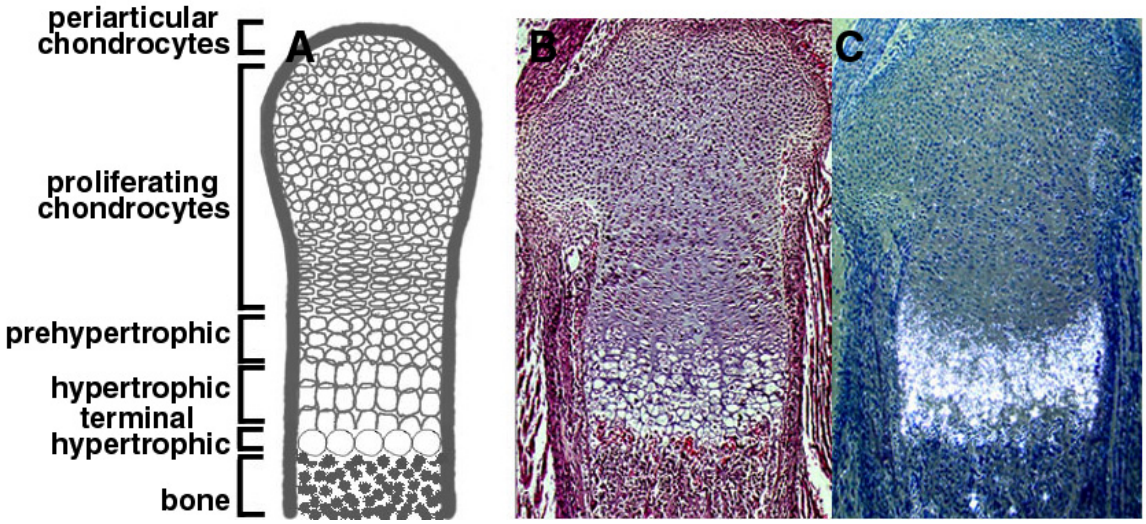

Fig. 4 Cellular organization of a cartilage in long bones

The developing cartilage is divided into the periarticular, proliferating, prehypertrophic/hypertrophic and terminal hypertrophic chondrocytes (A). A section of a femur from a newborn mouse was stained with Haemotoxilin-Eosin to visualize the cartilage zones, present in model (A, B). A parallel section was hybridized with antisense riboprobe for *ColX* (white staining) to demarcate the hypertrophic chondrocytes. The limb tissue was cointerstained with Toluidin blue (C).

around the differentiated chondrocytes is highly mineralized, this zone is also called mineralized cartilage. The last zone of cartilage consists of terminal hypertrophic chondrocytes that complete the differentiation process and die by apoptosis. The terminally differentiated chondrocytes are the only cells that are replaced by bone. Marker genes for terminal hypertrophic chondrocytes are matrix metalloproteinase 13 (MMP13) and osteopontin (secreted phosphoprotein 1, Spp1) (Gerstenfeld and Shapiro, 1996; Nakase et al., 1994; Yamagiwa et al., 1999).

1.4 Different signaling pathways regulating bone development

1.4.1 Human skeletal diseases

The regulation of endochondral ossification is a multifarious process that involves numerous signaling growth factors. First insight into the roles of different regulating pathways came from studies investigating human skeletal disorders. By biochemical analysis, Schipani et al. have demonstrated that Jansen metaphyseal chondrodysplasia, a rare form of short-limbed dwarfism, results from ligand-independent activation of the Parathyroid hormone receptor,

Pthr (Schipani et al., 1995). The studies of Parathyroid hormone receptor in mice and chicken led to the discovery of a central pathway, regulating endochondral ossification, the Indian hedgehog (Ihh) and Parathyroid hormone-like peptide (Pthlh) feed back loop (Karp et al., 2000; Vortkamp et al., 1996).

Other signaling factors, Bone morphogenetic proteins (BMP) have been implicated in regulation of cartilage growth and joint formation. Two human diseases, proximal symphalangism (SYM1) and multiple synostosis (SYNS1) syndromes, result from missense mutations in the *Noggin* gene, an antagonist of BMP signals (Gong et al., 1999; Marcelino et al., 2001). These mutations lead to haploinsufficiency of the Noggin protein and subsequent excess of BMP signaling. Heterozygous patients with SYM1 display fusion of carpal and tarsal bones in hands and feet, respectively, and sometimes deafness. Heterozygous patients with SYNS1 exhibit a more severe phenotype than SYM1 patients. They are characterized by progressive joint fusion in the hands, short digits, and deafness (Gong et al., 1999; Marcelino et al., 2001). At present, no mutations of BMPs have been described in human limb malformations, although the ear-patella-short-stature syndrome (EPS) might be a human equivalent of the short ear mice, homozygous mutant for the *Bmp5* gene (Cohen et al., 2002; King et al., 1994). Patients with EPS have very short external ears, small jaws, growth retardation and other skeletal abnormalities (Cohen et al., 2002).

The Fibroblast growth factors (FGF) and three of their four transmembrane FGF receptors (FGFR1, FGFR2, FGFR3) play critical roles in the control of bone formation. Mutations in FGFR1 and FGFR2 result in several craniosynostosis syndromes, Apert, Pfeiffer, Crouzon, Jackson-Weiss, Beare Stevenson (Kan Sh et al., 2002; Mulliken et al., 1999; Passos-Bueno et al., 1999; Wilkie, 1997). Patients with Apert's and Pfeiffer's syndromes have the most severe phenotype, exhibiting premature fusion of the calvarial bones leading to serious alterations in skull shape, and additional limb abnormalities (Kan Sh et al., 2002). FGFR3 seems to specifically regulate the endochondral ossification, as three inherited human dwarfism syndromes, hypochondroplasia, achondroplasia and thanatophoric dysplasia type I and type II, result from the mutations in the *Fgfr3* gene (Bellus et al., 1995; Rousseau et al., 1994; Shiang et al., 1994; Tavormina et al., 1995). These mutations lead to different levels of receptor activation, which correlates well with the severity of the human phenotypes. Hypochondroplasia patients have a mild dwarfism phenotype (Bellus et al., 1995). The clinical features of heterozygous achondroplasia include numerous skeletal defects such as proximal shortening of the limbs, depressed nasal bridge, narrowing of the spinal column and relative macrocephaly (Rousseau et al., 1994; Shiang et al., 1994). Thanatophoric dysplasias type I and type II clinically resemble the lethal phenotype of homozygous achondroplasia patients, which exhibit severely shortened limbs and generally die of

respiratory failure within a few hours after birth (Tavormina et al., 1995; Webster and Donoghue, 1996).

1.4.2 Ihh/Pthlh pathway directs endochondral ossification

Ihh, a member of the conserved Hedgehog family of signaling factors, regulates several aspects of endochondral ossification. During mouse embryonic development, *Ihh* expression is first detected at E11.5 in chondrocytes of the early cartilaginous condensation (Bitgood and McMahon, 1995). With the initiation of hypertrophic differentiation, *Ihh* expression becomes restricted to the prehypertrophic chondrocytes (Fig. 5A) (Bitgood and McMahon, 1995; Vortkamp et al., 1996; Vortkamp et al., 1998). Another secreted signaling molecule that regulates endochondral ossification is Pthlh, which is expressed in the periarticular region of the developing cartilage elements (Fig. 5C) (Lee et al., 1995; Schipani et al., 1997; Vortkamp et al., 1998). Pthlh signals through the Pthlh receptor (Pthr), which is expressed at low level throughout the growth plate and at high level in the prehypertrophic and hypertrophic chondrocytes (Fig. 5B) (Amizuka et al., 1994; Lee et al., 1995). Targeted disruption of *Pthlh* or *Pthr* in mice results in a dwarfism phenotype due to a reduced zone of proliferating cells and advanced hypertrophic differentiation (Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996). Conversely, activation of Pthlh signaling by overexpression of *Pthlh* or the expression of constitutively activated *Pthr*, leads to a delay in hypertrophic differentiation (Schipani et al., 1995; Schipani et al., 1997; Weir et al., 1996).

Several lines of evidence indicate that Ihh and Pthlh interact in a negative feedback loop regulating the onset of hypertrophic differentiation (Fig. 5). Virus-mediated expression of *Ihh* in developing limbs of chicken embryos results in a delay of hypertrophic differentiation and in strong upregulation of *Pthlh* in the periarticular region of the infected cartilage elements (Vortkamp et al., 1996). Furthermore, limb explants of *Pthr -/-* mice that were treated with Hedgehog protein in culture demonstrated that Pthlh is necessary to mediate the effect of Ihh on chondrocyte differentiation (Lanske et al., 1996; Vortkamp et al., 1996). It has been proposed that Ihh, which is expressed in the prehypertrophic chondrocytes, signals to the periarticular region and induces the expression of *Pthlh* (Fig. 5). Pthlh in turn signals back to its receptor and prevents hypertrophic differentiation of additional chondrocytes. The expression level of the Ihh/Pthlh system thus regulates the distance between the joint region and the onset of hypertrophic differentiation (Fig. 5D) (Vortkamp et al., 1996). This model has been supported by examination of *Ihh* deficient mice, which lack *Pthlh* expression and exhibit premature hypertrophic differentiation (St-Jacques et al., 1999). In addition to

regulating chondrocyte differentiation, the analysis of *Ihh* knockout mice has demonstrated that Ihh signaling regulates chondrocyte proliferation independent of Pthlh. Furthermore in *Ihh -/-* mice no endochondral bone formation was found, indicating that Ihh is required for the ossification process (Karp et al., 2000; St-Jacques et al., 1999).

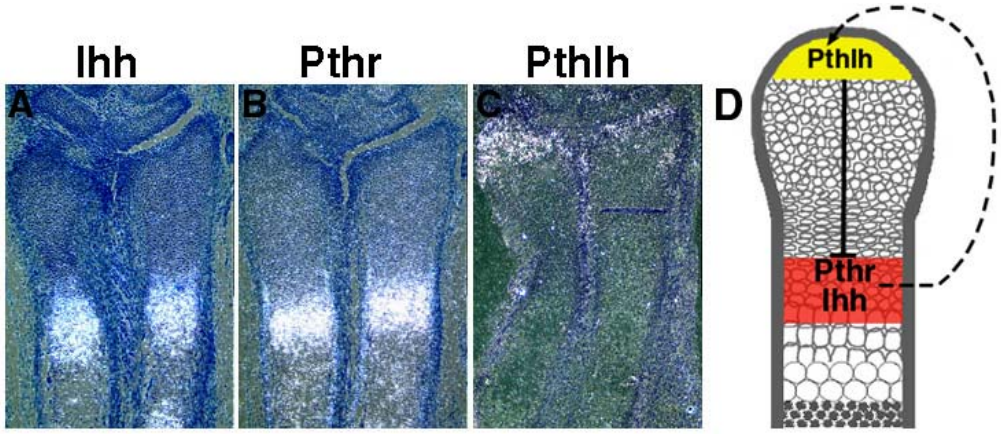

Fig. 5 The Ihh/Pthlp pathway regulates chondrocyte differentiation through a negative feed back loop

Forelimb of a mouse embryo at E14.5 was sectioned and hybridized with antisense riboprobes for *Ihh* (A), *Pthr* (B) and *Pthlh* (C). In cartilage at E14.5 *Ihh* (A) and *Pthr* (B) are expressed in prehypertrophic and hypertrophic chondrocytes. *Pthlh* (C) is expressed in the cells from the articular region. The model presents the Ihh/Pthlh pathway: Ihh, which is expressed in prehypertrophic chondrocytes (red), induces *Pthlh* expression in the periarticular region (yellow). Pthlh signals to its receptor, Pthr, to prevent a new portion of proliferating chondrocytes to undergo hypertrophy.

1.4.3 BMP family

Bone morphogenetic proteins (BMPs), which belong to the TGF-beta superfamily of secreted proteins, are another group of growth factors regulating bone development (Hogan, 1996; Kingsley, 1994a; Kingsley, 1994b). BMPs were identified due to their ability to induce ectopic bone formation when implanted under the skin of adult rats (Urist, 1965; Wozney et al., 1988). In addition, BMPs induce proliferation and differentiation of chondrocytes and osteoblasts in tissue culture experiments (Chen et al., 1995; Wu et al., 1997). BMPs signal through the serine/threonine kinase receptors type I and type II, which heterodimerize and transduce the BMP signal by phosphorylation of intracellular Smad proteins (Massague, 1998; von Bubnoff and Cho, 2001). BMP-specific Smad proteins, such as Smad 1, Smad 5 or Smad 8, heterodimerize with Smad 4 and enter to the nucleus, where the Smad-heterodimer directs the transcription of target genes (von Bubnoff and Cho, 2001). BMP signaling can be negatively regulated extracellularly by several BMP antagonists including the protein Noggin (Smith and Harland, 1992; Smith et al., 1993). Biochemical analysis has

demonstrated that Noggin directly interacts with various members of the BMP family including at least BMP2, BMP4 and BMP7, thus preventing them from binding to the BMP receptors (Holley et al., 1996; Kawabata et al., 1998; Smith, 1999; Zimmerman et al., 1996).

Several *Bmp* genes are expressed in the developing cartilage elements. In mice, *Bmp2*, *Bmp3*, *Bmp4*, *Bmp5*, and *Bmp7* are expressed in the perichondrium flanking the *Ihh* expression domain (Daluiski et al., 2001; Haaijman et al., 2000; Pathi et al., 1999; Zou et al., 1997). In addition, *Bmp7* is expressed in the proliferating chondrocytes (Haaijman et al., 2000). In the developing cartilage elements Bmp receptor-Ib *(Bmpr-Ib)* is expressed in the perichondrium whereas Bmp receptor-Ia *(Bmpr-Ia)* and Bmp receptor-II *(Bmpr-II)* are expressed in the prehypertrophic chondrocytes (Haaijman et al., 2000; Yi et al., 2000; Zou et al., 1997). In addition, *Bmp* receptors as well as *Bmp2*, *Bmp4* and *Bmp7* are expressed in the periarticular region (Macias et al., 1997; Pathi et al., 1999; Solloway et al., 1998; Zou et al., 1997).

1.4.4 BMPs and Ihh may interact to regulate chondrocyte maturation

Several lines of evidence indicate a possible interaction between two signaling systems, BMP signaling and the Ihh/Pthlh pathway, in regulating endochondral ossification. It has been shown in chicken embryos that ectopic expression of *Ihh* in developing cartilage elements upregulates *Bmp2* and *Bmp4* expression in the perichondrium (Pathi et al., 1999). Furthermore, retroviral ectopic expression of a constitutively activated *Bmpr-Ia* during chick limb development results in an upregulation of *Pthlh* expression and a block of chondrocyte differentiation, thus mimicking the effect of retroviral misexpression of *Ihh*. These results led to the hypothesis that Ihh signaling regulates *Bmp* expression locally, which in turn influences Pthlh production at a distance, thereby regulating the rate of chondrocyte differentiation (Zou et al., 1997). However, it was not shown whether BMPs mediate the Ihh signals in mice in a similar way, which was proposed for chicken. Analysis of BMP mutant mice has not given insight into this question. These mice display only milder defects in chondrocyte differentiation, as for example *Bmp5, Bmp6 or Bmp7* mutant mice, or the phenotype results in neonatal lethality before initiation of bone formation, as *Bmp2* and *Bmp4* knockout mice (Dunn et al., 1997; Jena et al., 1997; King et al., 1994; Solloway et al., 1998; Zhang and Bradley, 1996).

1.4.5 FGF signaling controls bone development

Fibroblast growth factor (FGF) signaling is an additional pathway that has been shown to play a critical role in controlling bone development. In the genome of mouse and human at least 22 *Fgf* genes have been identified (GenBank). FGFs signal through four tyrosine kinase transmembrane receptors (Ornitz and Itoh, 2001). Three of them, *Fgfr1*, *Fgf2* and *Fgf3* are expressed in the skeletal tissues of developing limbs and skull (Fig. 6) (Delezoide et al., 1998; Iseki et al., 1997). *Fgfr1* is expressed in periarticular region and in terminal hypertrophic chondrocytes of developing cartilage (Fig. 6A,B). *Fgfr2* is expressed in proliferating chondrocytes and in perichondrium flanking the hypertrophic cells (Fig. 6A,C) (Delezoide et al., 1998). Interestingly, *Fgfr3* expression overlaps with expression of *Fgfr1* and *Fgfr2*. It is weakly expressed in the periarticular region and strongly in proliferating and hypertrophic chondrocytes (Fig. 6D) (Delezoide et al., 1998; Naski et al., 1998). Analyses of *Fgfs* expression have demonstrated that only few of them, such as *Fgf8*, *Fgf17* and *Fgf18*, are weakly expressed in perichondrium/periosteum of developing limb bones (Liu et al., 2002; Xu et al., 1999).

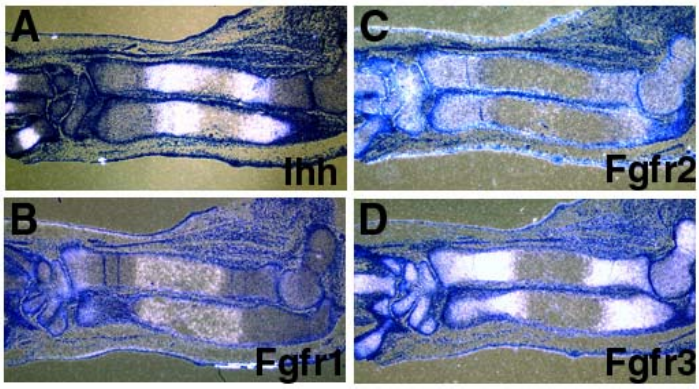

Fig. 6 Expression pattern of the *Fgf* receptors

Forelimbs of E14.5 embryos were hybridized with antisense riboprobes for *Ihh* (A), *Fgfr1* (B), *Fgfr2* (C) and *Fgfr3* (D). *Fgfr1* is expressed in the periarticular region and in the zones of hypertrophic and terminal hypertrophic chondrocytes (B). *Fgfr2* is expressed in proliferating chondrocytes bordering the *Ihh*-expressing cells (C,A). *Fgfr3* is expressed gradually, with the strongest expression in the hypertrophic cells and weak expression in the periarticular region (D). In all panels radius is up and ulna is down.

During recent years several mouse models for human dwarfism syndromes have been generated by misexpressing mutated forms of *Fgfr3* in the developing cartilage anlagen (Chen et al., 1999; Chen et al., 2001; Iwata et al., 2001; Li et al., 1999; Naski et al., 1998; Segev et al., 2000; Wang et al., 1999). These mouse models exhibit a severe shortening of the skeletal elements, a narrow thorax and a slightly larger skull thus mimicking the human dwarfism phenotypes (Fig. 7). On the molecular level, the regions of proliferating and hypertrophic

chondrocytes are significantly reduced in these mice. In contrast, mice carrying a targeted deletion of FGFR3 are characterized by enlarged skeletal elements due to increased regions of proliferating and hypertrophic chondrocytes (Colvin et al., 1996; Deng et al., 1996). These studies have led to the conclusion that FGF signaling is a negative regulator of chondrocyte proliferation and differentiation. Both, *Ihh* and *Bmp4* expression are altered in mouse models for achondroplasia and in FGFR3-/- mice, implicating an interaction between the different signaling pathways (Naski et al., 1998). However, the epistatic relationship of the three signaling systems has not been analyzed in detail.

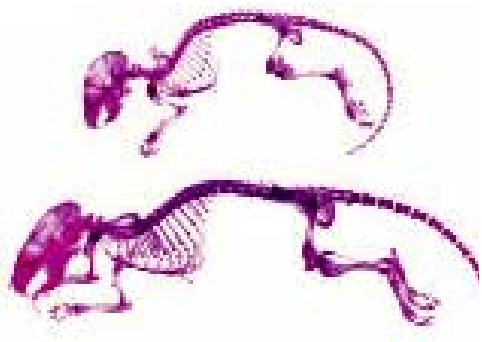

Fig. 7 Shortening of the skeletal elements in a mouse model for achondroplasia

Skeletons of one month old FGFr3ach (above) and wild type (below) mice were stained with Alizarin Red. The skeleton of FGFr3ach mouse exhibits the shortening of the skeletal elements and the kyphosis in the spine compared with skeleton of wild type littermates (accepted from Naski, et al. 1998).

1.5 The aim of the study

A desire to understand the origins of human dwarfism prompted a number of studies during the last decade to identify signaling pathways regulating bone development. Disturbed endochondral ossification, the process by which the bones of the limbs are formed, results in the shortness of limbs in dwarfism syndromes. One of the proteins, regulating endochondral ossification, is Parathyroid hormone-like peptide (Pthlh). Another secreted factor, Indian hedgehog (Ihh), has been shown to activate Pthlh signaling, which delays the onset of hypertrophic differentiation of chondrocytes (Karp et al., 2000; Lanske et al., 1996; St-Jacques et al., 1999; Vortkamp et al., 1996). In addition to the Ihh/Pthlh pathway other growth factors have been shown to regulate chondrocyte differentiation. Misexpression experiments in chick have demonstrated that BMPs regulate proliferation and differentiation of chondrocytes (Enomoto-Iwamoto et al., 1998; Zou et al., 1997). Viral-mediated activation of BMP signaling in chick embryos led to upregulation of *Pthlh* expression and block of hypertrophic differentiation (Zou et al., 1997). Thus BMPs were implicated to act downstream of Ihh signaling in regulating *Pthlh* expression. Another family of secreted proteins regulating proliferation and differentiation of chondrocytes is the Fibroblast growth

factor (FGF) family. Activation of FGF signaling due to mutations in the FGF receptor 3 gene results in dwarfism phenotype in human and in mouse (Bellus et al., 1995; Chen et al., 1999; Chen et al., 2001; Iwata et al., 2001; Li et al., 1999; Naski et al., 1998; Naski et al., 1996; Rousseau et al., 1994; Segev et al., 2000; Shiang et al., 1994; Tavormina et al., 1995; Wang et al., 1999). It has been shown that on the molecular level FGF signaling reduces chondrocyte proliferation and differentiation. In addition, it regulates the expression of *Ihh* and *Bmp4* (Naski et al., 1998). However, the interactions of the Ihh/Pthlh, the BMP and the FGF signaling pathways have not been analyzed in details.

The aim of this study was to analyze a potential interaction and an epistatic relationship between these three signaling pathways. As an organ culture is a promising *in vitro* approach for investigation of the multifactorial interactions, it was necessary to establish the organ culture system for embryonic limb explants, in which chondrocytes could proliferate and differentiate similar to the *in vivo* conditions. To analyze the functions of each signaling in the limb cultures it was essential at first to find an optimal concentration and a culture time for the specific growth factors. As a second step it was necessary to identify the specific functions of the BMP and the FGF signaling pathways during chondrocyte proliferation and differentiation. An important aim of this study was to analyze the interaction of each pathway with the Ihh/Pthlh system. Particularly, it was interesting to test the hypothesis that BMP signaling transduces the Ihh signals to induce *Pthlh* expression. The last aim of this study was to investigate an interaction of the BMP and the FGF signaling pathways. The results of this study should clarify how the Ihh/Pthlh, the BMP and the FGF signaling pathways may integrate into one common network that controls cartilage development.