3 Introduction

3.1 Hedgehog signaling

3.1.1 Function of the hedgehog gene family during development

The Hedgehog (Hh) family of secreted proteins control patterning, growth, and morphogenesis of many tissues in both vertebrates and invertebrates (Ingham and McMahon, 2001). Hh was first identified in *Drosophila* (hh) as a member of the segment polarity class of genes (Nusslein-Volhard and Wieschaus, 1980). In the hh mutant, the entire larval cuticle is covered with denticles and was therefore called 'hedgehog'. Unlike one Hedgehog gene found in *Drosophila*, three Hedgehog genes, first identified in the mouse, are found in vertebrates. These include Desert hedgehog (Dhh), Sonic hedgehog (Shh) and Indian hedgehog (Ihh). Dhh is most closely related to hh; Ihh and Shh are more closely related to each other and represent a more recent gene duplication (Cohen, 2003). Shh activity at the midline patterns the overlying ventral neural tube and adjacent ventral somites, and participates in the regulation of left-right asymmetry. Furthermore Shh exerts polarizing activity in the developing limb bud and regulates morphogenesis of a variety of organs including the brain, eye, hair, and lungs. Dhh and Ihh play more restricted roles: Dhh acts in the regulation of spermatogenesis and organization of the perineurum, which ensheats peripheral nerves. *Ihh* is expressed in extraembryonic endoderm (Becker et al., 1997) and at later stages in the gut, mammary gland and lung (Bitgood and McMahon, 1995). Furthermore *Ihh* is expressed in the cartilage anlagen where it coordinates proliferation and differentiation of chondrocytes and the ossification during the development of the endochondral skeleton (Lanske et al., 1996; Long et al., 2001; St-Jacques et al., 1999; Vortkamp et al., 1996). Hedgehog signals are thought to act as morphogens to induce distinct cell fates at specific concentration thresholds (Briscoe et al., 2001; Gritli-Linde et al., 2001; McMahon, 2000).

3.1.2 Hedgehog processing and signal transduction

Hh proteins are transcribed as pre-proproteins with a signal sequence attached to the N-terminus of the protein. This signal sequence is cleaved in the secretory pathway (Lee et al., 1992; Tabata et al., 1992), which generates a 19 kDa N-terminal peptide (Hh-N) and a 26 kDa C-terminal peptide (Bumcrot et al., 1995; Lee et al., 1994; Mann and Beachy,

2004). During this process a cholesterol moiety is attached to the C-terminus of Hh-N (Porter et al., 1996). Subsequently, an acetyltransferase catalyzes the addition of a palmitate to the N-terminus of Hh-N, producing a double lipid modified protein (Hh-Np, 'p' stands for processed) (Chamoun et al., 2001; Pepinsky et al., 1998). Release of this molecule from the cell requires the function of dispatched (Burke et al., 1999). On the receiving cells Hh signals through a receptor complex consisting of the 12-transmembrane receptor Patched (Ptch) and the G-protein-coupled 7-transmembrane protein Smoothened (Smo). In the absence of Hh signaling, Smo is repressed by Ptch. Binding of Hh to Ptch relieves the repression of Smo, which regulates the transcription factor Cubitus interruptus (Ci) in flies and the Gli-family of transcription factors in vertebrates (Ingham and McMahon, 2001).

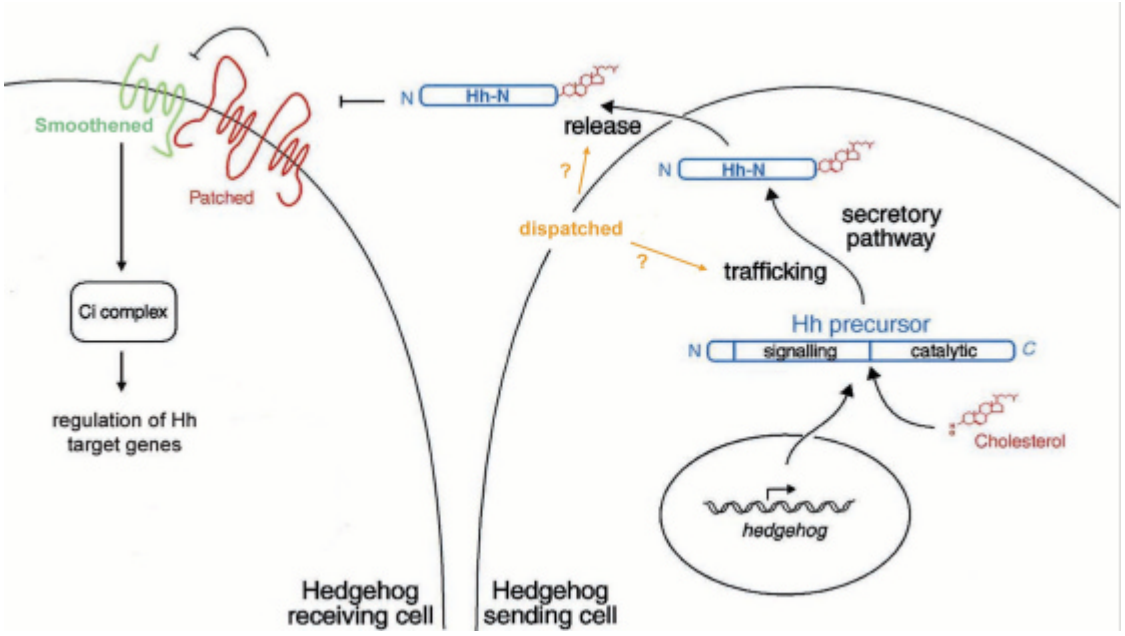

Fig. 1. Schematic representation of the hedgehog signaling pathway. See text for details (modificated after McMahon 2000)

3.1.3 The morphogen concept

Many of the molecules regulating organogenesis during development, have been demonstrated to work in a concentration dependent manner. These findings are in agreement with previous theoretical concepts of pattern formation, which postulated the existence of form giving substances, or "morphogens" (Lawrence, 2001). A true morphogen is a molecule that spreads out from a localized source to form a concentration gradient over a field of cells. The local concentration of the morphogen directs the fate of

nearby cells. Cells receiving a low concentration of the signaling molecule respond by expressing low threshold genes, while others, receiving a higher concentration respond by expressing high threshold genes (Entchev and Gonzalez-Gaitan, 2002). This concept provides an attractive explanation of how cells in the embryo acquire positional information and how the multitude of cellular differences can be coordinated by a much lower number of signaling molecules. Several molecules have been proven to function as morphogens during fly and vertebrate development. These molecules include Hh, fibroblast growth factors (Fgf), Wnt family members and members of the transforming growth factor (Tgf) family (Tabata and Takei, 2004).

3.2 The vertebrate skeleton

The skeleton is a complex organ, which fulfills various functions during life of a vertebrate organism. Unlike other organs the skeleton consist of more than 200 individual elements, each with a distinct location and shape. These skeletal elements ensure mechanical stability and simoultaniously allow the flexibilty for movement. They are needed for support and protection of internal organs and the regulation of calcium homeostasis. Furthermore, the bone marrow of the long bones presents the primary site of hematopoiesis in adults.

The development of the mammalian skeleton is a complex process, which continous until adolescence when growth ceases. 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). The skeleton is made out of two tissues, cartilage and bone. Each tissue consists of specific cell types: chondrocytes in cartilage and osteoblasts and osteoclasts in bone. When development is complete, most of the skeleton is made of bone. Some cartilage, however, remains throughout life, for example the articular cartilage in joints, the fibrocartilage in the intervertebral disks, and elastic cartilage in the external ear (Cormack, 1987). Tendons and ligaments serve as connections between the skeletal elements and muscles, or between different bones respectively. Tendons mediate muscular contractions to bones or cartilage structures, whereas ligaments hold the bones together at the joint region (Cormack, 1987; Seibel and Woitge, 1999).

Skeletal malformations belong to the most common forms of inherited human disorders. Mutation in genes, which are essential for bone development, can result in extreme changes of cartilage or bone differentiation. Such defects produce misshaped or strongly shortened bones like in many chondrodysplasias. Some mutations lead to size changes of the whole skeleton, resulting in dwarfism syndromes. Other mutations, which affect the structure of the developing bone, lead to increased fragility of this tissue like in osteogenesis imperfecta, a genetic disorder characterized by bones, which break easily. During the last years the identification of genes involved in development of skeletal diseases gave new insights into the developmental processes controlling bone formation. A rapidly increasing number of transgenic mice have been generated to serve as models, enabling function analysis of these genes during skeletogenesis.

The genetic basis for many of these skeletal disorders lies in impaired signaling of different growth factors like Hedgehog proteins, bone morphogenetic proteins (Bmps), Fgfs and Tgf family members. Additionally, transcription factors play key roles in the process of endochondral ossification as seen by mutations in *Sox9* and *Runx2*. Last not least, more and more attention is directed towards extracellular matrix (ECM)-components. The collagen and proteoglycan network not only provides a scaffold for chondrocytes, but seems to be involved in the control of chondrocyte differentiation by regulating receptor binding and signaling of important growth factors.

3.3 The process of endochondral ossification

Bone development is initiated by the aggregation of mechenchymal cells into condensations, which prefigure the future skeletal elements. There are two processes, by which bone can be formed in these condensations: during intramembranous ossification, a process by which the bones of the skull, some facial bones and parts of the clavicles are formed, mesenchymal cells directly differentiate into bone producing osteoblasts. In contrast, the majority of the skeletal elements, is formed by endochondral ossification, a multistep process during which a cartilaginous template is replaced by bone. During this process the mesenchymal cells in the center of the condensations differentiate into chondrocytes, whereas the cells on the outside form the perichondrium, a thin sheet of of fibroblastic cells that surrounds the cartilage anlagen. Chondrocytes secrete a defined

extracellular matrix (ECM) rich of collagen type II, collagen type IX, collagen type XI and aggrecan (Erlebacher et al., 1995). The chondrocytes proliferate to enlarge the cartilage model. Starting from the center, they differentiate into flat, columnar chondrocytes, which serve as a pool of regenerating cells, from which chondrocytes are continuoulsy released. These exit the cell cycle and differentiate into prehypertrophic, hypertrophic and terminal hypertrophic chondrocytes. During hypertrophic differentiation chondrocytes increase in size, which contributes significantly to the longitudinal growth of the bone. Hypertrophic chondrocytes secrete a different ECM, consisting mainly of collagen type X, which allows the mineralization of the matrix. In a parallel process perichondrial cells adjacent to the hypertrophic region, differentiate into osteoblasts, which produce the bone collar (periost). Hypertrophic chondrocytes also produce angiogenic factors like vascular endothelial growth factor (Vegf), which attracts blood vessels together with osteogenic cells. Initially the blood vessels invade the forming periosteum and later the terminal hypertrophic region. Terminal hypertrophic chondrocytes express matrix metalloprotease 13 (Mmp13), a collagenase responsible for the initial ECM degradation, which facilitates invasion of the blood vessels (Ortega et al., 2004). Finally, terminal hypertrophic cells undergo apoptosis leaving behind a scaffold of mineralized matrix, on which osteoblasts start to form trabecular bone. In parallel osteoclasts and chondroclasts, which are bone and chondrocyte resorbing cells respectively, degrade the cartilage matrix facilitating the deposition of bone matrix by the accompanying osteoblasts.

Bone lengthening, which is particularly rapid during fetal life, is driven primarily by the rate of chondrocyte proliferation and hypertrophic differentiation. Hypertrophic differentiation and ossification of the perichondrium are initiated in the center of the developing cartilage models and spreads toward their distal ends. Postnatally, so-called secondary ossification centers are established at the end of long bones. Thereby the zone of proliferating and hypertrophic chondrocytes is reduced to a narrow band, termed the growth plates, located between the regions of the primary and secondary ossification. The remaining chondrocytes in the growth plate, continue to proliferate until at the time of adolescence the growth plate close and chondrocytes disappear.

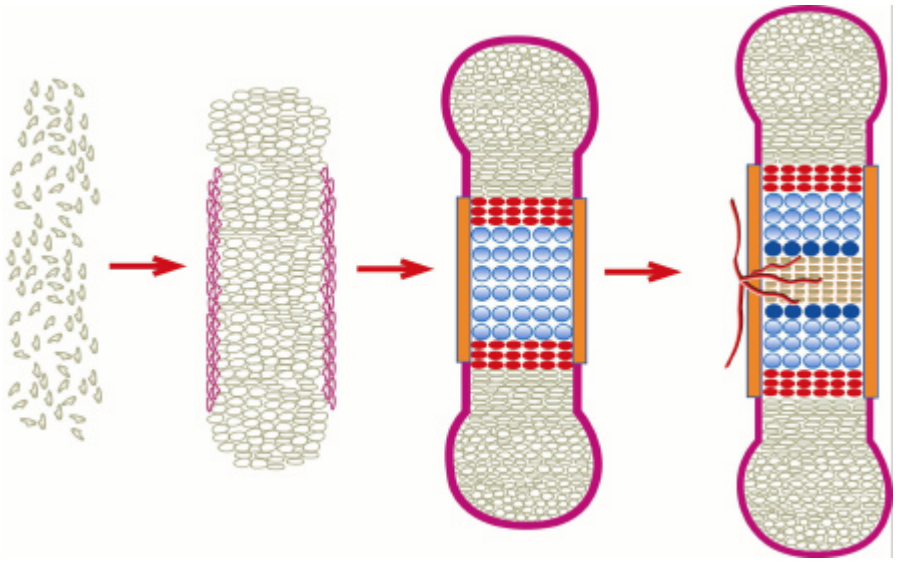

Fig. 2. Schematic diagram of the process of endochondral ossification.
Endochondral ossification starts with the aggregation of mesenchymal cells that condense and differentiate into chondrocytes (grey) and perichondral cells (pink), surrounding the cartilage model. In the center of the skeletal element the chondrocytes undergo hypertrophy (prehypertrophic chondrocytes, red; hypertrophic chondrocytes, blue) and secrete a different extracellular matrix. Perichondrial cells adjacent to the hypertrophic zone differentiate into osteoblasts and form the bone collar (orange). Terminal hypertrophic chondrocytes undergo apoptosis, which allows the invasion of blood vessels and osteogenic cells. Osteoblasts and osteoclasts replace the cartilaginous structure by bone and bone matrix (beige).

3.4 The Ihh/Pthlh feedback loop controls chondrocyte differentiation

The secreted signaling factor Indian Hedgehog (Ihh) is a key regulator of endochondral ossification. Ihh coordinates chondrocyte proliferation, the onset of hypertrophic differentiation and osteoblast differentiation. Studies in chicken and mice revealed that Ihh interacts with a second secreted factor, parathyroid hormone-like hormone (Pthlh), which is expressed in periarticular chondrocytes, to regulate the onset of hypertrophic differentiation (Lanske et al., 1996; Vortkamp et al., 1996). Pthlh signals through the Pth/Pthlh receptor (Pthr1), a Gprotein coupled 7 transmembrane receptor, which is expressed at low levels throughout the growth plate and at high levels in prehypertrophic chondrocytes (Amizuka et al., 1994; Lee et al., 1995). It has been shown that Ihh signaling induces the expression of *Pthlh*, which in turn acts back on the proliferating chondrocytes inhibiting the transition from the proliferating into the *Ihh* expressing prehypertrophic cell type. Boths signals thus interact in a negative feed back

mechanism. This model has been supported by several transgenic mouse lines: Loss of either *Ihh*, *Pthlh* or *Pthr1* in mice results in a dwarfism phenotype due to a reduced zone of proliferating chondrocytes and an advanced onset of hypertrophic differentiation (Amizuka et al., 1994; Lanske et al., 1996; St-Jacques et al., 1999). In contrast overexpression of *Ihh*, *Pthlh* or a constitutively activated *Pthr1* in chondrocytes, causes a delay in hypertrophic differentiation (Long et al., 2001; Schipani et al., 1997; Weir et al., 1996). Although both signals delay the onset of hypertrophic differentiation, several lines of evidence show that Pthlh is the executing molecule acting downstream of Ihh: (1) *Pthlh* expression is lost in *Ihh* deficient mice whereas *Pthlh* or *Pthr1* deficient animals still express *Ihh*, (2) Treatment of limb explant cultures with Shh, which activates the Ihh signaling pathway to a similar degree as Ihh (Pathi et al., 2001), only delays hypertrophic differentiation in wild-type but not in *Pthlh* or *Pthr1* deficient mice. (3) Constitutive activation of *Pthr1* delays the onset of hypertrophic differentiation in *Ihh* deficient mice (Amizuka et al., 1994; Lanske et al., 1996; St-Jacques et al., 1999). Pthr1 is, thus, necessary and sufficient to inhibit the differentiation of hypertrophic chondrocytes.

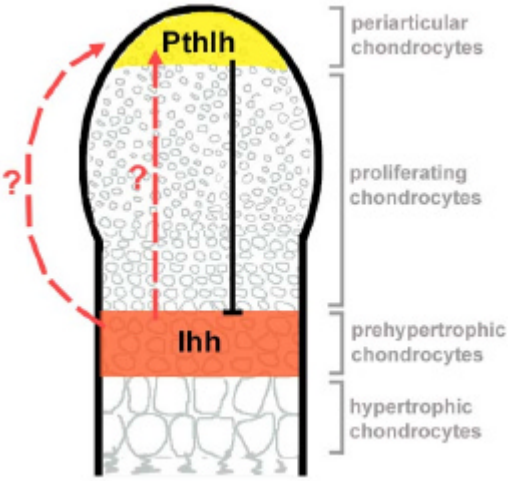

Fig. 3. The Ihh/Pthlh pathway regulates chondrocyte differentiation through a negative feed back loop. *Ihh*, which is expressed in prehypertrophic chondrocytes (red), induces *Pthlh* expression in the periarticular region (yellow). Pthlh in turn signals back to its receptor, Pthr1, which is expressed at low levels in proliferating and at high levels in prehypertrophic chondrocytes. The activated Pthr1 prevents hypertrophic differentiation of additional chondrocytes.

Interestingly limbs of *Ihh* deficient mice are severly shortened even in comparison with *Pthlh* or *Pthr1* mutant mice. Analysis of early stages of bone development revealed that the initial condensations are of normal size in *Ihh* mutants. However chondrocyte proliferation is severely reduced after the condensation stage. Due to the difference of the phenotypes the regulation of chondrocyte proliferation seems to be independent of Pthlh regulation. Correspondingly, overexpression of a constitutive active *Pthr1* in *Ihh* deficient mice can rescue the onset of hypertrophic differentiation but does not increase the proliferation rate (Karp et al., 2000). The regulation of the rate of chondrocyte proliferation seems to be a direct function of Ihh signaling to the neighbouring chondrocytes. These cell express high levels of *Ptch*, which is a direct target of Ihh and thus demarcates chondrocytes receiving a Ihh signal. Furthermore targeted deletion of *Smo* in chondrocytes results in a strong decrease of chondrocyte proliferation, whereas overexpression of *Ihh* or a constitutive activation of *Smo* in chondrocytes leads to an increased proliferation rate (Long et al., 2001; Minina et al., 2001).

A third striking phenotype of *Ihh* deficient mice is the lack of osteoblasts in endochondral bones. Although all chondrocytes undergo premature differentiation into hypertrophic cells, these are not surrounded by a bone collar. As the ossification of intramembranous bones occurs normally, Ihh does not seem to be necessary for the differentiation procedure in general but rather provides an inductive signal specifically in endochondral bones. Recently, analysis of mice carrying a targeted deletion of *Smo* in the perichondrium supported this idea as *Smo-/-* perichondrial cells did not differentiate into osteoblasts, whereas mesenchymal cells outside the deletion area formed an abnormal bone collar (Long et al., 2004).

It is an interesting question, how the Ihh signal reaches the far distant periarticular cells to activate *Pthlh* expression. Theoretically the Ihh signal could be mediated by a secondary factor, and Bmps or Tgfs have been hypothesized to act as those (Alvarez et al., 2002; Minina et al., 2001; Zou et al., 1997). However recent experiments have excluded Bmps as acting as secondary mediators: Limbs treated with cyclopamine, an inhibitor of Ihh signaling, and Bmps did not restore expression of *Pthlh*, which is lost after cyclopamine treatment (Minina et al., 2001). Alternatively, Ihh could act as a long range signal, forming a morphogen gradient, where different levels of Ihh protein lead to

activation of target genes. Such a model has been suggested for Shh signaling in the neural tube (Ingham and McMahon, 2001).

3.5 Ext dependent heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPG) are abundant cell surface glycoproteins that bear long, unbranched polymers of modified sugar residues, called glycosaminoglycans (GAG). Synthesis of HS takes place in the Golgi compartment. It is initiated by the assembly of a linkage tetrasaccharide - xylose, galactose, galactose, glucoronic acid (GlcA) – on conserved serine residues in the PG core polypeptide (Fig. 4) (Zhang and Esko, 1994). Since the same linkage region is found in chondroitin sulfate (CS) and HS, the committing step for HS synthesis is the addition of a N-acetylglucosamine (GlcNAc), a sugar specific for HS. The enzyme that determines this critical step is encoded by the *exostosis like gene 2* (*Extl2*) (Kitagawa et al., 1999). Polymerization then takes place by adding alternating units of GlcA and GlcNAc residues. This reaction is catalyzed by the glycosyltransferases, Exostosin1 (Ext1) and Exostosin2 (Ext2) in vertebrates and tout-velu (ttv) in *Drosophila* (Lind et al., 1998; McCormick et al., 1998). *Ext1* and *Ext2* as well as *Extl1*, *Extl2* and *Extl3* are members of the hereditary multiple exostosis (HME) gene family of tumor suppressors (Van Hul et al., 1998; Wise et al., 1997; Wuyts et al., 1997). *Ext1* and *Ext2* form a hetero-oligomeric complex in vivo that is accumulated in the Golgi apparatus (Kobayashi et al., 2000; McCormick et al., 2000). The *Ext1/Ext2* protein complex possesses substantially higher glycosyltransferase activity than *Ext1* alone, suggesting that this complex represents the biologically relevant form of the HS polymer modification unit (McCormick et al., 2000; Senay et al., 2000).

Differences in the composition of the sugars define the different types of GAG chains. Heparan and the structurally closely related Heparin molecules consists of disaccharide repeats of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), whereas chondroitin consists of N-acetylgalactosamine and GlcA disaccharides (Nybakken and Perrimon, 2002). Subsequent to polymerization of the GAG chains, the individual modified sugar residues undergo further modification, including N-deacetylation/N-sulfation, epimerization, and O-sulfation (Esko and Lindahl, 2001; Lander and Selleck, 2000; Lindahl et al., 1998). These modifications result in an ample spectrum of structural heterogenic GAG chains (Fig. 4).

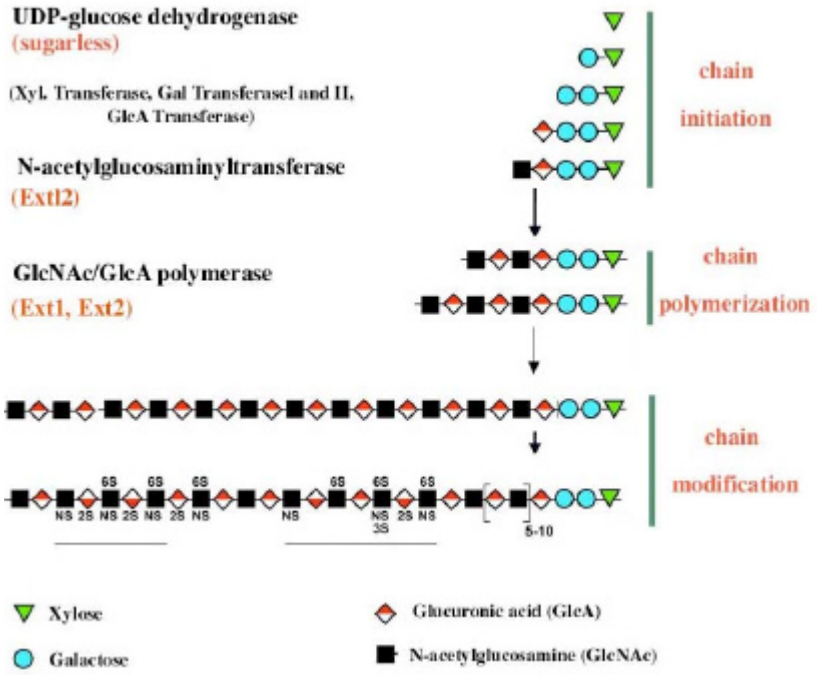

Fig. 4. Heparan sulfate synthesis. The chains are synthesized on the core protein by the sequential action of individual glycosyltransferases. A common tetra-saccharide linkage region is formed, followed by the addition of alternating GlcA and GlcNAc residues, producing the precursor chain. This chain is then enzymatically modified by deacetylation and N-sulfation, epimerization and O-sulfation, yielding individual chains (modified after (Esko and Selleck, 2002).

3.6 Biological role of HS

In human, mutations of genes affecting HS synthesis cause the autosomal dominant inherited syndrome HME, which is characterized by reduced skeletal size and multiple, cartilage-capped, benign bone tumors (exostoses) that arise from the growth plate of endochondral bones (Ahn et al., 1995; Stickens et al., 1996). Genetic linkage of this disorder has been ascribed to three independent loci on chromosomes 8q24.1 (*EXT1*), 11p11-13 (*EXT2*), and 19p (Cook et al., 1993; Le Merrer et al., 1994; Wu et al., 1994). Until today the putative *EXT3* gene at the 19p locus has not been isolated, and genetic linkage to this locus has only been identified in a few pedigrees to date. However, HME has not been linked to mutations in *EXTL* genes. Exostoses often cause pressure on

neighboring tissues, nerves or blood vessels and can lead to inhibition of joint movement. The most severe complication is the transformation of an exostosis into a malignant chondrosarcoma, although this complication is observed in only 2%-5% of HME patients. Sporadic and exostoses-derived chondrosarcomas are attributable to the loss of heterozygosity for the markers in *EXT1* and *EXT2* loci (Hecht et al., 1995; Raskind et al., 1995), indicating that these genes encode tumor suppressors. Until today, the molecular mechanisms leading to the formation of exostosis have remained unclear.

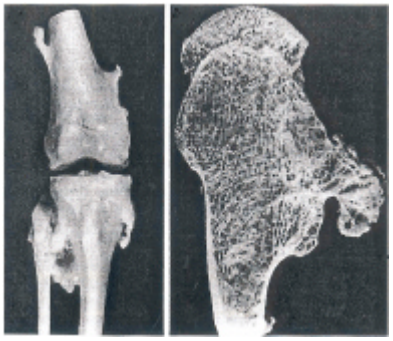

Fig. 5. Human Hereditary Multiple Exostosis disease. Dissected knee joint displaying exostoses (McCormick et al., 1999).

It has been shown that HS is critical for transport and receptor binding of several signaling molecules (Esko and Selleck, 2002). The interaction of HS with Fgf, for example, is a requirement for enabling the growth factor to activate its cell surface tyrosine kinase receptor (Pye et al., 2000; Schlessinger et al., 2000). Fgfs, Tgfs, and Hh proteins bind to HS with conserved motifs characterized by clusters of basic amino acids (Cardin and Weintraub, 1989; Rubin et al., 2002).

Genetic analyses have pointed to an important role for Ext1 in regulating Hh transport. In mice, targeted deletion of *Ext1* leads to a complete lack of HS synthesis. Homozygous embryos fail to gastrulate and lack embryonic mesoderm and extraembryonic tissues. Although *Ihh* mRNA is expressed in mutant embryos, Ihh protein fails to associate with *Ext1* deficient cells and cannot be detected by immunohistochemistry (Lin et al., 2000). In *Drosophila*, hh signals over several cell diameters in the wing imaginal disc. Mutation of the HS synthezising enzyme *ttv* results in a phenotype similar to the *hh* mutation. Importantly, hh movement in the wing disc is defective in clones of cells carrying

a somatic mutation of *ttv* (Bellaiche et al., 1998), suggesting that long-range hh-signaling depend upon HS.

Interestingly, HME patients only develop new exostoses until the growth plate closes, implicating misregulation of chondrocyte differentiation as a likely cause for the development of exostoses. In vertebrates, *Ext* genes are expressed throughout the body, however it has been shown that in the developing growth plate *Ext1* and *Ext2* are strongly expressed in chondrocytes and bone in mice (Stickens et al., 2000). The expression pattern of both genes flank the *Ihh* expression domain. *Ext1* and *Ext2* are therefore interesting candidates, to regulate the transport of the Ihh protein. Together, the proposed relationship of *ttv*-dependent HS and hh transport in *Drosophila* in combination with the bone defects observed in HME patients and the expression pattern of the *Ext* genes implicate a role for Ext1 in regulating Ihh signaling.

3.7 The *Gli* family of transcription factors

3.7.1 Cytoplasmic components of the Hh signaling pathway

In *Drosophila*, the Hh pathway is relatively simple since there is only one Hh protein and the cellular response to Hh signaling is mediated by the single transcription factor *Ci*, the homologe of the *Gli* gene family of transcription factors in vertebrates. In the absence of Hh signaling Ci lies within a multiprotein complex containing the ser/thr kinase Fused, the tumor suppressor protein Su(fu) and the kinesin-related protein Costal2. Within this complex Ci is phosphorylated by PKA, GSK3ß and CK1 kinases and subsequently proteolytically processed into a short 75 kDa small repressor protein. Processed Ci retains the DNA-binding motif, translocates to the nucleus and acts as a transcriptional repressor of Hh target genes (Dominguez et al., 1996; Methot and Basler, 2000). In the presence of Hh signaling, processing of Ci into the N-terminal repressor is inhibited and full-length Ci is turned into a labile activator (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). Interestingly, the activator function of Ci requires higher levels of Hh than the inhibition of processing. Thus, specific concentrations of Hh produce different responses due to the relative amounts of active Ci repressor, inactive full-lenght Ci and Ci activator (Aza-Blanc and Kornberg, 1999).

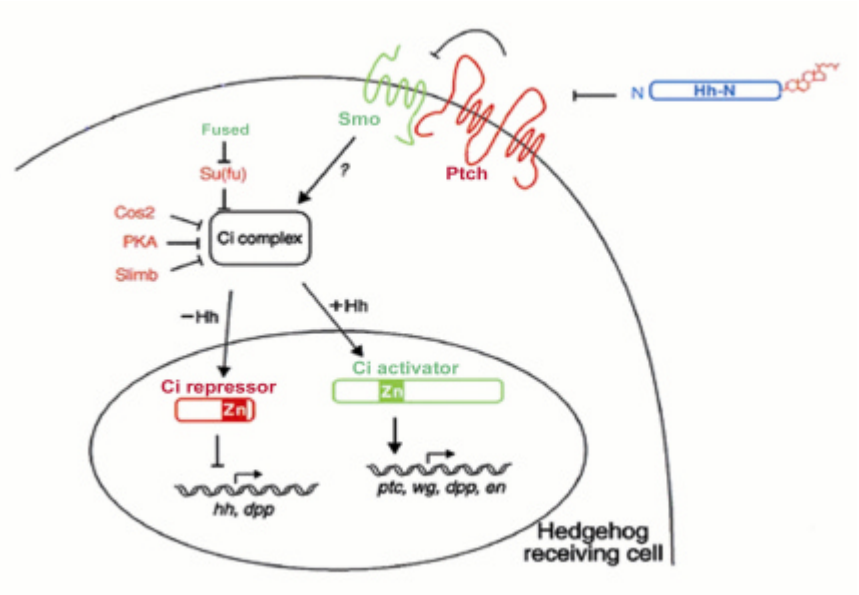

Fig. 6. Intracellular transduction of the hedgehog signal. In the absence of Hh signal, full-lenght Ci is anchored to microtubules via its interaction with Costal2 (Cos2) protein. Phosphorylation of several sites of Ci leads to processing of full-lenght Ci into the small repressor form, which enters the nucleus and inhibits Hh target genes. Upon stimulation of cells with high levels of Hh, full-lenght Ci is dephosphorylated, dissociates from the complex and is imported into the nucleus, where it activates Hh target genes.

3.7.2 Mouse mutations of the vertebrate *Gli* genes

In vertebrates, the Hh pathway is more complicated, in part because there are three *Gli* transcription factors, which mediate the activator and repressor functions of Ci to mediate Hh signaling. Furthermore unlike Ci, *Gli1* and *Gli3* are themselves transcriptional targets of Hh signaling: *Gli1* is activated and *Gli3* is repressed in response to Hh signals (Lee et al., 1997; Marigo et al., 1996). Of greatest difference from Ci, mouse *Gli1* does not undergo proteolytic processing and has therefore only activator function (Dai et al., 1999; Park et al., 2000). In contrast, like Ci, mouse *Gli2* and *Gli3* are processed and can theoretically act as both, activators and repressors (Dai et al., 1999; Sasaki et al., 1999). It is thought that the activator and repressor forms of the three different Gli genes have overlapping and specific functions in mediating the Hh signal in vertebrates.

Although *Gli1* is upregulated in cells receiving a hedgehog signal, mice homozygous for *Gli1* mutations, do not have developmental defects or a decrease in Hh signaling, unless one copy of *Gli2* is also removed (Bai et al., 2002; Park et al., 2000). *Gli2*-/- mice die at birth and lack floor plate and the most ventral neurons of the neural tube, indicating that

Gli2 positively functions downstream of Shh (Ding et al., 1998; Matise et al., 1998). In addition *Gli2-/-* mice display a mild skeletal phenotype (Mo et al., 1997). *Gli1*/*Gli2* compound mutants have normal digit number and pattern, whereas *Gli3* homozygous mutants show a dramatic polydactylous phenotype (Bai et al., 2002; Park et al., 2000).

In human *Gli3* plays an important role in embryonic patterning of limbs and other structures, as illustrated by two autosomal dominant syndromes associated with different types of mutations at the *Gli3* locus. Haploinsufficiency of the zinc finger gene *Gli3* causes the human Greig Cephalopolysyndactyly Syndrome (GCPS) as well as the homologous mouse mutation *'extra toes'* (*Gli3-Xt*) (Vortkamp et al., 1991). GCPS is characterized by polysyndactylies of hands and feet and mild craniofacial abnormalities. Pallister Hall Syndrome (PHS), which is caused by a frameshift mutation, leads to polydactyly and often to the formation of benign midline tumors of the ventral forebrain, known as hypothalamic hamartomas (Kang et al., 1997).

3.7.3 Gli3-repressor plays a major role during limb patterning

During limb bud development Shh acts as the polarizing activity establishing the anterior-posterior (AP) limb axis. It has been shown that loss of *Shh* results in skeletal elements without AP polarity, and only one rudimentary digit is formed. *Ptch* and *Gli1* are induced by Shh in the posterior limb bud, whereas *Gli2* and *Gli3* form a gradient of expression from anterior to posterior (Marigo et al., 1996; Schweitzer et al., 2000). A key finding is the demonstration of a gradient of Gli3 processing: the ratio of full-length Gli3 to its processed repressor form decreases from posterior to anterior (Wang et al., 2000).

Removal of *Gli3* function in *Shh* mutants partially rescues the *Shh* mutant phenotype: Limbs are distally complete and polydactylous, displaying a *Gli3* phenotype (Litingtung et al., 2002; Wijgerde et al., 2002). In the absence of *Gli3* and *Shh* neither the Gli3-activator nor the Gli3-repressor is present and therefore target genes are released from repression. Therefore *Gli3* is the main transcription factor downstream of Shh in patterning the AP axis of the developing zeugopod and autopod. If *Gli3* also exerts repressor functions downstream of Ihh during the process of endochondral ossification remains to be analysed.

3.7.4 *Gli* genes acting downstream of Shh in the neural tube

The spinal cord has been used as a model to study the function of the different Gli genes downstream of Hh, since it is a relatively simple structure. Six distinct classes of neurons are generated in the spinal cord from ventral to dorsal flanking the *Shh* expression domain (reviewed in Jessell, 2000; Gritli-Linde et al., 2001). Shh is sufficient in vitro to induce at least five of the ventral cell types in a concentration-dependent manner (reviewed in (Jessell, 2000)). *Gli1* and *Gli2* are highly expressed in the ventral part, whereas *Gli3* is expressed in an inverse gradient from dorsal to ventral.

Studies of neural tube development have led to the following conclusions: Gli1 and Gli2 are Hh-dependent activators, whereas Gli3 acts mainly as a repressor of Hh target genes. Gli3 is thought to act mainly as repressor, because loss of *Gli3* results in many phenotypes reminiscent of ectopic activation of the Shh pathways (Hui and Joyner, 1993). *Gli3* expression in place of *Gli2* revealed that Gli3 has only weak activator function. In contrast a Gli2-repressor does not seem to be required, since *Gli1*, which only acts as an activator, expressed in place of *Gli2* can rescue the spinal cord phenotype of *Gli2* mutants (Bai and Joyner, 2001). Gli1 enhances the activator function of Gli2 and transcription of *Gli1* was shown to dependent on both Gli2- and Gli3-activator (Bai et al., 2004). On the basis of these results the analysis of *Gli* genes acting as repressors or activators downstream of Ihh during bone formation is critical to understand the role of Ihh in this process.

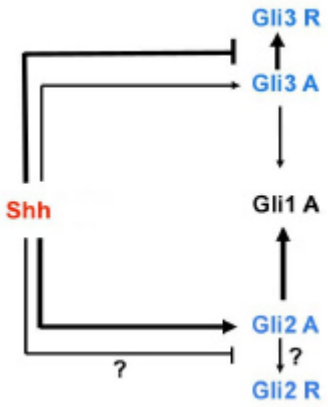

Fig. 7. Model of Shh signaling in vertebrates. During neural tube development Gli2 acts as the main activator of Shh signaling whereas Gli3 has only weak activator function and acts mainly as a repressor of Shh target genes. Formation of the Gli3-repressor is inhibited by Shh. Gli1 expression is activated by Gli2-A and Gli3-A.

3.8 Aim of the study

Considerable efforts have been made in elucidating the molecular mechanisms controlling chondrocyte differentiation in endochondral bones. A key molecule in this process is the secreted factor Indian hedgehog (Ihh). Ihh regulates the onset of hypertrophic differentiation by activating the expression of another secreted factor in the periarticular chondrocytes, *Parathyroid hormone-like hormone* (*Pthlh*). Pthlh diffuses through the growth plate and activates the *PTH/Pthlh* receptor (*Pthr1*), which inhibits the differentiation of proliferating chondrocytes into hypertrophic chondrocytes. This negative feedback loop is critical for the balanced growth of the developing skeletal elements (Amizuka et al., 1994; Lanske et al., 1996; Lee et al., 1995; Vortkamp et al., 1996). *Ihh* is expressed in prehypertrophic chondrocytes and one of the critical questions to understand it's function is, if Ihh can travel over long distances to directly induce the expression of *Pthlh* in the joint region or if secondary mediators are necessary. As Ihh signaling induces the expression of various members of the Transforming growth factor beta (Tgf) family, Bone morphogenetic proteins (Bmp) and other Tgfs have long been hypothesized to act as secondary signals downstream of Ihh to induce the expression of *Pthlh* (Alvarez et al., 2002; Zou et al., 1997). However, Bmps have been excluded from acting as such mediators (Minina et al., 2001). In contrast the proposed relationship of *ttv* dependent heparan sulfates (HS) and hedgehog transport in *Drosophila* in combination with the bone defects observed in HME patients and the expression pattern of *Ext1* and *Ext2* flanking the *Ihh* expression domain in murine limbs, implicate a role for Ext1 in regulating Ihh signaling (Ahn et al., 1995; Bellaiche et al., 1998; Stickens et al., 2000; Stickens et al., 1996; The et al., 1999). This study was therefore aimed to analyse the role of *Ext1* dependent HS on transport of the Ihh signal in the growth plate. As deletion of *Ext1* is gastrula lethal, we used a 'gene trap' mouse line carrying a hypomorphic allele of *Ext1* ($Ext1^{Gt/Gt}$) to analyse the effect of reduced amounts of HS on chondrocyte differentiation (Mitchell et al., 2001). In contrast, the influence of high levels of HS was analysed by use of an organ culture system. Treatment of wild-type, $Ext1^{Gt/Gt}$ and limbs from *Ihh* overexpressing mice, with different amounts of HS pointed to a role of these extracellular matrix molecules in regulating Ihh transport. Furthermore $Ext1^{Gt/Gt}$ limbs were treated with an inhibitor of hedgehog signaling, cyclopamine. To investigate a possible interaction between Ext1 and Fgf signaling, the $Ext1^{Gt/Gt}$ mouse line

was crossed to mouse lines with disturbed Fgf signaling and *Ext1*$^{Gt/Gt}$ limbs were treated with Fgf.

During the process of endochondral ossification Ihh regulates not only the onset of hypertrophic differentiation but also chondrocyte proliferation and ossification of the periosteum (Long et al., 2001; St-Jacques et al., 1999). To understand how the Ihh signal is translated into these different functions by the downstream transcription factors of the *Gli* gene family, we analysed the expression pattern of these genes at different stages in wild-type and *Ihh* deficient mice. As during neural tube and early limb bud development, Gli3 was found to act mainly as a repressor, opposing the role of Shh (Litingtung et al., 2002; Wijgerde et al., 2002), we aimed to elucidate the specific role for Gli3 acting downstream of Ihh during the process of endochondral ossification. Downstream targets of Ihh signaling and specific molecular chondrocyte markers were therefore analysed in *Gli3* deficient mice. Furthermore generation of *Ihh-/-;Gli3-/-* compound mutants revealed new insights into a possible repressor function of Gli3 during the process of endochondral ossification.