The role of EXT and growth signalling pathways in osteochondroma and its progression towards secondary peripheral chondrosarcoma

Hameetman, L.

Citation

Hameetman, L. (2007, April 26). *The role of EXT and growth signalling pathways in osteochondroma and its progression towards secondary peripheral chondrosarcoma*. Department Pathology, Faculty of Medicine / Leiden University Medical Center (LUMC), Leiden University. Retrieved from https://hdl.handle.net/1887/11865

General Introduction

1

CONTENTS

I. Bone tumours: general introduction

II. Cartilaginous tumours

- a. Peripheral cartilaginous tumours
  - i. Osteochondroma
  - ii. Secondary peripheral chondrosarcoma
  - iii. Multiple Osteochondromas
- b. Central chondrosarcoma and enchondroma

III. The epiphyseal growth plate

IV. Growth signalling in the growth plate and neoplastic cartilage

- a. EXT and heparan sulphate proteoglycans (HSPGs)
  - i. *EXT1*
  - ii. *EXT2*
  - iii. *EXT-like* genes
  - iv. Function *EXT* genes in HSPG biosynthesis
  - v. EXT and HSPG in the growth plate
  - vi. EXT and HSPG in Multiple Osteochondromas
  - vii. Animal models for EXT function
- b. IHH/PTHLH signalling
- c. BMP and TGF-β signalling
- d. WNT signalling

V. Aims of the study and outline of the thesis

I. BONE TUMOURS: GENERAL INTRODUCTION

The involvement of bone in metastasis of epithelial tumours is quite common, whereas primary bone sarcomas account for only 0.2% of all neoplasms regardless of having their origin in the skeletal system or not [1]. Nevertheless 32 different histological types of primary bone tumours (both benign and malignant) are distinguished by the World Health Organization (WHO) [2]. The most common primary malignant tumours of bone are osteosarcoma (35%), chondrosarcoma (25%), Ewing sarcoma (16%) and malignant fibrous histiocytoma (MFH; 5%) [3] (table I.I). Both osteosarcoma and Ewing sarcoma have a peak incidence around adolescence, whereas for chondrosarcoma the incidence is most frequent in the fourth decade of life [5]. Osteosarcoma has a second peak of incidence over 60 years of age, coinciding with the peak incidence of MFH. Both these tumours at older age arise frequently secondary to pre-existing bone abnormalities like Paget disease, radiation damage, bone infarction or fibrous dysplasia.

Table I.I. Overview of the most common primary bone tumours.

A. Benign primary bone tumours		
	Incidence (% all benign bone tumours)	Age at diagnosis (years)
Osteochondroma	> 35%	10 - 30
Giant cell tumour	20%	20 - 45
Enchondroma	10-25%	20 - 40
Osteoid osteoma	10%	5 - 25
Aneurysmal bone cyst	7%	0 - 20
Chondromyxoid fibroma	2%	10 - 30
Osteoblastoma	2%	10 - 30
Chondroblastoma	1%	10 - 25 and 40 - 45
Periosteal chondroma	<1%	All
B. Bone sarcomas		
	Incidence (% all malignant bone sarcomas)	Age at diagnosis (years)
Osteosarcoma	35%	5 - 25 and > 40
Chondrosarcoma	26%	30 – 70
Ewing sarcoma	16%	5 – 25
Malignant Fibrous Histiocytoma	6%	> 40
Chordoma	1-4%	> 30
Fibrosarcoma	2%	35 - 60
Angiosarcoma	1%	10 - 80
Adamantinoma	<1%	5 - 85

Overview of the most frequent benign and malignant primary bone tumours. Osteochondroma is the most frequent benign bone tumour. Chondrosarcoma is the most frequent bone sarcoma in adult patients [2,4].

In addition to bone sarcomas several benign bone neoplasms are known (table I.I). From some of these lesions little aetiology and epidemiologic information is available from literature, because many of the lesions are asymptomatic and therefore diagnosed only incidentally. Nonetheless are benign bone tumours more frequent than bone sarcomas.

Most bone tumours are considered to be of mesenchymal origin; however for some tumours this is still unclear, like Ewing sarcoma, which might also originate from neuroectodermal precursor cells.

II. CARTILAGINOUS BONE TUMOURS

Cartilaginous bone tumours are characterized by production of a characteristic chondroid matrix. They are classified based on their histological features and the location within the bone and can be clinically divided according to their behaviour into benign and malignant tumours [6] (table I.II).

II.a. Peripheral cartilaginous tumours

As the name of this subgroup of cartilaginous tumours already implies, these tumours are located at the periphery of bone. Most are benign tumours (osteochondroma and periosteal chondroma), but also malignant tumours can occur (secondary peripheral chondrosarcoma and periosteal chondrosarcoma) (table I.IIB). Osteochondroma and secondary peripheral chondrosarcoma are the focus of this thesis.

II.a.i. Osteochondroma

Osteochondroma is the most common benign bone tumour arising at the periphery of long bones preformed by endochondral ossification [7,25]. It consists of a cartilage cap, a perichondrium (a thin fibrous layer that covers the cartilage cap and is continuous with the periost of the underlying bone) and a bony stalk consisting of cortex and medulla, that is continuous with that of the underlying bone (figures 1.1 and 2.1). In the cartilage cap the chondrocytes show a spatial organization as seen in the epiphyseal growth plate and undergo endochondral ossification [7].

Osteochondromas develop in the first decade of life and cease to grow at puberty when the skeleton matures. Most of the lesions occur in a solitary (nonhereditary) setting; however 15% of the patients have multiple lesions, usually in the context of the hereditary syndrome known as Multiple Osteochondromas (see section *II.a.iii*).

In a small percentage of osteochondromas, the cartilage cap transforms into its malignant counterpart, secondary peripheral chondrosarcoma. For solitary osteochondroma malignant transformation is estimated to occur in less than 1%, whereas for hereditary lesions the risk of malignant transformation is estimated at 0.5-3% [7]; however exact numbers are unknown.

II.a.ii. Secondary peripheral chondrosarcoma

Secondary peripheral chondrosarcomas arise in the cartilage cap of osteochondromas (figures 1.1 and 2.1). They constitute approximately 17% of all conventional chondrosarcomas [11]. Chondrosarcomas are characterized by the production of hyaline cartilage and comprise a

Table I.II. Cartilaginous tumours and related syndromes.

A. Benign tumours

	Incidence (% all benign bone tumours)	Age (yrs)	Sex distribution (M:F)	Common sites of involvement	Reference
Osteochondroma	> 35%	10 - 30	1 : 1	Metaphysis femur, humerus, tibia, fibula	7
Enchondroma	10-25%	20 - 40	1 : 1	Tubular bones hand and feet, humerus, femur	8
Chondromyxoid fibroma	2%	10 - 30	1.5 : 1	Proximal tibia, distal femur, ilium	9
Chondroblastoma	1%	10 - 25	2 : 1	Epiphysis femur, tibia, humerus	10
Periosteal chondroma	>1%	all	1 : 1	Proximal humerus	8

B. Malignant tumours

			Incidence (% all chondrosarcomas)	Age (yrs)	Sex distribution (M:F)	Common sites of involvement	Reference
Chondrosarcoma							
Conventional			80 - 85%				
	Central	Primary	50 - 81% of conventional chondrosarcoma	30 - 70	1.5 : 1	Trunk, upper ends femur and humerus	11
		Secondary	2 - 33% of conventional chondrosarcoma	30 - 70	1.5 : 1	Pelvic, shoulder girdle bones	11
	Secondary Peripheral		17 % of conventional chondrosarcoma	30 - 70	1.5 : 1	Pelvic, shoulder girdle bones	11
Dedifferentiated			10%	50 - 60	1 : 1	Pelvis, femur, humerus	12
Mesenchymal			3 - 10%	20 - 30	1 : 1	Jawbones, ribs, ilium, vertebrae	13
Periosteal			2%	30 - 70	2 : 1	Metaphyses distal femur	11
Clear cell			2%	25 - 50	3 : 1	Humeral and femoral head	14

C. Syndromes with cartilaginous tumours

	Incidence	Tumours	Sex distribution (M:F)	Hereditary	Genes	Reference
Multiple Osteochondromas	1: 50 000	Osteochondromas	1.5 : 1	Yes	EXT1, EXT2	15
Enchondromatosis						
Ollier Disease	Unknown, rare	Enchondromas	1 : 1	No	Unknown	16
Mafucci syndrome	Unknown, very rare	Enchondromas, heamangiomas	1 : 1	No	Unknown	16
Spondyloenchondrodysplasia	Unknown, very rare	Enchondromas	Unknown	Yes	Unknown	17,18
Generalized enchondromatosis	Very rare	Enchondromas	Unknown	Yes	Unknown	19,20
Dysplasia epiphysealis hemimelica	1: 1 000 000	Osteochondra-like lesions	1.5 : 1	No	Unknown	21,22
Metachondromatosis	Very rare	Osteochondroma + enchondromas	1 : 1	Yes	Unknown	23,24

Overview of the most frequent benign and malignant cartilaginous tumours and syndromes characterized by the formation of cartilaginous tumours.No epidemiological data is available for central and peripheral chondrosarcoma separately.

heterogeneous group of lesions with diverse morphological features and clinical behaviour (table I.II.B).

Like all conventional chondrosarcomas, secondary peripheral chondrosarcomas are graded based upon several histological features [26] (table I.III, figure 1.1). Histological grading is still the most important predictor of clinical behaviour and prognosis for chondrosarcoma. Grade I chondrosarcomas rarely metastasize, but the risk increases to 10-33% and 70% for grade II and grade III lesions, respectively [26,27].

Secondary peripheral chondrosarcomas are usually low-grade tumours and in daily practice, it can be difficult to distinguish these lesions from osteochondroma both radiologically[28] and histologically. So far, the diagnosis is based on a combination of clinical, radiological and

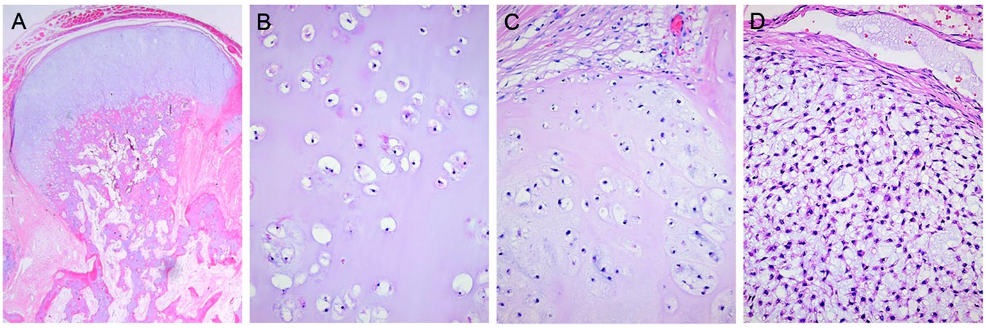

Figure 1.1 Histology of osteochondroma and peripheral secondary chondrosarcoma. In (A) a micrograph of an osteochondroma is shown. The cartilage cap has low cellularity and a large amount of chondroid matrix. A thin perichondrium covers the cartilage cap. (B) Micrograph of a grade I secondary peripheral chondrosarcoma showing low cellularity, limited cytonuclear atypia and large amount of extracellular matrix. Binucleated cells are present, whereas mitoses are not. A micrograph of a grade II chondrosarcoma is shown in (C), displaying increased cellularity and diminished amounts of matrix. The tumour cells show increased cytonuclear atypia and mitoses can be present. (D) Micrograph of a grade III chondrosarcoma demonstrating high cellularity and cytonuclear atypia.

histological findings. Though secondary peripheral chondrosarcomas are usually low-grade lesions, which is favourable for the prognosis, they can recur with a higher histological grade, suggesting progression in malignancy with time [26,27].

II.a.iii. Multiple Osteochondromas

Multiple Osteochondromas (MO, hereditary multiple exostoses, multiple hereditary osteochondromatosis, diaphyseal aclasis) is an autosomal dominant disorder, characterized by the presence of multiple osteochondromas of which the number can vary significantly between and within families [15,29]. Clinicopathological features, genetic spectrum and basic scientific understanding of Multiple Osteochondromas are reviewed in detail in **chapter 2**.

Multiple Osteochondromas is a heterogeneous disorder for which two causative genes have been identified, *Exostosin-1* (*EXT1*) located at 8q24.11-q24.13 and *Exostosin-2* (*EXT2*) located at 11p11-p12 [30-32]. Most germ-line mutations are non-sense, frame shift or splice-site mutations and cause loss of EXT protein function [33,34] (figure 2.2). Loss of the remaining wild type allele of *EXT1* has been demonstrated in osteochondroma [35], proving that *EXT1* acts as a classical tumour suppressor gene in osteochondroma formation in Multiple Osteochondromas patients. Thus, *EXT1* acts in line with Knudson's two-hit model for tumour suppressor genes [36]. However, in other studies loss of the wild type allele could not be demonstrated in hereditary osteochondromas [37-39], which led the investigators to suggest that haploinsufficiency via mutational inactivation of one allele, is sufficient for osteochondroma formation [37]. Molecular investigation of cartilaginous tumours however is challenged by excess of extracellular matrix, poor cellularity, both hampering DNA and RNA isolation, and small sample size and therefore such negative result of LOH detection should be handled with caution.

In solitary osteochondromas somatic *EXT1* mutations are extremely rare [40-42]. However, loss of heterozygosity (LOH) and clonal rearrangement of 8q24 in non-hereditary osteochondroma are frequently found [35,43,44]. No somatic *EXT2* mutations have been reported in solitary osteochondroma and LOH at the *EXT2* locus has been shown only once [44]. Therefore, the mechanism of *EXT* inactivation in solitary osteochondroma was a subject of investigation. Multiple Osteochondroma patients usually also suffer from a variety of orthopaedic deformities, including shortening of the ulna with secondary bowing of the radius (39-60%) and inequality of the limbs (10-50%) [15]. It is still debated whether these deformities are a result of skeletal dysplasia due to *EXT1* or *EXT2* haploinsufficiency [45], or the result of local effects on the growth plate by the developing osteochondromas [46].

There are two rare skeletal disorders considered in the clinical and radiological differential diagnosis of solitary and hereditary osteochondromas, namely Dysplasia epiphysealis hemimelica (Trevor's disease, tarso-epiphyseal aclasis) and metachondromatosis (table I.IIC). Dysplasia epiphysealis hemimelica is a developmental disorder with cartilaginous overgrowth (osteochondroma-like lesion) of a part of one or more epiphyses or their equivalents predominantly affecting the lower extremity on one side of the body [21]. Metachondromatosis is a rare autosomal dominant disorder exhibiting synchronously both multiple osteochondromas and enchondromas [23,24]. Histological, radiological and molecular characteristics of both disorders are discussed in detail in **chapter 7**.

Table I.III. Histological grading criteria of conventional chondrosarcoma [26].

Grade	Histological features
I	Marked preponderance of small densely-straining nuclei Calcification and bone formation are frequent Multiple nuclei within one lacuna, sometimes infrequent Occasionally small number or larger, somewhat pleomorphic nuclei are present Background varies from chondroid to myxiod
II	Proportion of nuclei is of moderate size Low mitotic rate (< 2 mitosis per 10 high power fields) Increased cellularity, specifically toward periphery of tumour lobules Nuclei are paler and have visible intranuclear detail Background in more cellular areas tends to be more myxoid
III	2 mitoses per 10 high power fields in most active areas Increased cellularity in periphery of tumour lobules Larger nuclei in areas with increased cellularity as compared to grade II chondrosarcomas Spindle cell shaped cells in high cellular areas, no appreciable chondroid/myxiod matrix

Although these criteria were formulated over 30 years ago, they are still optimal for grading and have the best correlation with progression and prognosis.

II.b. Central chondrosarcoma and enchondroma

The majority (90%) of conventional chondrosarcoma arise centrally in the medullar cavity of bone, either as a primary lesion or secondary to a pre-existing benign enchondroma [11]. Approximately 75% of primary central chondrosarcoma arise in the pelvis, scapulae and upper part of the femur and humerus. Histologically central chondrosarcomas are similar to secondary peripheral chondrosarcoma and are also graded into three grades for malignancy using the same criteria [26].

Enchondroma (central chondroma) is the benign counterpart of central chondrosarcoma[8]. Most enchondromas occur in the medullar cavity of small tubular bones of the hand and feet, but also the long tubular bones are regularly affected. Unlike osteochondroma, chondrocytes in enchondroma do not display any longitudinal organization.

Enchondromas occur both solitary or in the context of several rare developmental disorders that are classified as enchondromatosis and include Ollier disease and Mafucci syndrome[16,47,48] (table I.IIC). The malignant transformation of solitary enchondroma is rare (<1%), whereas in enchondromatosis the risk of malignant transformation can be as high as 15-30%.

III. THE EPIPHYSEAL GROWTH PLATE

Since the cartilage cap of osteochondroma morphologically resembles the epiphyseal growth plate, the growth signalling pathways involved in the growth plate, are thought to be affected in these lesions. Growth signalling pathways have been extensively studied in the growth plates of normal and transgenic animal models (mostly rats and mice). The growth plate is a cartilaginous structure entrapped between the epiphysis and metaphysis at the ends of long bones. It functions as scaffold and is replaced by bone in a coordinated fashion [49,50]. Most of the skeleton develops via this so-called endochondral ossification, except for the cranial vault, the facial bones and the clavicles. These bones develop via intramembranous ossification, where osteoblasts differentiate directly from embryonic mesoderm without a cartilaginous intermediary [51].

The growth plate is a highly organized structure, in which different morphological zones of chondrocytes at different stages of differentiation can be distinguished [52] (figure 1.2). At the epiphyseal part of the growth plate resides the resting or germinal zone, which contains the resting chondrocytes. The resting chondrocytes enter the proliferative zone upon a yet unknown trigger. The flat proliferating chondrocytes assemble in orderly, longitudinal columns and start producing extracellular matrix proteins (e.g. collagen II). Longitudinal bone growth depends on the length of the columns, thus the number of proliferating cells [53]. Eventually these chondrocytes loose their proliferative capacity and start to differentiate into hypertrophic chondrocytes, either by a finite number of cell divisions or by changes in exposure to a local growth factor (e.g. parathyroid hormone-like hormone (PTHLH))[52]. The chondrocytes in de hypertrophic zone increase in size, obtain a more rounded appearance and start producing more and different matrix proteins (e.g. collagen X). The extracellular matrix around the hypertrophic chondrocytes is finally calcified and the hypertrophic chondrocytes undergo apoptosis. The calcified matrix is resorbed by osteoclasts and osteoblasts enter the area to form trabecular bone [49]. In humans, fusion of the growth plates at the end of puberty induced by oestrogen stops this process of longitudinal growth [54].

The process of endochondral ossification is maintained by growth factors [55], but is also dependent on hormonal factors, like oestrogen, as well as environment and nutrition[49,51,52].

IV. GROWTH SIGNALLING IN THE GROWTH PLATE AND NEOPLASTIC CARTILAGE

IV.a. EXT and heparan sulphate proteoglycans (HSPGs)

IV.a.i. EXT1

The *EXT1* gene was first identified as a gene involved in the development of Multiple Osteochondromas in 1995 by positional cloning [30]. The gene on chromosome 8q24.11-q24.13 is composed of 11 exons (figure 2.3) that give rise to a coding sequence of 2,238 bp [30]. The mRNA was found to be ubiquitously expressed [30]. *EXT1* seems to be highly conserved since orthologues have been identified in *Drosophila melanogaster* (fruitfly; *tout-velu* (*ttv*)) [56], *Caenorhabditis elegans* (worm; *rib-1*) [57] and *mus Musculus* (mouse; *Ext1*) [58].

The human *EXT1* mRNA encodes a 746 amino acids type II transmembrane glycosyltransferase, Exostosin-1 (EXT1) [59]. The three-dimensional (3D) structure of the protein still needs to be elucidated.

IV.a.ii. EXT2

In 1996, two research groups independently identified the *EXT2* gene as the second gene that gives rise to Multiple Osteochondromas when mutated, by positional cloning on chromosome 11p12-p11 [31,32]. The gene consists of 16 exons (figure 2.3) and has an open reading frame of 2,154 bp. The mRNA is ubiquitously expressed and the C-terminus shows high similarity with *EXT1*. Like for *EXT1*, orthologues of *EXT2* have been identified in several other organisms including *mus Musculus* (*Ext2*) [57,60], *Drosophila melanogaster* (*sister of tout-velu*, *stv*) [61] and *Danio rerio* (zebrafish; *dackel*) [62].

The Exostosin-2 (EXT2) protein contains 718 amino acids and like EXT1 it is a type II transmembrane glycosyltransferase with a yet unknown 3D structure [33,63].

In the endoplasmatic reticulum EXT1 forms a hetero-oligomeric protein complex with EXT2, which after formation transfers to the Golgi apparatus where it is involved in the heparan sulphate proteoglycan (HSPG) biosynthesis [64] (see section *IV.a.4*). The Golgi-localized EXT1/EXT2 complex possesses substantially higher enzyme activity than EXT1 or EXT2 alone[64].

IV.a.iii. EXTL-genes

In addition to *EXT1* and *EXT2*, the exostosin family of genes has three other known members; the *EXT-like* genes, *EXTL1*, *EXTL2* and *EXTL3* [65-68], located at 1p36.1, 1p11-p12 and 8p12-p22, respectively. All *EXTL*-genes share sequence similarities with *EXT1* and *EXT2* and based on the level of amino acid conservation, they possess similar enzyme activities as EXT1 and EXT2 proteins [69]. For *EXTL3*, orthologues have been identified in several other organisms, among which are *brother of tout-velu* (*btv*) in *Drosophila melanogaster* [61] and *boxer* in *Danio rerio* [62].

No linkage with Multiple Osteochondromas or other bone diseases has been documented for the *EXTL*-genes [70]. Since the *EXTL*-genes function upstream of EXT1 and EXT2 in the heparan sulphate biosynthesis, mutations in *EXTL*-genes might have a much more severe result.

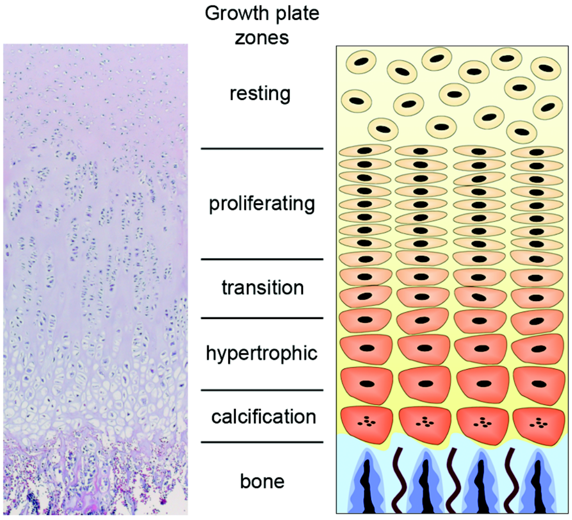

Figure 1.2 The epiphyseal growth plate. Micrograph (left) and schematical representation (right) in which the different zones of the growth plate are represented. The resting zone contains stem-cell like chondrocytes. When these cells start proliferating upon a yet unknown trigger they enter the proliferative zone and assemble in orderly, longitudinal columns. At a certain point these chondrocytes stop proliferating and start to differentiate into hypertrophic chondrocytes in the transition zone. Hypertrophic chondrocytes increase in size, obtain a more rounded appearance in the hypertrophic zone. Finally the hypertrophic chondrocytes undergo apoptosis and the extracellular matrix calcifies. This leaves a scaffold for new bone formation.

IV.a.iv. Function of EXT genes in HSPG biosynthesis

All EXT family members are involved in the attachment and polymerization of heparan sulphate (HS) chains to HSPG core proteins [71] (figure 2.4). HSPGs are large macromolecules present at the membrane or residing in the extracellular matrix and have many different functions. They are involved in several growth signalling pathways, anchorage of cells to the extracellular matrix and sequestering of growth factors [72]. HSPGs can be subdivided into several families among which are the syndecans, glypicans, perlecan and CD44 isoforms.

EXTL2 and EXTL3 initiate polymerization of the HS chain by addition of N-acetylglucosamine on a tetrasaccharide attached to the HSPG core protein [69,73]. The elongation of the HS chain is catalyzed by the EXT1/EXT2 protein complex, which alternatively adds units of N-acetylglucosamine and glucoronic acid [63,64,74]. The HS chain is subsequently modified by de-acetylases, epimerases and sulphotransferases to create a large spectrum of structural heterogenic HS chains [71]. Different sulphation patterns of the HS chains are important for the binding of specific growth factors [71], which in turn can have conserved patterns of basic amino acids for binding to HSPGs, crucial for proper signalling [75,76].

The expression of HSPG core proteins is both cell- and tissue type specific. However, the different structures of HS chains do not appear to correlate with the core protein they attach to, but more with the cell-type of origin [77]. The specific sulphation patterns can also be influenced by aging and disease [78].

IV.a.v. EXT and HSPG in the growth plate

Both *EXT1* and *EXT2* have been described to be ubiquitously expressed, however Stickens et al. described differential expression of three *EXT* genes (*EXT1*, *EXT2* and *EXTL1*) during mouse embryogenesis [79]. The *EXT* genes display the highest expression in the limbs throughout all embryonic stages tested. *EXT1* and *EXT2* were shown to be expressed in both bone and cartilage, mainly the proliferating and pre-hypertrophic chondrocytes, whereas *EXTL1* was only expressed in growth plate, but not restricted to a specific zone.

HSPGs are important for proper growth signalling in the growth plate. In both murine and chick growth plate, syndecan-2 and syndecan-3 were shown to be involved in signalling pathways in proliferating chondrocytes, like Indian Hedgehog (IHH)/parathyroid hormone-like hormone (PTHLH) signalling and fibroblast growth factor (FGF) signalling [80-83]. The expression of glypican has been demonstrated in the perichondrium, the developing limb and mesenchymal tissues of the developing mouse embryo [84]. Perlecan, the most common proteoglycan of the basement membrane is expressed throughout the rat growth plate [85].

Not much is known about HSPGs in human growth plates. The expression of HS chains and perlecan has been investigated in one normal human growth plate that served as control sample for a series of osteochondromas [40]. Both the HS chains and perlecan were strongly expressed around the chondrocyte lacunae. At present, in literature no data are available on the *EXT* genes or proteins neither in human growth plates nor in cartilaginous tumours.

IV.a.vi. EXT and HSPG in Multiple Osteochondromas

Hecht and colleagues were able to demonstrate greatly diminished protein levels of EXT1 in cultured osteochondroma chondrocytes, which was often accompanied by loss of EXT2 protein expression [41]. This study was followed by two publications in which they were able to identify complete loss of heparan sulphate in osteochondroma as well as diminished and abnormal distribution of perlecan [40,86]. However, no second mutational event to inactivate the remaining wild type allele could be detected. They therefore concluded that loss of one copy of either *EXT1* or *EXT2* disables the function of EXT1/2 complex sufficient to induce osteochondroma formation. Their conclusion conflicts with Knudson's two-hit model for the *EXT1* gene demonstrated in osteochondromas from Multiple Osteochondromas patients [35].

IV.a.vii. Animal models for EXT function

The first data suggesting a role for *EXT* genes in growth signalling pathways came from *Drosophila* studies. Mutants of the *EXT1* orthologue, *ttv*, showed that it is required for the diffusion of Hedgehog (*Hh*, orthologue of mammalian IHH) [56,87]. Also *Drosophila* mutants for the two other *EXT* orthologues, *sotv* (*EXT2*) and *botv* (*EXTL3*) showed impaired gradient formation of the different morphogens including *Hh*, *Wingless (Wg, WNT)* and decapentaplegic (*Dpp*, TGF-β/BMP) [61,88,89]. Table I.IV summarizes the different phenotypes of these *Drosophila* mutants and the different other animal models that have been developed, including several mouse models.

EXT1 null ($EXT1^{-/-}$) mice were embryonic lethal and despite the claim that osteochondroma can develop as a result of *EXT1* haploinsufficiency [41], the *EXT1* heterozygous ($EXT1^{+/-}$) mice did not develop osteochondromas, nor did they present any significant skeletal abnormalities [93]. However, more detailed examination of the long bones of $EXT1^{+/-}$ mice revealed increased proliferation and delayed hypertrophic differentiation in the growth plate due to increased diffusion of IHH [94].

Another group was able to demonstrate that mice carrying a hypomorphic mutation in *EXT1* ($EXT1^{\Delta GT/\Delta GT}$) produced shorter HS chains [97], which increased the range of IHH signalling in a concentration dependent manner during embryonic chondrocyte differentiation [95].

Like *EXT1* null mice, *EXT2* null ($EXT2^{-/-}$) mice are embryonic lethal, but *EXT2* heterozygous ($EXT2^{+/-}$) mice had a normal lifespan. Analysis of the skeleton revealed abnormalities in cartilage differentiation and one-third of the mice formed one or more ectopic bone growths that resembled osteochondromas [96]. However these bone growths still produced HS, in contrast to the osteochondromas in humans [40,86].

Table I.IV. Animal models for Multiple Osteochondromas

Model	Mutation in	Phenotype	Reference
***Drosophila melanogaster* (fruitfly)**	*ttv* (*EXT1*)	Impaired HS biosynthesis Wing imaginal disc: defective *Hh*, *Wg* and *Dpp* distribution and signalling activity Adult wing narrower in anterior/posterior orientation Mutant embryos die at pupal stage	56,61,87-89
	sotv (*EXT2*)	Impaired HS biosynthesis Wing imaginal disc: defective *Hh*, *Wg* and *Dpp* distribution and signalling activity Adult wing not as affected as *ttv* and *botv* mutants	61,88,89
	botv (*EXTL3*)	Impaired HS biosynthesis Wing imaginal disc: defective *Hh*, *Wg* and *Dpp* distribution and signalling activity Adult wing narrower in anterior/posterior orientation	61,88,89
***Danio rerio* (zebrafish)**	*dackel* (*EXT2*)	All arches short and thick and strongly reduced No pectoral fins; tail often curls up Jaw not extended Impaired HS synthesis	62,90
	boxer (*EXTL3*)	All arches short and thick and strongly reduced No pectoral fins; tail often curls up Jaw not extended Impaired HS synthesis	62,90
***Caenorhabditis elegans* (worm)**	*rib-1* (*EXT1*)	Embryonic lethality Short, thick isthmus Impaired HS synthesis	91
	rib-2 (*EXTL3*)	Egg laying defects Increased body width Reduced activity in movement Impaired HS synthesis	92

***Mus Musculus* (mouse)**

Model	General phenotypes	Skeletal phenotypes	Growth plate phenotypes	Reference
$EXT1^{-/-}$	Fail to gastrulate Lack organized mesoderm and extraembryonic tissues Smaller embryos Resorbed at E8.5			93
$EXT1^{+/-}$	Phenotypically normal Fertile No increase in tumour incidence	No osteochondromas Mild reduction in humerus and femur bone density	Enlarged proliferative zone Reduced hypertrophic zone Reduction of HS to near 50% Increased range IHH diffusion	93,94
$EXT1^{''GT/''GT}$	Survive until E14.5 Embryos are small and appear oedematous Heart defects	No osteochondromas Reduced size Fused vertebrae Shortened limbs Fusion elbow and knee joints	Expanded proliferating zone Loss of columnar organization in proliferating zone Severely delayed bone formation Delay in hypertrophic differentiation Reduced amounts of HS Elevated range IHH signalling	95
$EXT2^{-/-}$	Die at E6.5 Fail to gastrulate Fail to form primitive streak or mesoderm			96
$EXT2^{+/-}$	Normal lifespan Fertile	Ectoptic bone growths of ribs (osteochondroma-like) No gross skeletal abnormalities	Misplaced chondrocytes near perichondrium in ribs Loss of columnar organization in proliferating zone	96

IV.b. IHH/PTHLH signalling

The HSPGs have a crucial role in the long distance diffusion of Indian Hedgehog (IHH) to its receptor as demonstrated in *Drosophila* [56,61,87-89].

IHH belongs to the hedgehog (HH) protein family, which contains morphogens that play a crucial role during embryonic and post-embryonic development. The other two family members are Sonic Hedgehog (SHH) and Desert Hedgehog (DHH). HH proteins are known to be involved in the regulation of both cell proliferation and differentiation [98]. Binding of HH to its receptor Patched (PTCH), leads to the activation of the membrane protein Smoothened (SMO), which activates GLI transcription factor family members (GLI1-3) (figure 1.3). This leads to activation of target genes, including *GLI1* and *PTCH* itself [100,101].

In the growth plate IHH is one of the most important regulators of chondrocyte proliferation and differentiation as part of a tightly regulated paracrine feedback loop (figure 1.4), together with parathyroid hormone-like hormone (PTHLH or PTHrP) [51,103,104] and it induces ossification of the perichondrium independent of PTHLH [55]. It has to be noted that most of the IHH mediated signal transduction involved in growth plate regulation and endochondral ossification has been investigated in model organisms and may not be entirely representative for humans.

In the embryonic growth plate (figure 1.4A) IHH is secreted by chondrocytes in the transition zone and diffuse to PTCH in the lateral perichondrium. The subsequently PTHLH at the apical perichondrium, diffuses to its receptor expressed in the late proliferating chondrocytes [55], stimulating proliferation and inhibiting the terminal differentiation via upregulation of BCL2 [103], thereby reducing the number of IHH secreting chondrocytes. In this feedback loop progression of chondrocyte differentiation towards the hypertrophic zone is delayed by PTHLH and BCL2, allowing longitudinal bone growth [103]. Recently, it was shown that GLI3 represses PTHLH expression in the growth plate, which is antagonized by IHH[105,106]. This results in a restricted zone of PTHLH expression in the growth plate. In the rat post-natal growth plate the feedback loop is confined to the transition and hypertrophic zone [104] (figure 1.4B). The co-expression of PTCH and PTHLH expression in resting and hypertrophic chondrocytes suggested the existence of two growth restraining feedback loops in the post-natal growth plate [104] (figure 1.4B).

Immunohistochemical evaluation of human post-natal growth plate has demonstrated IHH expression in the prehypertrophic and hypertrophic chondrocytes, similar to the expression found in rat. However, PTCH and PTHLH expression was found in the proliferating and hypertrophic chondrocytes [107,108] and not the resting chondrocytes.

In the transgenic *EXT1*$^{\Delta GT/\Delta GT}$ mice, the shorter HS chains resulted in an elevated range of IHH signalling [95]. This is in contrast with the results found in *Drosophila*, where in *ttv* (*EXT1*) mutants HH diffusion was impaired due to complete loss of HS chains, resulting in a shorter range of HH signalling [56,87,88].

In osteochondroma, a different effect of possible disrupted HSPG synthesis due to loss of *EXT* gene function was observed. All chondrocytes in the cartilage cap of hereditary osteochondromas expressed IHH [109], in contrast to the expression restricted to the transition zone as normally seen in normal growth plate [110]. Despite the presence of IHH in osteochondroma, it was previously demonstrated that PTHLH signalling downstream of IHH is absent [111], suggesting that the IHH/PTHLH feedback loop is disrupted in osteochondroma.

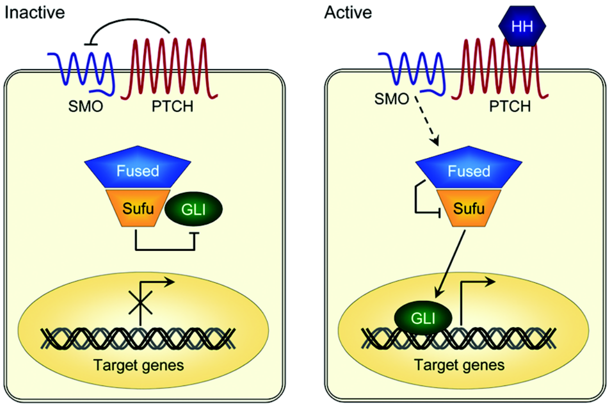

Figure 1.3 Hedgehog signalling. Left: In the absence of ligand, Hedgehog (HH) signalling is inactive. The transmembrane receptor Patched (PTCH) inhibits another transmembrane protein Smoothened (SMO). This prevents the transcription factor GLI to enter the nucleus through interactions with cytoplasmic proteins, including Fused and Suppressor of fused (Sufu). Right: HH signalling is initiated upon binding of a ligand, e.g. IHH, to PTCH. This results in the release of SMO by PTCH, thereby activating a cascade that leads to the translocation of GLI to the nucleus where it activates transcription of target genes. These genes include *PTCH* and *GLI1* itself. Adapted from Pasca di Magliano et al [99].

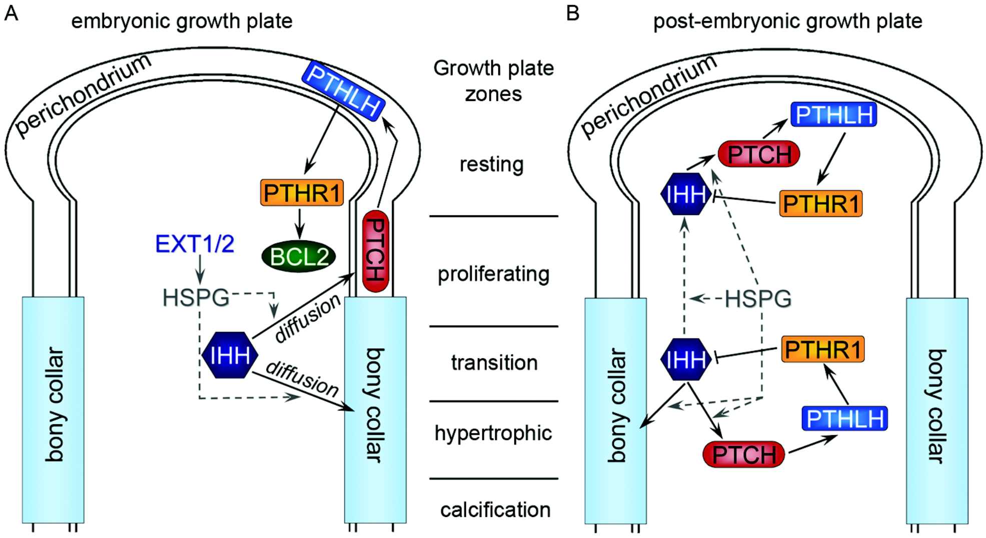

Figure 1.4 Indian Hedgehog signalling in the epiphyseal growth plate. (A) EXT1 and EXT2 are expressed in the proliferative and transition zone. IHH is secreted by chondrocytes in the transition zone and diffuses to PTCH in the lateral perichondrium, presumably coordinated by HSPGs. Upon binding of IHH, PTCH will relieve its inhibitory effect on SMO, activating GLI2, the GLI family member that transduces the IHH signal during endochondral bone development [102]. The subsequently induced expression of PTHLH at the apical perichondrium, diffuses to its receptor (PTHR1) expressed in the late proliferating chondrocytes [55], stimulating proliferation and inhibiting terminal differentiation via upregulation of BCL2. This reduces the number of IHH secreting chondrocytes, thereby closing the feedback loop [55,103]. In this feedback loop progression of chondrocyte differentiation towards the hypertrophic zone is delayed by PTHLH and BCL2, allowing longitudinal bone growth [103]. (B) In the rat post-natal growth plate the feedback loop is confined to the transition and hypertrophic zone. IHH expression was found in the prehypertrophic and hypertrophic chondrocytes and co-expression of PTCH and PTHLH expression in resting and hypertrophic chondrocytes, suggesting the existence of two growth restraining feedback loops in the post-natal growth plate [104]

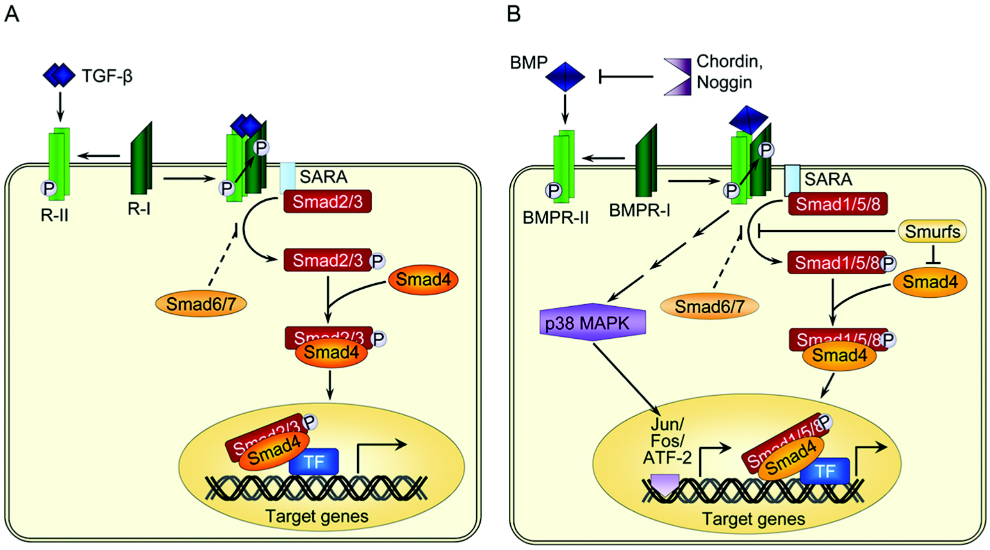

Figure 1.5 TGF-β and BMP signalling. (A) The canonical TGF-β/Smad signalling pathway. TGF-β binds to and stabilizes heteromeric complexes of type I (R-I) and type II (R-II) serine/threonine kinase receptors. R-II has constitutively active kinase activity and phosphorylates R-I on specific serine and threonine residues in the juxtamembrane region. Upon this activation, R-I propagates the signal in the cytoplasm by phoshorylating the receptor Smad2 or Smad3 at two serine residues at the C-terminus. Smad2/3 can be recruited to the activated R-I trough auxiliary proteins, such as Smad anchor for receptor activation (SARA). Phosphorylation of Smad2/3 can be inhibited by inhibitory Smad6 and Smad7. Activated Smad2/3 form a heteromeric complex with Smad4 and translocates to the nucleus. There, in combination with transcription factors (TF), this complex can bind to promoters of target genes. Adapted from ten Dijke et al. [120]. (B) The BMP signalling pathway. BMPs bind to BMP receptor type I (BMPR-I)/type II (BMPR-II) heteromeric receptor complexes, after which BMPR-II phosphorylates BMPR-I in the juxtamembrane region. Upon this activation, Smad1, Smad5 or Smad8 are activated by BMPR-I via phoshorylating. Activated Smad1/5/8 form a heteromeric complex with Smad4 and translocates to the nucleus. There, in combination with transcription factors (TF), this complex can bind to promoters of target genes. BMP signalling is inhibited by BMP antagonists (e.g. Chordin, Noggin), which prevent binding of BMPs to the receptors or by Smad6/7, thereby blocking intracellular signalling by Smads. Adapted from ten Dijke et al [121]. BMP signalling can also activate transcription of target genes independent of Smad, via the p38 MAPK pathway using the transcription factors Jun/Fos and ATF-2. Adapted from Nohe et al. [122].

Upregulation of PTHLH and BCL2 characterized malignant transformation towards secondary peripheral chondrosarcoma [111]. However for central chondrosarcomas, upregulation of BCL2 was only seen in high-grade tumours [111,112].

Three other groups investigated the protein expression of PTHLH in cartilaginous tumours [113-115], all showing that chondrosarcomas expressed PTHLH, which increased with increasing histological grade. The study of Amling et al. also demonstrated that only high-grade chondrosarcomas expressed BCL2 protein [113], which is in concordance with the results found in the central chondrosarcomas [111,112], but not with the results of peripheral chondrosarcoma [111]. All three studies were conducted before it became clear that central and peripheral chondrosarcomas genetically are two separate tumour types [116]. Since central chondrosarcomas are far more frequent than peripheral chondrosarcomas [11], most of the tumours from the study by Amling et al. were most likely to be central chondrosarcomas [113].

Recently, active HH signalling accompanied with increased proliferation, was demonstrated in both chondrosarcoma and the benign cartilaginous tumours enchondroma and chondroblastoma [117]. In both enchondroma and chondroblastoma, but also in chondromyxoid fibroma, PTHLH signalling is known to be active [112,118,119].

IV.c. BMP and TGF-β signalling

Apart from *Hh*, other morphogens have been shown to be dependent on heparan sulphate synthesis [88], including *Dpp*, the *Drosophila* orthologue of transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMPs). Members of the TGF-β superfamily regulate numerous cellular responses, like proliferation, differentiation, migration and apoptosis. Currently, 34 members of the TGF-β superfamily have been identified in the human genome, including TGF-β1-3, activins and BMPs [120].

Members of the TGF-β superfamily signal through type I and type II serine/threonine kinase receptors and subsequent intracellular signal transducers, the Smad proteins, which upon activation translocate to the nucleus and promote transcription of target genes (figure 1.5A, reviewed by ten Dijke and Heldin [120]). The so-called receptor-regulated Smads have chondrosarcomas and increased with increasing histological grade, whereas expression was absent or low in benign tumours [131]. Expression of the TGF-β receptors was restricted to chondrosarcomas.

The expression of BMP signalling molecules has been mostly investigated in large immunohistochemical studies on a non-selected series of bone sarcomas, showing that conventional chondrosarcomas did not express BMP2, BMP4 and BMP6 and BMP receptor II, in contrast to dedifferentiated chondrosarcomas that expressed all four proteins [132,133]. In the cartilage cap of three osteochondromas BMP2 and BMP4, as well as BMP receptor IB, were detected [134].

IV.d. WNT signalling

A third growth signalling pathway important for skeletogenesis for which it was shown that HPSGs are required, is WNT signalling. HSPGs facilitate the diffusion of *wingless* (*Wg*, the *Drosophila* orthologue of WNT) during *Drosophila* wing-development [88]. To date, 19 *WNT* genes in the vertebrate genome and 7 *Wg* genes in *Drosophila* have been identified, which participate in three distinguished types of WNT signalling, the classical canonical pathway, the JNK (planar polarity) pathway and the WNT/Ca^{2+} pathway [135]. The canonical pathway signals via β-catenin (figure 1.6), which is stabilized and accumulated in the cytoplasm of WNT-activated cells and is translocated to the nucleus. There it acts as activator in a transcription factor complex together with a member of the LEF1/TCF transcription factor family to activate transcription of WNT target genes [136].

The canonical WNT signalling acts at different levels during skeletogenic differentiation. It inhibits chondrocyte differentiation from osteochondro-progenitor cells in favour of osteoblast development [137] (figure 1.7). However, nuclear β-catenin expression has been found in hypertrophic chondrocytes [139], suggesting a role for WNT signalling in terminal hypertrophic chondrocyte maturation.

The expression of several WNTs and WNT signalling components and their putative functions have been studied during skeletal development (reviewed by Church et al. [140]).

Their different spatial and temporal expression patterns suggest that distinct WNT family members have specific functions during chondrogenesis, bone formation and joint development.

When β-catenin is in its cadherin-bound form a the cell membrane, it regulates cell-cell adhesion [136]. No membranous expression of β-catenin was found in eight chondrosarcoma[141]. WNT signalling, both canonical and non-canonical, has not been investigated in cartilaginous tumours.

V. Aims of the study and outline of the thesis

In the past decade our knowledge on cartilaginous tumours has increased. The identification of *EXT*-genes as causative genes for Multiple Osteochondromas has contributed to the molecular background of peripheral cartilaginous tumours. The distinction recently between peripheral and central chondrosarcomas based upon clinocoradiological as well as tumour genetic differences was another major finding contributing to the tumorigenesis of cartilaginous tumours [116]. Based upon the genetic and protein studies performed thus far a multi-step genetic model for peripheral cartilaginous tumorigenesis was introduced (figure 2.6). However, it is still unclear whether similar or different molecular mechanisms and signal transduction pathways underlie the development of solitary versus hereditary osteochondroma. We first need to assess this in order to conduct experiments in relatively large series of osteochondromas and peripheral chondrosarcomas. If in solitary osteochondromas *EXT* genes are also inactivated, this enables us to combine both hereditary and solitary tumours, allowing the formation of larger study groups for better statistical power.

A clinically important issue is that most secondary peripheral chondrosarcomas are well-differentiated low-grade tumours and it can be difficult to distinguish them from benign osteochondroma. Our studies aim at elucidating the molecular processes involved in malignant transformation of osteochondroma to chondrosarcoma. This could lead to the identification of possible biological markers that differentiate benign from low-grade malignant tumours and enable the development of diagnostic tools.

These two issues were investigated in a well-documented series using

1) a hypothesis-driven approach, to study the role of *EXT* genes, HSPGs and the IHH/PTHLH growth signalling pathway. Since IHH/PTHLH signalling depends on HPSGs, *EXT* inactivation could affect this pathway.
2) a genome-wide approach using cDNA microarray analysis to identify possible other genes and pathways involved.

Chapter 2 is a detailed review, introducing the hereditary syndrome Multiple Osteochondromas. It summarizes the most important clinical and histological aspects of the disorder and the tumours but also elaborates on the genes and growth signalling pathways involved. Finally, suggestions for patient management focusing on the establishment of the diagnosis Multiple Osteochondromas and proposed screening methods are presented.

Multiple Osteochondroma patients harbour germ line mutations in the *EXT* genes and loss of the wild type allele has been demonstrated in the cartilage cap of hereditary osteochondroma[35].

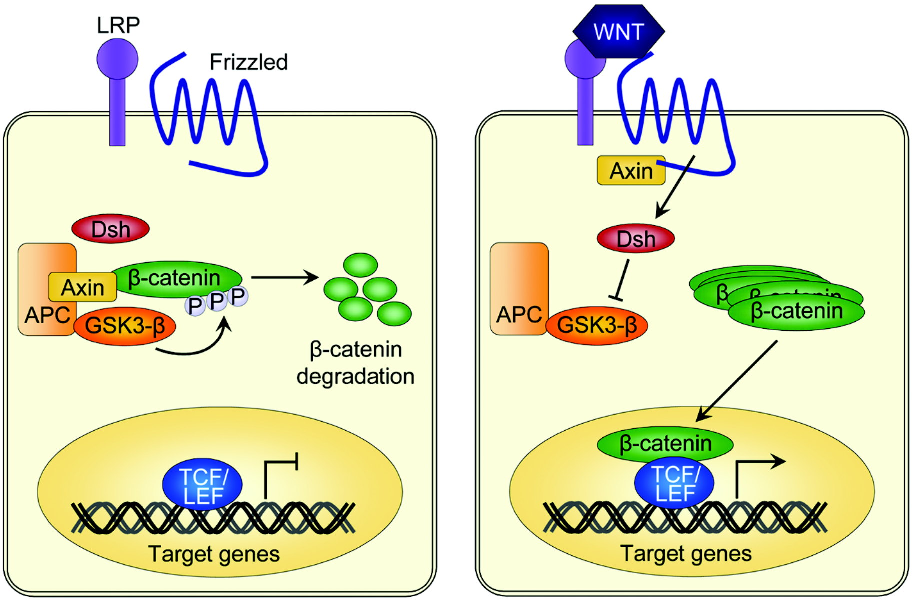

Figure 1.6 The canonical WNT signalling pathway. In the absence of WNT ligand (left), β-catenin is in a complex with Axin, APC and GSK3-β, and is phosphorylated and targeted for degradation. Upon binding of a WNT ligand to the Frizzled receptor (right), β-catenin is uncoupled from the degradation complex, accumulates in the cytoplasm and translocates to the nucleus, where it binds TCF/LEF transcription factors to activate transcription of target genes. Adapted from Reya and Clevers [136].

Mutational inactivation of *EXT* genes in sporadic osteochondromas is very rare, but LOH of 8q24, including the *EXT1* locus, is frequently found. In **chapter 3** the role of *EXT1* as possible tumour suppressor gene for sporadic (non-hereditary) osteochondroma is investigated to asses whether inactivation of both alleles of *EXT1* is necessary in sporadic osteochondromas. For this study we used array-based comparative genomic hybridization (array-CGH) analysis using a chromosome 8q tile BAC-array, multiplex ligation-dependent probe amplification (MLPA), locus specific fluorescent *in situ* hybridization (FISH) and mutation analysis by direct sequencing.

The EXT proteins are involved in the biosynthesis of HSPG. To investigate the influence of the mutational inactivation of *EXT* genes on the HSPG biosynthesis, **Chapter 4** describes the expression of the *EXT* genes at the mRNA level and protein expression of the HS chains and HSPG core proteins in a large series of osteochondromas and secondary peripheral chondrosarcomas. The results of the tumours are compared with a series of normal epiphyseal growth plates.

PTHLH signalling is absent in osteochondromas and re-expressed in secondary peripheral chondrosarcomas. This suggested that IHH signalling is also disturbed in osteochondromas, due to the loss of *EXT* genes and that PTHLH signalling is regulated in an autocrine fashion or perhaps by other signalling pathways. **Chapter 5** approaches these hypotheses. First, the expression of IHH signalling molecules was investigated by quantitative RT-PCR. Second, a genome-wide approach was used to identify other signalling pathways involved in osteochondroma and chondrosarcoma development.

The distinction between osteochondroma and grade I secondary peripheral chondrosarcoma is considered difficult both at the radiological and the histological level. Immunohistochemical or molecular markers could be useful to improve the accuracy of the diagnosis. A previous immunohistochemical study on a pilot series of osteochondroma and secondary peripheral chondrosarcoma indicated that upregulation of PTHLH and BCL2 characterizes progression of osteochondroma towards grade I secondary peripheral chondrosarcoma [111]. **Chapter 6** describes the immunohistochemical analysis of a large nation-wide series of osteochondromas and grade I secondary peripheral chondrosarcoma to asses the diagnostic value of BCL2 and PTHLH.

Dysplasia epiphysealis hemimelica and metachondromatosis are two very rare skeletal disorders that are considered in the differential diagnosis of solitary and hereditary osteochondromas. In **chapter 7** lesions from these two disorders are characterized at the radiological and histological level and compared with solitary and hereditary osteochondromas. Also expression profiles of dysplasia epiphysealis hemimelica and metachondromatosis are compared with those of osteochondromas using cDNA microarray analysis, quantitative RT-PCR and immunohistochemistry.

In **chapter 8** all results are summarized and discussed to postulate a model for the genes and molecular pathways involved in osteochondroma formation and subsequent malignant transformation and tumour progression.

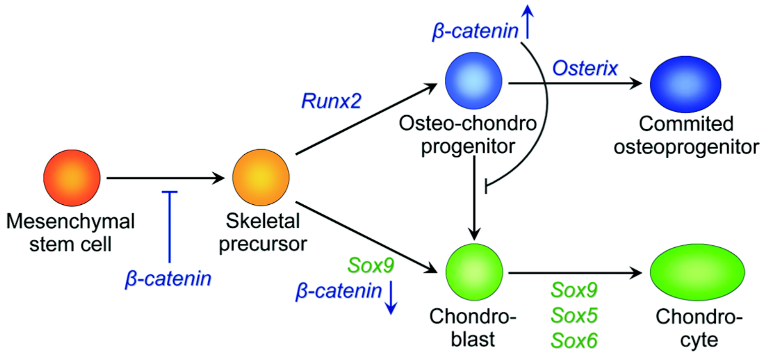

Figure 1.7 The canonical WNT signalling pathway in differentiation of skeletal progenitors. β-catenin is highly expressed in mesenchymal stem cells negatively regulates the differentiation of mesenchymal cells into a common skeletal precursor[138]. Skeletal precursor cells downregulate β-catenin and upregulated transcription factors SOX9 and, subsequently SOX5 and SOX6 to differentiate into chondrocytes. In contrast if the precursors upregulate Runx2 and elevate β-catenin levels they commit to differentiation towards osteoprogenitor cells. High levels of β-catenin are necessary to suppress the chondrogenic potential of these progenitor cells. Osterix is required for final commitment of progenitors to osteoblasts. Adapted from Hartman et al.[138].

References

1. Huvos AG. (1991) *Bone tumors. Diagnosis, treatment, and prognosis* (2 edn). W.B. Saunders Company: Philadelphia
2. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone* (2002) IARC Press: Lyon
3. Dorfman HD, Czerniak B, Kotz R, Vanel D, Park YK, Unni KK. (2002) WHO classification of tumours of bone: Introduction. In *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 226-232
4. Dorfman HD, Czerniak B. (1995) Bone cancers. *Cancer* **75**:203-210
5. Mulder JD, Schütte HE, Kroon HM, Taconis WK. (1993) *Radiologic atlas of bone tumors* (2 edn). Elsevier: Amsterdam
6. Fletcher CDM, Unni KK, Mertens F. (2002) Cartilage tumours. In *World health organization classification of tumours. Pathology and genetics. Tumours of soft tissue and bone*, Fletcher C.D.M., Unni KK, Mertens F (eds). pp. 234-257
7. Khurana J, Abdul-Karim F, Bovée JVMG. (2002) Osteochondroma. In *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 234-236
8. Lucas DR, Bridge JA. (2002) Chondromas: enchondroma,periosteal chondroma,and enchondromatosis. In *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 237-240
9. Ostrowski ML, Spjut HJ, Bridge JA. (2002) Chondromyxoid fibroma. In *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 243-245
10. Kilpatrick SE, Parisien M, Bridge JA. (2002) Chondroblastoma. In *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher C.D.M., Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 241-242
11. Bertoni F, Bacchini P, Hogendoorn PCW. (2002) Chondrosarcoma. In *World Health Organisation classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 247-251
12. Milchgrub S, Hogendoorn PCW. (2002) Dedifferentiated chondrosarcoma. In *World health organization classification of tumours. Pathology and genetics. Tumours of soft tissue and bone*, Fletcher C.D.M., Unni KK, Mertens F (eds). pp. 252-254
13. Nakashima Y, Park YK, Sugano O. (2002) Mesenchymal chondrosarcoma. In *World health organization classification of tumours. Pathology and genetics. Tumours of soft tissue and bone*, Fletcher C.D.M., Unni KK, Mertens F (eds). pp. 255-256
14. McCarthy EF, Freemont A, Hogendoorn PCW. (2002) Clear cell chondrosarcoma. In *World health organization classification of tumours. Pathology and genetics. Tumours of soft tissue and bone*, Fletcher C.D.M., Unni KK, Mertens F (eds). pp. 257-258
15. Bovée JVMG, Hogendoorn PCW. (2002) Multiple osteochondromas. In *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 360-362
16. Mertens F, Unni KK. (2002) Enchondromatosis: Ollier disease and Maffucci syndrome. In *World Health Organization Classification of Tumours. Pathology and genetics of tumours of soft tissue and bone*, Fletcher CDM, Unni KK, Mertens F (eds). IARC Press: Lyon. pp. 356-357
17. Schorr S, Legum C, Ochshorn M. (1976) Spondyloenchondrodysplasia. Enchondromatomosis with severe platyspondyly in two brothers. *Radiology* **118**:133-139

18. Menger H, Kruse K, Spranger J. (1989) Spondyloenchondrodysplasia. *J Med Genet* **26**:93-99
19. Kaibara N, Mitsuyasu M, Katsuki I, Hotokebuchi T, Takagishi K. (1982) Generalized enchondromatosis with unusual complications of soft tissue calcifications and hemangiomas. Follow-up for over a twelve-year period. *Skeletal Radiol* **8**:43-46
20. Halal F, Azouz EM. (1991) Generalized enchondromatosis in a boy with only platyspondyly in the father. *Am J Med Genet* **38**:588-592
21. Murphey MD, Choi JJ, Kransdorf MJ, Flemming DJ, Gannon FH. (2000) Imaging of osteochondroma: variants and complications with radiologic-pathologic correlation. *RadioGraphics* **20**:1407-1434
22. Silverman FN. (1989) Dysplasia epiphysealis hemimelica. *Semin Roentgenol* **24**:246-258
23. Bassett GS, Cowell HR. (1985) Metachondromatosis. Report of four cases. *J Bone Joint Surg Am* **67**:811-814
24. Kennedy LA. (1983) Metachondromatosis. *Radiology* **148**:117-118
25. Cooper A. (1818) Exostosis. In *Surgical Essays*, (3 edn), Cooper A, Travers B (eds). Cox&Son: London. pp. 169-226
26. Evans HL, Ayala AG, Romsdahl MM. (1977) Prognostic factors in chondrosarcoma of bone. A clinicopathologic analysis with emphasis on histologic grading. *Cancer* **40**:818-831
27. Bjornsson J, McLeod RA, Unni KK, Ilstrup DM, Pritchard DJ. (1998) Primary chondrosarcoma of long bones and limb girdles. *Cancer* **83**:2105-2119
28. Geirnaerdt MJA, Hogendoorn PCW, Bloem JL, Taminiau AHM, Van der Woude HJ. (2000) Cartilaginous tumors: Fast contrast-enhanced MR imaging of cartilaginous tumors. *Radiology* **214**:539-546
29. Wicklund LC, Pauli RM, Johnston D, Hecht JT. (1995) Natural history study of hereditary multiple exostoses. *Am J Med Genet* **55**:43-46
30. Ahn J, Ludecke H-J, Lindow S, Horton WA, Lee B, Wagner MJ, Horsthemke B, Wells DE. (1995) Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (EXT1). *Nature Genet* **11**:137-143
31. Wuyts W, Van Hul W, Wauters J, Nemtsova M, Reyniers E, Van Hul E, De Boulle K, De Vries BBA, Hendrickx J, Herrygers I, *et al.* (1996) Positional cloning of a gene involved in hereditary multiple exostoses. *Hum Mol Genet* **5**:1547-1557
32. Stickens D, Clines G, Burbee D, Ramos P, Thomas S, Hogue D, Hecht JT, Lovett M, Evans GA. (1996) The EXT2 multiple exostoses gene defines a family of putative tumour suppressor genes. *Nature Genet* **14**:25-32
33. Zak BM, Crawford BE, Esko JD. (2002) Hereditary multiple exostoses and heparan sulfate polymerization. *Biochim Biophys Acta* **1573**:346-355
34. Cheung PK, McCormick C, Crawford BE, Esko JD, Tufaro F, Duncan G. (2001) Etiological point mutations in the hereditary multiple exostoses gene EXT1: a functional analysis of heparan sulfate polymerase activity. *Am J Hum Genet* **69**:55-66
35. Bovée JVMG, Cleton-Jansen AM, Wuyts W, Caethoven G, Taminiau AHM, Bakker E, Van Hul W, Cornelisse CJ, Hogendoorn PCW. (1999) EXT-mutation analysis and loss of heterozygosity in sporadic and hereditary osteochondromas and secondary chondrosarcomas. *Am J Hum Genet* **65**:689-698
36. Knudson AG, Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* **68**:820-823
37. Hall CR, Cole WG, Haynes R, Hecht JT. (2002) Reevaluation of a genetic model for the development of exostosis in hereditary multiple exostosis. *Am J Med Genet* **112**:1-5

38. Bernard MA, Hogue DA, Cole WG, Sanford T, Snuggs MB, Montufar-Solis D, Duke PJ, Carson DD, Scott A, Van Winkle WB, *et al*. (2000) Cytoskeletal abnormalities in chondrocytes with EXT1 and EXT2 mutations. *J Bone Miner Res* **15**:442-450
39. Legeai-Mallet L, Rossi A, Benoist-Lasselin C, Piazza R, Mallet JF, Delezoide AL, Munnich A, Bonaventure J, Zylberberg L. (2000) EXT 1 gene mutation induces chondrocyte cytoskeletal abnormalities and defective collagen expression in the exostoses. *J Bone Miner Res* **15**:1489-1500
40. Hecht JT, Hall CR, Snuggs M, Hayes E, Haynes R, Cole WG. (2002) Heparan sulfate abnormalities in exostosis growth plates. *Bone* **31**:199-204
41. Bernard MA, Hall CE, Hogue DA, Cole WG, Scott A, Snuggs MB, Clines GA, Ludecke HJ, Lovett M, Van Winkle WB, *et al*. (2001) Diminished levels of the putative tumor suppressor proteins EXT1 and EXT2 in exostosis chondrocytes. *Cell Motil Cytoskeleton* **48**:149-162
42. Hecht JT, Hogue D, Wang Y, Blanton SH, Wagner M, Strong LC, Raskind W, Hansen MF, Wells D. (1997) Hereditary multiple exostoses (EXT): mutational studies of familial EXT1 cases and EXT-associated malignancies. *Am J Hum Genet* **60**:80-86
43. Mertens F, Rydholm A, Kreicbergs A, Willen H, Jonsson K, Heim S, Mitelman F, Mandahl N. (1994) Loss of chromosome band 8q24 in sporadic osteocartilaginous exostoses. *Genes Chromosomes Cancer* **9**:8-12
44. Bridge JA, Nelson M, Orndal C, Bhatia P, Neff JR. (1998) Clonal karyotypic abnormalities of the hereditary multiple exostoses chromosomal loci 8q24.1 (EXT1) and 11p11-12 (EXT2) in patients with sporadic and hereditary osteochondromas. *Cancer* **82**:1657-1663
45. Schmale GA, Conrad EU, Raskind WH. (1994) The natural history of hereditary multiple exostoses. *J Bone Joint Surg [Am]* **76A**:986-992
46. Porter DE, Emerton ME, Villanueva-Lopez F, Simpson AHRW. (2000) Clinical and radiographic analysis of osteochondromas and growth disturbance in hereditary multiple exostoses. *J Pediatr Orthop* **20**:246-250
47. Ollier M. (1899) De la dyschondroplasia. *Bull Soc Chir Lyon* **3**:22-23
48. Maffucci A. (1881) Di un caso encondroma ed angioma multiplo. *Movimento medico-chirurgico, Napoli* **3**:399-412; 565-575
49. Cancedda R, Descalzi CF, Castagnola P. (1995) Chondrocyte differentiation. *Int Rev Cytol* **159**:265-358
50. Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R. (1995) Toward a molecular understanding of skeletal development. *Cell* **80**:371-378
51. Hogendoorn PCW, Bovée JVMG, Karperien M, Cleton-Jansen AM. (2003) Skeletogenesis: Genetics. In *Nature Encyclopedia of the Human Genome*, Cooper DN (ed). Nature Publishing Group: London. pp. 306-313
52. van der Eerden BC, Karperien M, Wit JM. (2003) Systemic and local regulation of the growth plate. *Endocr Rev* **24**:782-801
53. Hunziker EB. (1994) Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microsc Res Tech* **28**:505-519
54. Van der Eerden BCJ, Karperien M, Wit JM. (2001) The estrogen receptor in the growth plate: implications for pubertal growth. *J Pediatr Endocrinol Metab* **14** (Suppl 6):1527-1533
55. Kronenberg HM. (2003) Developmental regulation of the growth plate. *Nature* **423**:332-336
56. Bellaiche Y, The I, Perrimon N. (1998) Tout-velu is a drosophila homologue of the putative tumour suppressor EXT1 and is needed for Hh diffusion. *Nature* **394**:85-88
57. Clines GA, Ashley JA, Shah S, Lovett M. (1997) The structure of the human multiple exostoses 2 gene and characterization of homologs in mouse and caenorhabditis elegans. *Genome Res* **7**:359-367

58. Lohmann DR, Buiting K, Ludecke H-J, Horsthemke B. (1997) The murine Ext1 gene shows a high level of sequence similarity with its human homologue and is part of a conserved linkage group on chromosome 15. *Cytogenet Cell Genet* **76**:164-166
59. McCormick C, Leduc Y, Martindale D, Mattison K, Esford LE, Dyer AP, Tufaro F. (1998) The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nature Genet* **19**:158-161
60. Stickens D, Evans GA. (1997) Isolation and characterization of the murine homolog of the human EXT2 multiple exostoses gene. *Biochem Mol Med* **61**:16-21
61. Han C, Belenkaya TY, Khodoun M, Tauchi M, Lin X, Lin X. (2004) Distinct and collaborative roles of Drosophila EXT family proteins in morphogen signalling and gradient formation. *Development* **131**:1563-1575
62. Lee JS, von der HS, Rusch MA, Stringer SE, Stickney HL, Talbot WS, Geisler R, Nusslein-Volhard C, Selleck SB, Chien CB, *et al*. (2004) Axon sorting in the optic tract requires HSPG synthesis by ext2 (dackel) and extl3 (boxer). *Neuron* **44**:947-960
63. Lind T, Tufaro F, McCormick C, Lindahl U, Lidholt K. (1998) The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *J Biol Chem* **273**:26265-26268
64. McCormick C, Duncan G, Goutsos KT, Tufaro F. (2000) The putative tumor suppressors EXT1 and EXT2 form a stable complex that accumulates in the golgi apparatus and catalyzes the synthesis of heparan sulfate. *Proc Natl Acad Sci USA* **97**:668-673
65. Wise CA, Clines GA, Massa H, Trask BJ, Lovett M. (1997) Identification and localization of the gene for EXTL, a third member of the multiple exostoses gene family. *Genome Res* **7**:10-16
66. Wuyts W, Van Hul W, Hendrickx J, Speleman F, Wauters J, De Boulle K, Van Roy N, Van Agtmael T, Bossuyt P, Willems PJ. (1997) Identification and characterization of a novel member of the EXT gene family, EXTL2. *Eur J Hum Genet* **5**:382-389
67. Van Hul W, Wuyts W, Hendrickx J, Speleman F, Wauters J, De Boulle K, Van Roy N, Bossuyt P, Willems P. (1998) Identification of a third EXT-like gene (EXTL3) belonging to the EXT gene family. *Genomics* **47**:230-237
68. Saito T, Seki N, Yamauchi M, Tsuji S, Hayashi A, Kozuma S, Hori T. (1998) Structure, chromosomal location, and expression profile of EXTR1 and EXTR2, new members of the multiple exostoses gene family. *Biochem Biophys Res Commun* **243**:61-66
69. Kitagawa H, Shimakawa H, Sugahara K. (1999) The tumor suppressor EXT-like gene EXTL2 encodes an alpha1, 4-N-acetylhexosaminyltransferase that transfers N-acetylglucosamine to the common glycosaminoglycan-protein linkage region. *J Biol Chem* **274**:13933-13937
70. Arai T, Akiyama Y, Nagasaki H, Murase N, Okabe S, Ikeuchi T, Saito K, Iwai T, Yuasa Y. (1999) EXTL3/EXTR1 alterations in colorectal cancer cell lines. *Int J Oncol* **15**:915-919
71. Esko JD, Selleck SB. (2002) Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem* **71**:435-471
72. Knudson CB, Knudson W. (2001) Cartilage proteoglycans. *Semin Cell Dev Biol* **12**:69-78
73. Kim BT, Kitagawa H, Tamura J, Saito T, Kusche-Gullberg M, Lindahl U, Sugahara K. (2001) Human tumor suppressor EXT gene family members EXTL1 and EXTL3 encode alpha 1,4- N-acetylglucosaminyltransferases that likely are involved in heparan sulfate/ heparin biosynthesis. *Proc Natl Acad Sci U S A* **98**:7176-7181
74. McCormick C, Duncan G, Tufaro F. (1999) New perspectives on the molecular basis of hereditary bone tumours. *Mol Med Today* **5**:481-486
75. Rubin JB, Choi Y, Segal RA. (2002) Cerebellar proteoglycans regulate sonic hedgehog responses during development. *Development* **129**:2223-2232

76. Cardin AD, Weintraub HJ. (1989) Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* **9**:21-32
77. Kato M, Wang H, Bernfield M, Gallagher JT, Turnbull JE. (1994) Cell surface syndecan-1 on distinct cell types differs in fine structure and ligand binding of its heparan sulfate chains. *J Biol Chem* **269**:18881-18890
78. Selleck SB. (2000) Proteoglycans and pattern formation. Sugar biochemistry meets developmental genetics. *T I G* **16**:206-212
79. Stickens D, Brown D, Evans GA. (2000) EXT genes are differentially expressed in bone and cartilage during mouse embryogenesis. *Dev Dyn* **218**:452-464
80. David G, Bai XM, Van der Schueren B, Marynen P, Cassiman JJ, Van den Berghe H. (1993) Spatial and temporal changes in the expression of fibroglycan (syndecan-2) during mouse embryonic development. *Development* **119**:841-854
81. Zimmermann P, David G. (1999) The syndecans, tuners of transmembrane signaling. *FASEB J* **13** (Suppl):S91-S100
82. Seghatoleslami MR, Kosher RA. (1996) Inhibition of in vitro limb cartilage differentiation by syndecan-3 antibodies. *Dev Dyn* **207**:114-119
83. Shimo T, Gentili C, Iwamoto M, Wu C, Koyama E, Pacifici M. (2004) Indian hedgehog and syndecans-3 coregulate chondrocyte proliferation and function during chick limb skeletogenesis. *Dev Dyn* **229**:607-617
84. Veugelers M, De Cat B, Ceulemans H, Bruystens AM, Coomans C, Durr J, Vermeesch J, Marynen P, David G. (1999) Glypican-6, a new member of the glypican family of cell surface heparan sulfate proteoglycans. *J Biol Chem* **274**:26968-26977
85. SundarRaj N, Fite D, Ledbetter S, Chakravarti S, Hassell JR. (1995) Perlecan is a component of cartilage matrix and promotes chondrocyte attachment. *J Cell Sci* **108**:2663-2672
86. Hecht JT, Hayes E, Haynes R, Cole WG, Long RJ, Farach-Carson MC, Carson DD. (2005) Differentiation-induced loss of heparan sulfate in human exostosis derived chondrocytes. *Differentiation* **73**:212-221
87. The I, Bellaiche Y, Perrimon N. (1999) Hedgehog movement is regulated through tout velu - dependant synthesis of a heparan sulfate proteoglycan. *Mol Cell* **4**:633-639
88. Takei Y, Ozawa Y, Sato M, Watanabe A, Tabata T. (2004) Three Drosophila EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development* **131**:73-82
89. Bornemann DJ, Duncan JE, Staatz W, Selleck S, Warrior R. (2004) Abrogation of heparan sulfate synthesis in Drosophila disrupts the Wingless, Hedgehog and Decapentaplegic signaling pathways. *Development* **131**:1927-1938
90. Schilling TF, Piotrowski T, Grandel H, Brand M, Heisenberg CP, Jiang YJ, Beuchle D, Hammerschmidt M, Kane DA, Mullins MC, *et al.* (1996) Jaw and branchial arch mutants in zebrafish I: branchial arches. *Development* **123**:329-344
91. Franks DM, Izumikawa T, Kitagawa H, Sugahara K, Okkema PG. (2006) C. elegans pharyngeal morphogenesis requires both de novo synthesis of pyrimidines and synthesis of heparan sulfate proteoglycans. *Dev Biol* **296**:409-420
92. Morio H, Honda Y, Toyoda H, Nakajima M, Kurosawa H, Shirasawa T. (2003) EXT gene family member rib-2 is essential for embryonic development and heparan sulfate biosynthesis in Caenorhabditis elegans. *Biochem Biophys Res Commun* **301**:317-323
93. Lin X, Wei G, Shi Z, Dryer L, Esko JD, Wells DE, Matzuk MM. (2000) Disruption of gastrulation and heparan sulfate biosynthesis in EXT1- deficient mice. *Dev Biol* **224**:299-311
94. Hilton MJ, Gutierrez L, Martinez DA, Wells DE. (2005) EXT1 regulates chondrocyte proliferation and differentiation during endochondral bone development. *Bone* **36**:379-386

95. Koziel L, Kunath M, Kelly OG, Vortkamp A. (2004) Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. *Dev Cell* **6**:801-813
96. Stickens D, Zak BM, Rougier N, Esko JD, Werb Z. (2005) Mice deficient in Ext2 lack heparan sulfate and develop exostoses. *Development* **132**:5055-5068
97. Yamada S, Busse M, Ueno M, Kelly OG, Skarnes WC, Sugahara K, Kusche-Gullberg M. (2004) Embryonic fibroblasts with a gene trap mutation in EXT1 produce short heparan sulphate chains. *J Biol Chem* **279**:32134-32141
98. Nybakken K, Perrimon N. (2002) Hedgehog signal transduction: recent findings. *Curr Opin Genet Dev* **12**:503-511
99. Pasca dM, Hebrok M. (2003) Hedgehog signalling in cancer formation and maintenance. *Nat Rev Cancer* **3**:903-911
100. Mullor JL, Sanchez P, Altaba AR. (2002) Pathways and consequences: Hedgehog signaling in human disease. *Trends Cell Biol* **12**:562-569
101. Ingham PW. (1998) Transducing hedgehog: the story so far. *EMBO J* **17**:3505-3511
102. Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Cheng SH, *et al*. (1997) Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* **124**:113-123
103. Amling M, Neff L, Tanaka S, Inoue D, Kuida K, Weir E, Philbrick WM, Broadus AE, Baron R. (1997) Bcl-2 lies downstream of parathyroid hormone related peptide in a signalling pathway that regulates chondrocyte maturation during skeletal development. *J Cell Biol* **136**:205-213
104. Van der Eerden BCJ, Karperien M, Gevers EF, Lowik CWGM, Wit JM. (2000) Expression of Indian Hedgehog, PTHrP and their receptors in the postnatal growth plate of the rat: evidence for a locally acting growth restraining feedback loop after birth. *J Bone Miner Res* **15**:1045-1055
105. Koziel L, Wuelling M, Schneider S, Vortkamp A. (2005) Gli3 acts as a repressor downstream of Ihh in regulating two distinct steps of chondrocyte differentiation. *Development* **132**:5249-5260
106. Hilton MJ, Tu X, Cook J, Hu H, Long F. (2005) Ihh controls cartilage development by antagonizing Gli3, but requires additional effectors to regulate osteoblast and vascular development. *Development* **132**:4339-4351
107. Nakase T, Miyaji T, Kuriyama K, Tamai N, Horiki M, Tomita T, Myoui A, Shimada K, Yoshikawa H. (2001) Immunohistochemical detection of parathyroid hormone-related peptide, Indian hedgehog, and patched in the process of endochondral ossification in the human. *Histochem Cell Biol* **116**:277-284
108. Kindblom JM, Nilsson O, Hurme T, Ohlsson C, Savendahl L. (2002) Expression and localization of Indian hedgehog (Ihh) and parathyroid hormone related protein (PTHrP) in the human growth plate during pubertal development. *J Endocrinol* **174**:R1-R6
109. Benoist-Lasselin C, de Margerie E, Gibbs L, Cormier S, Silve C, Nicolas G, Lemerrer M, Mallet JF, Munnich A, Bonaventure J, *et al*. (2006) Defective chondrocyte proliferation and differentiation in osteochondromas of MHE patients. *Bone* **39**:17-26
110. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. (1996) Regulation of rate of cartilage differentiation by indian hedgehog and PTH-related protein. *Science* **273**:613-622
111. Bovée JVMG, Van den Broek LJCM, Cleton-Jansen AM, Hogendoorn PCW. (2000) Up-regulation of PTHrP and Bcl-2 expression characterizes the progression of osteochondroma towards peripheral chondrosarcoma and is a late event in central chondrosarcoma. *Lab Invest* **80**:1925-1933
112. Rozeman LB, Hameetman L, Cleton-Jansen AM, Taminiau AHM, Hogendoorn PCW, Bovée JVMG. (2005) Absence of IHH and retention of PTHrP signalling in enchondromas and central chondrosarcomas. *J Pathol* **205**:476-482

113. Amling M, Posl M, Hentz MW, Priemel M, Delling G. (1998) PTHrP and Bcl-2: essential regulatory molecules in chondrocyte differentiation and chondrogenic tumors. *Verh Dtsch Ges Path* **82**:160-169
114. Kunisada T, Moseley JM, Slavin JL, Martin TJ, Choong PF. (2002) Co-expression of parathyroid hormone-related protein (PTHrP) and PTH/PTHrP receptor in cartilaginous tumours: a marker for malignancy? *Pathology* **34**:133-137
115. Pateder DB, Gish MW, O'Keefe RJ, Hicks DG, Teot LA, Rosier RN. (2002) Parathyroid hormone-related Peptide expression in cartilaginous tumors. *Clin Orthop*:198-204
116. Bovée JVMG, Cleton-Jansen AM, Kuipers-Dijkshoorn N, Van den Broek LJCM, Taminiau AHM, Cornelisse CJ, Hogendoorn PCW. (1999) Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in the origin of peripheral and central chondrosarcoma. *Genes Chrom Cancer* **26**:237-246
117. Tiet TD, Hopyan S, Nadesan P, Gokgoz N, Poon R, Lin AC, Yan T, Andrulis IL, Alman BA, Wunder JS. (2006) Constitutive hedgehog signaling in chondrosarcoma up-regulates tumor cell proliferation. *Am J Pathol* **168**:321-330
118. Romeo S, Bovée JVMG, Jadnanansing NAA, Taminiau AHM, Hogendoorn PCW. (2004) Expression of cartilage growth plate signalling molecules in chondroblastoma. *J Pathol* **202**:113-120
119. Romeo S, Bovée JVMG, Grogan S, Taminiau AHM, Eilers PHC, Cleton-Jansen AM, Mainil-Varlet P, Hogendoorn PCW. (2005) Chondromyxoid fibroma resembles *in vitro* chondrogenesis, though differs in expression of signalling molecules. *J Pathol* **206**:135-142
120. ten Dijke P, Heldin CH. (2006) The Smad Family. In *Smad Signal Transduction*, ten Dijke P, Heldin CH (eds). Springer: Dordrecht, Netherlands. pp. 1-13
121. ten Dijke P, Fu J, Schaap P, Roelen BA. (2003) Signal transduction of bone morphogenetic proteins in osteoblast differentiation. *J Bone Joint Surg Am* **85-A** (Suppl 3):34-38
122. Nohe A, Keating E, Knaus P, Petersen NO. (2004) Signal transduction of bone morphogenetic protein receptors. *Cell Signal* **16**:291-299
123. Shi Y, Massague J. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**:685-700
124. Pizette S, Niswander L. (2000) BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev Biol* **219**:237-249
125. Kobayashi T, Lyons KM, McMahon AP, Kronenberg HM. (2005) BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc Natl Acad Sci U S A* **102**:18023-18027
126. Serra R, Karaplis A, Sohn P. (1999) Parathyroid hormone-related peptide (PTHrP)-dependent and -independent effects of transforming growth factor beta (TGF-beta) on endochondral bone formation. *J Cell Biol* **145**:783-794
127. Sakou T, Onishi T, Yamamoto T, Nagamine T, Sampath T, ten Dijke P. (1999) Localization of Smads, the TGF-beta family intracellular signaling components during endochondral ossification. *J Bone Miner Res* **14**:1145-1152
128. Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Reynolds PR, Rosier RN, O'Keefe RJ. (1999) BMP-6 is an autocrine stimulator of chondrocyte differentiation. *J Bone Miner Res* **14**:475-482
129. Alvarez J, Sohn P, Zeng X, Doetschman T, Robbins DJ, Serra R. (2002) TGFbeta2 mediates the effects of hedgehog on hypertrophic differentiation and PTHrP expression. *Development* **129**:1913-1924
130. Ferguson CM, Schwarz EM, Puzas JE, Zuscik MJ, Drissi H, O'Keefe RJ. (2004) Transforming growth factor-beta1 induced alteration of skeletal morphogenesis in vivo. *J Orthop Res* **22**:687-696

131. Masi L, Malentacchi C, Campanacci D, Franchi A. (2002) Transforming growth factor-beta isoform and receptor expression in chondrosarcoma of bone. *Virchows Arch* **440**:491-497
132. Guo W, Gorlick R, Ladanyi M, Meyers PA, Huvos AG, Bertino JR, Healey JH. (1999) Expression of bone morphogenetic proteins and receptors in sarcomas. *Clin Orthop*:175-183
133. Yoshikawa H, Rettig WJ, Lane JM, Takaoka K, Alderman E, Rup B, Rosen V, Healey JH, Huvos AG, Garin-Chesa P. (1994) Immunohistochemical detection of bone morphogenetic proteins in bone and soft-tissue sarcomas. *Cancer* **74**:842-847
134. Nakase T, Myoui A, Shimada K, Kuriyama K, Joyama S, Miyaji T, Tomita T, Yoshikawa H. (2001) Involvement of BMP-2 signaling in a cartilage cap in osteochondroma. *J Orthop Res* **19**:1085-1088
135. Montcouquiol M, Crenshaw EB, III, Kelley MW. (2006) Noncanonical Wnt signaling and neural polarity. *Annu Rev Neurosci* **29**:363-386
136. Reya T, Clevers H. (2005) Wnt signalling in stem cells and cancer. *Nature* **434**:843-850
137. Day TF, Guo X, Garrett-Beal L, Yang Y. (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* **8**:739-750
138. Hartmann C. (2006) A Wnt canon orchestrating osteoblastogenesis. *Trends Cell Biol* **16**:151-158
139. Tamamura Y, Otani T, Kanatani N, Koyama E, Kitagaki J, Komori T, Yamada Y, Costantini F, Wakisaka S, Pacifici M, *et al*. (2005) Developmental regulation of Wnt/beta -catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification. *J Biol Chem* **280**:19185-19195
140. Church VL, Francis-West P. (2002) Wnt signalling during limb development. *Int J Dev Biol* **46**:927-936
141. Naka T, Iwamoto Y, Shinohara N, Chuman H, Fukui M, Tsuneyoshi M. (1997) Cytokeratin subtyping in chordomas and the fetal notochord: an immunohistochemical analysis of aberrant expression. *Mod Pathol* **10**:545-551