General Introduction
Chapter 1

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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. Nevertheless, 32 different histological types of primary bone tumours (both benign and malignant) are distinguished by the World Health Organization (WHO). The most common primary malignant tumours of bone are osteosarcoma (35%), chondrosarcoma (25%), Ewing sarcoma (16%) and malignant fibrous histiocytoma (MFH; 5%) (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. 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.

<table>
<thead>
<tr>
<th>A. Benign primary bone tumours</th>
<th>Incidence (% all benign bone tumours)</th>
<th>Age at diagnosis (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteochondroma</td>
<td>&gt; 35%</td>
<td>10 - 30</td>
</tr>
<tr>
<td>Giant cell tumour</td>
<td>20%</td>
<td>20 - 45</td>
</tr>
<tr>
<td>Enchondroma</td>
<td>10-25%</td>
<td>20 - 40</td>
</tr>
<tr>
<td>Osteoid osteoma</td>
<td>10%</td>
<td>5 - 25</td>
</tr>
<tr>
<td>Aneurysmal bone cyst</td>
<td>7%</td>
<td>0 - 20</td>
</tr>
<tr>
<td>Chondromyxoid fibroma</td>
<td>2%</td>
<td>10 - 30</td>
</tr>
<tr>
<td>Osteoblastoma</td>
<td>2%</td>
<td>10 - 30</td>
</tr>
<tr>
<td>Chondroblastoma</td>
<td>1%</td>
<td>10 - 25 and</td>
</tr>
<tr>
<td>Periosteal chondroma</td>
<td>&lt;1%</td>
<td>All</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Bone sarcomas</th>
<th>Incidence (% all malignant bone sarcomas)</th>
<th>Age at diagnosis (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteosarcoma</td>
<td>35%</td>
<td>5 - 25 and</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>26%</td>
<td>30 - 70</td>
</tr>
<tr>
<td>Ewing sarcoma</td>
<td>16%</td>
<td>5 - 25</td>
</tr>
<tr>
<td>Malignant Fibrous Histiocytoma</td>
<td>6%</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>Chordoma</td>
<td>1-4%</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>2%</td>
<td>35 - 60</td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>1%</td>
<td>10 - 80</td>
</tr>
<tr>
<td>Adamantinoma</td>
<td>&lt;1%</td>
<td>5 - 85</td>
</tr>
</tbody>
</table>

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.
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 (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 performed by endochondral ossification. 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.

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%; 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. Chondrosarcomas are characterized by the production of hyaline cartilage and comprise a
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Table I.II. Cartilaginous tumours and related syndromes.

A. Benign tumours

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Incidence (% all benign bone tumours)</th>
<th>Age (yrs)</th>
<th>Sex distribution (M:F)</th>
<th>Common sites of involvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteochondroma</td>
<td>&gt; 35%</td>
<td>10 - 30</td>
<td>1:1</td>
<td>Metaphysis femur, humerus, tibia, fibula Tubular bones hand and feet, humerus, femur</td>
<td>7</td>
</tr>
<tr>
<td>Enchondroma</td>
<td>10-25%</td>
<td>20 - 40</td>
<td>1:1</td>
<td>Proximal tibia, distal femur, ilium</td>
<td>8</td>
</tr>
<tr>
<td>Chondromyxoid fibroma</td>
<td>2%</td>
<td>10 - 30</td>
<td>1.5:1</td>
<td>Epiphysis femur, tibia, humerus</td>
<td>9</td>
</tr>
<tr>
<td>Chondroblastoma</td>
<td>3%</td>
<td>10 - 25</td>
<td>2:1</td>
<td>Proximal humerus</td>
<td>10</td>
</tr>
<tr>
<td>Periosteal chondroma</td>
<td>&gt;1%</td>
<td>all</td>
<td>1:1</td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

B. Malignant tumours

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

C. Syndromes with cartilaginous tumours

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Incidence</th>
<th>Tumours</th>
<th>Sex distribution (M:F)</th>
<th>Hereditary</th>
<th>Genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple Osteochondromas</td>
<td>1: 50 000</td>
<td>Osteochondromas</td>
<td>1.5 : 1</td>
<td>Yes</td>
<td>EXT1, EXT2</td>
<td>15</td>
</tr>
<tr>
<td>Enchondromatosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ollier Disease</td>
<td>Unknown, rare</td>
<td>Enchondromas</td>
<td>1 : 1</td>
<td>No</td>
<td>Unknown</td>
<td>16</td>
</tr>
<tr>
<td>Maffucci syndrome</td>
<td>Unknown, very rare</td>
<td>Enchondromas, heamangiosmas</td>
<td>1 : 1</td>
<td>No</td>
<td>Unknown</td>
<td>16</td>
</tr>
<tr>
<td>Spondyloenchondrodysplasia</td>
<td>Unknown, very rare</td>
<td>Enchondromas</td>
<td>Unknown</td>
<td>Yes</td>
<td>Unknown</td>
<td>17,18</td>
</tr>
<tr>
<td>Generalized enchondromatosis</td>
<td>Very rare</td>
<td>Enchondromas</td>
<td>Unknown</td>
<td>Yes</td>
<td>Unknown</td>
<td>19,20</td>
</tr>
<tr>
<td>Dysplasia epiphysialis hemimelica</td>
<td>1: 1 000 000</td>
<td>Osteochondroma-like lesions Osteochondroma + enchondromas</td>
<td>1.5 : 1</td>
<td>No</td>
<td>Unknown</td>
<td>21,22</td>
</tr>
<tr>
<td>Metachondromatosis</td>
<td>Very rare</td>
<td>Osteochondroma + enchondroma</td>
<td>1 : 1</td>
<td>Yes</td>
<td>Unknown</td>
<td>23,24</td>
</tr>
</tbody>
</table>

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.

A 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 (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 radiologically28 and histologically. So far, the diagnosis is based on a combination of clinical, radiological and
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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. 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 35,41,44. No somatic EXT2 mutations have been reported
in solitary osteochondroma and LOH at the EXT2 locus has been shown only once. 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%). It is still debated whether these deformities are a result of skeletal dysplasia due to \( EXT1 \) or \( EXT2 \) haploinsufficiency, or the result of local effects on the growth plate by the developing osteochondromas.

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. Metachondromatosis is a rare autosomal dominant disorder exhibiting synchronously both multiple osteochondromas and enchondromas. Histological, radiological and molecular characteristics of both disorders are discussed in detail in chapter 7.

Table I.III. Histological grading criteria of conventional chondrosarcoma.

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

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 medullary cavity of bone, either as a primary lesion or secondary to a pre-existing benign enchondroma. 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.
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Enchondroma (central chondroma) is the benign counterpart of central chondrosarcoma. 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 Maffucci syndrome (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. 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.

The growth plate is a highly organized structure, in which different morphological zones of chondrocytes at different stages of differentiation can be distinguished (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.

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 (PTHLM)). 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. In humans, fusion of the growth plates at the end of puberty induced by oestrogen stops this process of longitudinal growth.

The process of endochondral ossification is maintained by growth factors, but is also dependent on hormonal factors, like oestrogen, as well as environment and nutrition.
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 \(^3^0\). 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 \(^3^0\). The mRNA was found to be ubiquitously expressed \(^3^0\). EXT1 seems to be highly conserved since orthologues have been identified in Drosophila melanogaster (fruitfly; tout-velu (ttv)) \(^5^6\), Caenorhabditis elegans (worm; rib-1) \(^5^7\) and mus Musculus (mouse; Ext1) \(^5^8\).

The human EXT1 mRNA encodes a 746 amino acids type II transmembrane glycosyltransferase, Exostosin-1 (EXT1) \(^5^9\). 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 \(^3^1,3^2\). 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) \(^5^7,6^0\), Drosophila melanogaster (sister of tout-velu, stv) \(^6^1\) and Danio rerio (zebrafish; dackel) \(^6^2\).

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 \(^3^3,6^3\).

In the endoplasmic 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 \(^6^4\) (see section IV.a.4). The Golgi-localized EXT1/EXT2 complex possesses substantially higher enzyme activity than EXT1 or EXT2 alone\(^6^4\).

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 \(^6^5,6^6\), 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 \(^6^9\). For EXTL3, orthologues have been identified in several other organisms, among which are brother of tout-velu (btv) in Drosophila melanogaster \(^6^1\) and boxer in Danio rerio \(^6^2\).

No linkage with Multiple Osteochondromas or other bone diseases has been documented for the EXTL-genes \(^7^0\). 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.
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**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 sequestration 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 glucuronic 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 \(^71\). 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. The expression of glypican has been demonstrated in the perichondrium, the developing limb and mesenchymal tissues of the developing mouse embryo. Perlecan, the most common proteoglycan of the basement membrane is expressed throughout the rat growth plate. 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. 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. 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. 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.

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 EXT orthologue, ttv, showed that it is required for the diffusion of Hedgehog (Hh, orthologue of mammalian IHH). 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). 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, the EXT1 heterozygous (EXT1+/-) mice did not develop osteochondromas, nor did they present any significant skeletal abnormalities. 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.

Another group was able to demonstrate that mice carrying a hypomorphic mutation in EXT1 (EXT1<sup>+<sub>AT/AT</sub></sup>) produced shorter HS chains, which increased the range of IHH signalling in a concentration dependent manner during embryonic chondrocyte differentiation.
Like *EXT1* null mice, *EXT2* null (*EXT2<sup>-/-</sup>*) mice are embryonic lethal, but *EXT2* heterozygous (*EXT2<sup>+/−</sup>*) 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. However, these bone growths still produced HS, in contrast to the osteochondromas in humans.

### Table I.IV. Animal models for Multiple Osteochondromas

<table>
<thead>
<tr>
<th>Model</th>
<th>Mutation in</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em> (fruitfly)</td>
<td><em>ttv</em> (<em>EXT1</em>)</td>
<td>Impaired HS biosynthesis, Wing imaginal disc: defective <em>Hh</em>, <em>Wg</em> and <em>Dpp</em> distribution and signaling activity</td>
<td>56,61,87-89</td>
</tr>
<tr>
<td></td>
<td><em>sobo</em> (<em>EXT2</em>)</td>
<td>Impaired HS biosynthesis, Wing imaginal disc: defective <em>Hh</em>, <em>Wg</em> and <em>Dpp</em> distribution and signaling activity, Adult wing not as affected as <em>ttv</em> and <em>sobo</em> mutants</td>
<td>61,88,89</td>
</tr>
<tr>
<td></td>
<td><em>botv</em> (<em>EXTL3</em>)</td>
<td>Impaired HS biosynthesis, Wing imaginal disc: defective <em>Hh</em>, <em>Wg</em> and <em>Dpp</em> distribution and signaling activity, Adult wing narrower in anterior/posterior orientation</td>
<td>61,88,89</td>
</tr>
<tr>
<td><em>Danio rerio</em> (zebrafish)</td>
<td><em>dackel</em> (<em>EXT2</em>)</td>
<td>All arches short and thick and strongly reduced, No pectoral fins; tail often curls up, Jaw not extended</td>
<td>62,90</td>
</tr>
<tr>
<td></td>
<td><em>boxer</em> (<em>EXTL3</em>)</td>
<td>Impaired HS synthesis, All arches short and thick and strongly reduced, No pectoral fins; tail often curls up, Jaw not extended</td>
<td>62,90</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em> (worm)</td>
<td><em>nb-1</em> (<em>EXT1</em>)</td>
<td>Embryonic lethality, Short, thick isthmus</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td><em>nb-2</em> (<em>EXTL3</em>)</td>
<td>Impaired HS synthesis, Egg laying defects, Increased body width, Reduced activity in movement, Impaired HS synthesis</td>
<td>92</td>
</tr>
<tr>
<td><em>Mus Musculus</em> (mouse)</td>
<td>General phenotypes</td>
<td>Skeletal phenotypes</td>
<td>Growth plate phenotypes</td>
</tr>
<tr>
<td><em>EXT1&lt;sup&gt;+/−&lt;/sup&gt;</em></td>
<td>No osteochondromas</td>
<td>Enlarged proliferative zone</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Mild reduction in humerus and femur bone density</td>
<td>Reduced hypertrophic zone</td>
<td>93,94</td>
</tr>
<tr>
<td><em>EXT1&lt;sup&gt;−&lt;sub&gt;−&lt;/sub&gt;&lt;/sup&gt;</em></td>
<td>Reduced size</td>
<td>Reduction of HS to near 50%</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>No osteochondromas</td>
<td>Increased range IHH diffusion</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Shortened limbs</td>
<td>Expanded proliferating zone</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Fusion elbow and knee joints</td>
<td>Severely delayed bone formation</td>
<td>95</td>
</tr>
<tr>
<td><em>EXT2&lt;sup&gt;−&lt;sub&gt;−&lt;/sub&gt;&lt;/sup&gt;</em></td>
<td>Die at E6.5</td>
<td>Reduced amounts of HS</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>No osteochondromas</td>
<td>Elevated range IHH signalling</td>
<td>96</td>
</tr>
</tbody>
</table>
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 *EXT1ΔGT/ΔGT* 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.
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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.

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. The subsequently induced expression of PTHLH at the apical perichondrium, diffuses to its receptor (PTHR1) expressed in the late proliferating chondrocytes, stimulating proliferation and inhibiting terminal differentiation via upregulation of BCL2. This reduces the number of IHH secreting chondrocytes, thereby closing the feedback loop. In this feedback loop progression of chondrocyte differentiation towards the hypertrophic zone is delayed by PTHLH and BCL2, allowing longitudinal bone growth. (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.
General Introduction

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 \(^{111,115}\), 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 HSPGs 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 \( \beta \)-catenin is in its cadherin-bound form a the cell membrane, it regulates cell-cell adhesion \(^{136}\). No membranous expression of \( \beta \)-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 assess 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. Chapter 6 describes the immunohistochemical analysis of a large nation-wide series of osteochondromas and grade I secondary peripheral chondrosarcoma to assess 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. Skeletal precursor cells downregulate β-catenin and upregulated transcription factors SOX9 and, subsequently SOX3 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.
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