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Author: Andrea, Carlos Eduardo de
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Chapter 1

General Introduction

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I. Developmental regulation of the growth plate

Skeletal development is a highly orchestrated process in which all the players involved ought to be perfectly co-ordinated and regulated in order to achieve harmonious and symmetrical growth. Most of the skeleton is formed by endochondral ossification, including the long, short, and irregular bones (1;2). In the process of endochondral ossification, mesenchymal cells differentiate into chondrocytes, which then provide a cartilage template for bone morphogenesis.

Endochondral ossification occurs at two distinct centres of ossification in the vertebrate long bone – the primary (diaphyseal) and the secondary (epiphyseal) centres (3). Bone development initiates at the primary centre. The secondary (epiphyseal) centre is ossified later. During this process, the segregation of chondrocytes at different stages of differentiation between the diaphysis and the epiphysis forms the growth plate.

The epiphyseal growth plate is regulated by a wide array of autocrine and paracrine molecules (3). Signalling through Indian hedgehog (Ihh), parathyroid hormone-related peptide (PTHrP; also known as PTHLH), bone morphogenetic proteins (BMPs), fibroblast growth factor (FGFs), and others, modulates and regulates chondrocyte proliferation and hypertrophy (3). Another important growth factor for both embryonic and post-natal development is insulin-like growth factors (IGF-1 and IGF-2). While IGF-2 is important for normal embryonic growth (4), IGF-1 appears to regulate post-natal growth (5). IGF-1 and IGF-2 are known to signal through the type I IGF receptor (IGF1R) in target tissues (6). Both circulating and locally produced IGF-1 stimulate chondrocyte proliferation in the growth plate (7).

The cartilaginous extracellular matrix produced by and surrounding the terminally differentiated hypertrophic chondrocytes is calcified. Following vascular invasion, osteogenic progenitors are recruited to this calcified area, replacing it by trabecular bone (3). Proteoglycans and collagens are the most abundant matrix components of the growth plate. Proteoglycans are localized intracellularly (usually in secretory granules), at the cell surface, or in the extracellular matrix (8). Their biological functions are highly diversified (8). Proteoglycans play an important role on various cellular processes, such as cell adhesion, motility, and proliferation, further to differentiation and tissue morphogenesis (8). Most of these effects depend on binding of signalling molecules to the glycosaminoglycan side chains. Proteoglycans influence the distribution of these signalling molecules in the extracellular matrix, their receptor binding affinity and the responses of cells to secreted protein factors (8;9).

Mutations affecting the biosynthesis of either proteoglycans or glycosaminoglycans alter the interaction between a cell and its micro-environment and are the cause of several human disorders. Several of these disorders are associated with a skeletal phenotype (10).
II. Peripheral cartilaginous bone tumours

Cartilaginous bone tumours are characterized by production of a chondroid matrix. They are classified based on their histological and radiological features, location, and clinical behaviour. Cartilaginous bone tumours can form on the surface of bones, as in sporadic or multiple osteochondromas, or within the bones, as in sporadic or multiple enchondromas. These originally benign tumours often carry significant morbidity and risk of developing chondrosarcoma.

Osteochondromas are the most common benign bone tumours of childhood and adolescence (11). They are characterized by sporadic (non-familial/solitary) or multiple (hereditary) cartilage-capped bony projections from the metaphyses of endochondral bones adjacent to the growth plate and develop during skeletal growth (12). Multiple osteochondromas, previously called hereditary multiple exostoses, is an autosomal dominant disorder with a prevalence of 1 in 18,000 (13). Patients with multiple osteochondromas are often short in stature and have bowed bones that can restrict movement and ultimately result in joint dislocation (13). In contrast, patients with sporadic lesions may develop symptoms on the affected side only. Sporadic and multiple lesions are histologically indistinguishable (12;14).

Multiple osteochondromas is characterized by genetic variability, which partially explains inter- and intra-familial phenotypic variation often found in these patients (15). The majority of the hereditary cases are caused by point mutations (70-75%). Small deletions involving single or multiple exons are found in about 10% of all hereditary cases (16-18). Large deletions have been identified in few cases (15). No genomic alterations are detected in about 10-15%. In some of these negative cases, somatic mosaicism with large genomic deletions of EXT1 and EXT2 has been described as the underlying mechanism of multiple osteochondromas formation (19). In sporadic osteochondromas, homozygous deletions of EXT1 are often identified (20).

EXT1 and EXT2 encode type II transmembrane glycosyltransferases (21;22), whose functions are not fully known. EXT1 and EXT2 form a hetero-oligomeric complex in the Golgi apparatus of most human cells that participate in chain elongation in heparan sulphate biosynthesis (23;24). During endochondral ossification, heparan sulphate regulates the range of Ihh signalling, and thus proliferation of growth plate chondrocytes (3). Albeit the genetic correlation between mutations in EXT1/EXT2 and osteochondromas, the mechanism by which alterations in heparan sulphate biosynthesis leads to osteochondroma is not entirely understood (25).
Neoplastic transformation of an osteochondroma occurs in less than 1% of patients with sporadic osteochondromas and 1-3% of patients with multiple osteochondromas (26). Neoplastic transformation usually occurs 20-60 years after the cessation of osteochondroma growth that happens at the time of the fusion of the epiphyseal growth plate at puberty (12;14).

III. Zebrafish as a model system to study human skeletal disorders
As a vertebrate, zebrafish, Danio rerio, is more closely related to humans than are yeast, worms or flies. Many features of zebrafish development have been characterized, including early embryonic patterning, development of the musculoskeletal system as well as aspects of cell fate and lineage determination. Zebrafish has several valuable features as a model organism for study of vertebrate development.

Zebrafish embryos are transparent and accessible throughout development. In live embryos, techniques for ablation and transplantation of individual cells have been used to explore questions about induction and cell fate (27). Because of their relatively short reproductive cycle, the large number of progeny that can be produced, and the relatively small space needed to maintain large numbers of offspring, zebrafish is an efficient model system for genetic analysis (28;29).

Mutations in exostosin genes, *dackel* (*dak/ext2*) and *boxer* (*box/ext3*), cause in zebrafish cartilage defects that strongly resemble those seen in patients with multiple osteochondromas (27). As zebrafish cartilaginous skeleton develops by similar mechanisms to that of humans, *dak/ext2* and *box* may be a powerful model for the study of osteochondromagenesis.

IV. Scope of the study and outline of the thesis
In the past decades, our knowledge on the epiphyseal growth plate regulation and peripheral cartilaginous tumour formation has increased significantly. Although some milestones have been achieved to date, our studies address many remaining questions on these topics:

1. Zebrafish as a model system to study human skeletal disorders.
2. Developmental regulation of the epiphyseal growth plate in relation to the formation of osteochondroma.
3. The role of *EXT1* and *EXT2* genes in the formation of a secondary peripheral chondrosarcoma.
4. Clues to the mechanisms of neoplastic transformation of osteochondroma towards secondary peripheral chondrosarcoma.
The most relevant literature on the epiphyseal growth plate and peripheral cartilaginous tumours is reviewed in Chapter 2. Chondrocytes interact with each other and with their micro-environment. These interactions are modulated by proteoglycans and other molecules and lead to the formation of a polarized tissue, such as the epiphyseal growth plate. The zebrafish (Danio rerio) exhibits fast development, a growth plate-like organization of its craniofacial skeleton and an availability of various mutants, making it a powerful model for the study of human skeletal disorders with unknown aetiology. Five zebrafish lines with known mutations in genes involved in proteoglycan synthesis were studied in Chapter 3. Each mutant displays different phenotypes related to: (a) cartilage morphology; (b) composition of the extracellular matrix; (c) ultrastructure of the extracellular matrix; and (d) the intracellular ultrastructure of chondrocytes. In addition, these zebrafish mutants might bring to light new candidate genes for human skeletal disorders.

Mutations in exostosin genes in zebrafish - dackel (dak/ext2) - cause cartilage defects that strongly resemble those seen in patients with multiple osteochondromas, and lead to a heparan sulphate deficiency (27). Heparan sulphate modulates receptor-ligand binding of many growth factors. The impact of mutations in the ext2 gene in the zebrafish craniofacial skeleton development was investigated in Chapter 4.

Several growth factors diffuse across the extracellular matrix creating short and long range signalling. The distribution of these signalling molecules in a gradient fashion is shown to be established by proteoglycans (30). Cytochemical staining with positively charged dyes (e.g., polyethyleneimine-PEI) allows visualisation of proteoglycans and provides a detailed description of how proteoglycans are distributed throughout the cartilage matrix. The distribution of proteoglycans was studied in the five zebrafish mutants described above and in the epiphyseal growth plate and osteochondroma (Chapter 5). In addition, the distribution of proteoglycans throughout the cartilage matrix might shed light on the regulation of the epiphyseal growth plate and the formation of osteochondroma.

Primary cilia are specialized cell surface projections present on most eukaryotic cells (31;32). They function as signalling apparatus of the cells that receives and transduces mechanical and chemical signals from the neighbouring cells and the extracellular matrix (33). Primary cilia have been associated with vertebrate planar cell polarity and loss of cell polarity has been hypothesised to be involved in osteochondroma formation. The link between primary cilia and cell polarity in the epiphyseal growth plate and osteochondroma was investigated in Chapter 6.
The role of *EXT1* and *EXT2* genes in secondary peripheral chondrosarcoma formation was studied in Chapter 7. The homozygous inactivation of the *EXT* genes required for osteochondromagenesis and the mixture of cells with distinct genetic background within the osteochondroma cap raised the possibility of non-involvement of the *EXT1/EXT2* genes in the genesis of peripheral chondrosarcoma. Moreover, the presence of wild-type cells in the osteochondroma cap is not just an incidental component (34) and they might play a role during neoplastic transformation of osteochondroma.

Clues to the mechanisms of neoplastic transformation of osteochondroma towards secondary peripheral chondrosarcoma may give reliable histological criteria to properly identify each tumour type. An initial step in the process of defining histological criteria for guiding the diagnosis of peripheral cartilaginous tumours is to assess diagnostic reliability among specialized bone-tumour pathologists, as measured by intraclass correlation coefficient (35). A second step is to identify common histological criteria among the concordant cases, aiming to have histological parameters that characterize each tumour type (Chapter 8).

Endochondral ossification is a multistep process in which a cartilaginous model is gradually replaced by bone (3). Like in the epiphyseal growth plate, endochondral ossification takes place deep to the cartilage cap of osteochondroma and secondary peripheral chondrosarcoma (12). Two critical steps of endochondral ossification are the deposition of collagen X-rich matrix and blood vessel attraction/invasion (3). Formation of a collagen X-rich matrix and vascularisation might be useful prognostic markers of neoplastic transformation of an osteochondroma and were studied in Chapter 9.

Finally, the achievements of the study are summarised and possible future directions of research are indicated in Chapter 10.

References

Chapter 1


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