Cell biology of osteochondromas: Bone morphogenic protein signalling and heparan sulphates

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Abstract Frequent benign outgrowths from bone known as osteochondromas, exhibiting typical endochondral ossification, are reported from single to multiple lesions. Characterised by a high incidence of osteochondromas and skeletal deformities, multiple hereditary exostoses (MHE) is the most common inherited musculoskeletal condition. While factors for severity remain unknown, mutations in exostosin 1 and exostosin 2 genes, encoding glycosyltransferases involved in the biosynthesis of ubiquitously expressed heparan sulphate (HS) chains, are associated with MHE. HS-binding bone morphogenetic proteins (BMPs) are multifunctional proteins involved in the morphogenesis of bone and cartilage. HS and HS proteoglycans are involved in BMP-mediated morphogenesis by regulating their gradient formation and activity. Mutations in exostosin genes disturb HS biosynthesis, subsequently affecting its functional role in the regulation of signalling pathways. As BMPs are the primordial morphogens for bone development, we propose the hypothesis that BMP signalling may be critical in osteochondromas. For this reason, the outcomes of exostosin mutations on HS biosynthesis and interactions within osteochondromas and MHE are reviewed. Since BMPs are HS binding proteins, the interactions of HS with the BMP signalling pathway are discussed. The impact of mouse models in the quest to better understand the cell biology of osteochondromas is discussed. Several challenges and questions still remain and further investigations are needed to explore new approaches for better understanding of the pathogenesis of osteochondromas.

Introduction

The differentiation of mesenchymal stem cells to bone can occur by one of two pathways. In the flat bones of the skull there is a direct development of bone from mesenchyme which is known as intramembranous ossification. On the other hand, in the majority of the long bones of the skeleton, there is an intervening stage of cartilage during bone morphogenesis which is referred to as endochondral bone formation [11, 29]. While neoplasias among bones are rare, it is common knowledge that bone tumours can be considered as normal bone development gone awry [3, 8, 40]. Frequent benign outgrowths known as osteochondromas are reported generally during the first two decades of human life. Osteochondromas exhibit typical endochondral ossification and are generally variable and asymptomatic. The incidence of osteochondromas declines following the closure of epiphysis during endochondral bone development. As bone morphogenetic proteins (BMPs) are the primordial morphogens for bone development, we will propose the hypothesis that BMP signalling may be critical in osteochondromas.

Multiple hereditary exostoses

Multiple hereditary exostoses (MHE) is an autosomal dominant inherited trait that is genetically heterogeneous [3, 33, 43]. It is the most common inherited musculoskeletal condition affecting 1 in 50,000 people [3, 28]. MHE is characterised by significant phenotypic variability, including variation in the size and number of osteochondromas, the number and location of bones involved and the degree of skeletal deformities. Common skeletal deformities associated with MHE include dislocation of the hand or valgus of the knee, reduced mobility in range of motion, bowed bones, limb length discrepancies and short stature [28, 41]. Skeletal deformities can lead to

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functional problems and morbidity due to the entrapment of blood vessels, nerves and tendons. The possibility of transformation into a low-grade chondrosarcoma increases to 5 % in MHE [4]. While the disease can occur spontaneously, it has been estimated that 80 % of affected individuals have a positive family history with varied penetrance among affected members [28, 34]. It has been suggested that genetic variation could account for the difference in severity of the disease [28]. Currently, the factors that determine the severity of the disease are unknown. However, loss-of-function mutations in exostosin 1 (EXT1) and exostosin 2 (EXT2) genes have been identified as the causative agents in MHE [1, 12, 28, 32, 34].

Other osteochondroma-related diseases

Metachondromatosis

Metachondromatosis (MC) is a rare autosomal dominant inherited disorder resulting in the formation of enchondromas and osteochondromas on the hands and feet [33]. In MC, osteochondroma outgrowths point toward the adjacent growth plate and predominantly occur on the digits and toes. Unlike MHE, MC lesions do not result in shortening or deformity of affected bones. In some cases MC lesions may suddenly decrease in size or resolve completely [3]. While no mutations in the EXT genes have been detected in MC, mutations in PTPN11 have been identified as the genetic aetiology of the disease [35].

Fibrodysplasia ossificans progressiva

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder resulting in progressive heterotopic ossification of skeletal muscle and soft connective tissue [7]. Dysregulation of the BMP signalling pathway is involved in the disease. FOP is known to be caused by a missense mutation in the ACVR1 gene resulting in the activation of BMP type I receptor ALK2 [7, 33]. Skeletal similarities between FOP and MHE include short broad femoral necks and metaphyseal widening. Like MHE, FOP patients have a high incidence of osteochondroma formation, especially on the proximal tibia [7]. These similarities may suggest that the two conditions may share overlapping pathophysiological mechanisms [7]. Therefore, it is sensible to suggest that the BMP signalling pathway may play a role in MHE and osteochondroma formation.

Exostosin and heparan sulfate interaction

As highly conserved tumour suppressor genes, the exostosin (EXT) gene family is ubiquitously expressed in a variety of tissues including the growth plate [12, 39]. EXT genes encode

type II transmembrane glycosyltransferases involved in the polymerisation of linear heparan sulphate (HS) chains on specific proteoglycans (PGs) [6, 25, 39]. HS chain biosynthesis is initiated by the covalent attachment of a tetrasaccharide linkage region to a specific serine residue in the core protein. EXT1 and EXT2 encode proteins with N-acetylglucosaminyltransferase II and glucuronyltransferase II activities [25]. EXT1 and EXT2 form a hetero-oligomeric glycoprotein complex localised in the Golgi apparatus where it catalyses the elongation of HS chains by the addition of alternating N-acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcAc) residues. The length of the HS chains can vary and appears to be cell specific [34]. EXT1 and EXT2 function as a complementary pair that forms a stable enzyme complex [25, 34]. Thus, functional HS synthesis requires both gene products. Mutations in either EXT1 or EXT2 genes lead to the synthesis of truncated EXT proteins, resulting in the formation of a defective EXT1/EXT2 complex that disturbs HS synthesis [1, 3, 12, 39]. Truncated EXT proteins result in reduced co-polymerase activity which leads to the production of short HS chains [6, 33]. Hence, HSPGs accumulate in the cytoplasm instead of being transported to the cell surface to be expressed [12]. In MHE, EXT1 and EXT2 mutations present the same phenotype. The deficiency in HS chain elongation is thought to be the initiating event for osteochondroma development [6]. While short HS chains can be found in all tissue types of individuals with an EXT mutation, there does not seem to be any other clinically obvious phenotype outside the skeleton [15].

BMP signalling and heparan sulfate interaction

BMPs are dimeric disulphide-linked proteins belonging to the TGF- β superfamily—which includes TGF- β , activins, inhibins, Müllerian duct inhibiting substance and growth/differentiation factors (GDFs)—and act in a concentration-dependent manner [18, 31]. BMPs are multifunctional proteins with chemotactic, mitogenic and differentiation-inducing properties; they are involved in the morphogenesis of a variety of tissues including bone and cartilage [30]. For instance, BMPs are involved in the regulation of skeletal homeostasis [2]. BMPs exert their effects by binding to both type I and type II serine/threonine kinase receptors (Fig. 1) [30]. So far, over 15 BMPs have been identified, cloned and expressed in human and mice (Table 1). BMP-2, BMP-4, BMP-6, and BMP-7 induce bone and cartilage formation *in vivo* while BMP-2, BMP-4 and BMP-7 regulate the growth and maturation of chondrocytes *in vitro* [10]. In addition, developmental abnormalities in the skeletal tissue of humans and animals are related to defects in BMP signalling [18, 27, 37]. BMP-2 and its receptor BMP receptor type 1B (BMP1B) are localised in the regions of the cartilage cap in osteochondroma [23].

Fig. 1 BMP signalling pathway. BMP binds to BMPR-I and BMPR-II to form a signal-transducing complex. BMPR-II phosphorylates the GS domain of BMPR-I. Subsequently, BMPR-I phosphorylates signal transduction proteins Smad 1, 5 or 8 (R-Smads). The phosphorylation of R-Smads is inhibited and modulated by inhibitory Smad 6 and 7 (I-Smads). R-Smads interact with Smad 4 (Co-Smad) to form a complex. The R-Smads/Co-Smad complex enters the nucleus and activates the transcription of BMP-response genes. HSPGs are found in the extracellular matrix or bound to the cell membrane. HSPGs are composed of HS chains attached to a protein core. HSPGs can bind and sequester BMPs

BMP binding proteins, such as noggin and chordin, act as extracellular antagonists of BMP signalling by binding directly to BMP, thus preventing them from interacting with their cognate receptors on the cell surface (Fig. 1). Noggin is a pleiotropic factor and binds to BMP-2, BMP-4, BMP-5, BMP-7, BMP-13 and BMP-14 in vitro [19]. Noggin expression is induced by Ihh (Indian hedgehog) in chondrocytes and skeletal dysplasia results from increased noggin activity [19]. Independent of its action as a BMP antagonist, noggin binds strongly to HSPGs on the surface of cultured cells [26]. Thus, HSPGs can regulate the cellular distribution of noggin, offering a possible mechanism through which HSPGs can regulate the responses of cells to BMPs in vivo [26]. Chordin is expressed predominantly in the epiphyseal regions of developing long bones and binds to BMP-2 and BMP-4 [24, 30, 42]. Chordin is capable of antagonising BMP-induced chondrocyte differentiation and is in turn proteolytically inactivated by BMP-1 [30]. Chordin expression is increased by PTHLH (parathyroid hormone-like hormone) and decreased by BMP-2 [42]. Membrane bound HSPGs bind to chordin, affect the retention of chordin at cell surfaces and are necessary for its cellular uptake [13]. Membrane bound HSPGs intensify chordin antagonism of BMP signalling by concentrating it at the cell surface [13]. Thus, HSPGs contribute to shape and stabilise the fields of BMP inhibition while at the same time shaping and stabilising reciprocal fields of BMP signalling [13].

Membrane bound and extracellular HS is known to bind to BMPs and is involved in BMP-mediated morphogenesis

by regulating their gradient formation and activity [14, 38]. HSPGs can sequester BMPs at the cell surface, directing their movement across the surface through restricted diffusion [14]. HS enhances BMP activity by continuously serving the ligands to their signalling receptors on the cell surface [38]. Furthermore, HSPGs regulate BMP-2 induction of osteogenic markers and internalisation in vitro [14]. In the absence of HSPGs, the spatial range of BMP signalling is found to be dysregulated in the developing limb [21]. HS-deficient cells in the limb were capable of transducing BMP signals but the tight spatial regulation of BMP signalling—as seen in wild-type cells—was disrupted [21]. Similarly, reduced HS levels in synovial joints led to an increase in BMP signalling activity which suggest signalling had been disrupted [22]. This demonstrates that HSPGs regulate BMP signalling during skeletal development. It is reasonable to propose that osteochondroma formation in MHE is mediated by a reduction in HS chain length and subsequent disruption of BMP signalling.

Mouse models for pathogenesis

Insights into the pathogenesis of MHE had revolved around the following question: is an osteochondroma the result of a haploinsufficiency-dependent misregulation of signalling or of clonally occurring second mutations in the EXT genes? Loss of heterozygosity (LOH) of EXT genes has been observed in both solitary osteochondromas and osteochondromas in MHE;

Table 1 Bone morphogenetic protein family

BMP designation	Generic name	BMP subfamily	Chromosome location
BMP-1	BMP-1	Not a BMP family member	8
BMP-2	BMP-2A	BMP 2/4	20
BMP-4	BMP-2B	BMP 2/4	14
BMP-3	Osteogenin	BMP-3	4
BMP-3B	Growth/differentiation factor-10 (GDF-10)	BMP-3B	10
BMP-5	BMP-5	OP-1/BMP-7	6
BMP-6	Vegetal related-1 (Vgr-1)	OP-1/BMP-7	6
BMP-7	Osteogenic protein-1 (OP-1)	OP-1/BMP-7	20
BMP-8	Osteogenic protein-2 (OP-2)	OP-1/BMP-7	1
BMP-8B	Osteogenic protein-3 (OP-3)	OP-1/BMP-7	1
BMP-9	Growth/differentiation factor-2 (GDF-2)	GDF	10
BMP-10	BMP-10		2
BMP-11	Growth/differentiation factor-11 (GDF-11)	GDF	12
BMP-14	Cartilage-derived morphogenetic protein-1 (CDMP-1) Growth/differentiation factor-5 (GDF-5)	CDMP/GDF	20
BMP-13	Cartilage-derived morphogenetic protein-2 (CDMP-2) Growth/differentiation factor-6 (GDF-6)	CDMP/GDF	8
BMP-12	Cartilage-derived morphogenetic protein-3 (CDMP-3) Growth/differentiation factor-7 (GDF-7)	CDMP/GDF	2
BMP-15	Growth/differentiation factor-9B (GDF-9B)	BMP	X
BMP-16	BMP-16	BMP	

but, in the majority of cases, the loss of the remaining wild-type allele has not been detected. A study conducted by Bovee et al. found LOH in six of 14 osteochondromas, indicating that inactivation of both copies of the *EXT1* gene is required for osteochondroma formation in a hereditary case [5]. However, Zuntini et al. found LOH in five of 35 osteochondromas from MHE and none in solitary osteochondromas, suggesting that biallelic inactivation of *EXT* genes does not account for osteochondroma formation in the majority of cases [43]. This led to contradictory reports in the literature regarding the requirement of a complete inactivation of *EXT* genes for osteochondroma formation. Thus, the development of genetic animal models helped explain why LOH is not identified in many patients.

When modelling the human MHE genotype, one third of mice heterozygous for *Ext2* develop small solitary osteochondroma-like structures on the ribs [36]. However, these mice did not display shortened long bones, bowing or growth of larger osteochondromas on other bones as in MHE in humans. By conditionally inactivating *Ext1* through head-

to-head *loxP* sites and temporally controlling *Cre*-recombinase in chondrocytes, Jones et al. generated a mouse to model the chimeric tissue genotype of somatic LOH [17]. The mouse model demonstrated that limited duration of *Cre*-mediated recombination in chondrocytes led to clonal inactivation of the *Ext1* gene with low prevalence. This gave rise to frequent osteochondromas on the appendicular skeleton, indicating that LOH is the inductive event for osteochondroma [17]. In addition, low-prevalence LOH for *Ext1* in physeal chondrocytes was enough to mimic a short-bone phenotype, indicating that LOH drives the short-bone phenotypes associated with MHE [16]. Furthermore, the model indicated that the physeal chondrocyte is the cell of osteochondroma origin, rather than the ossification groove of Ranvier as was previously speculated [17]. By stochastically inactivating *Ext1* in a minor fraction of chondrocytes using a *Cre* transgene with a low level of leakiness, Matsumoto et al. generated the *Ext1*-SKO mouse [20]. *Ext1*-SKO mice developed multiple bony protrusions similar to osteochondromas as well as skeletal deformities such as dislocation of the radial head, bowed bones and short stature,

thus mimicking the human MHE phenotype [20]. Matsumoto et al. also showed that osteochondromas originated from chondrocytes in the growth plate and contained a mixture of *Ext1* null and wild-type chondrocytes.

Similar to mouse models, Reijnders et al. found that the cartilaginous cap of osteochondroma is composed of a mixture of wild-type cells and HS-negative cells (presumed *EXT*^{-/-}), displaying a heterogeneous composition [32]. Using polyethyleneimine (PEI) staining, de Andrea et al. demonstrated that human osteochondromas have a heterogeneous distribution of both normal and PG-deficient cells [6]. The presence of PG gradients within the growth plate and their absence around a group of osteochondroma cells was demonstrated. These studies suggest that osteochondromas recruit wild-type chondrocytes from the neighbouring tissue to generate a non-clonal population of chondrocytes. This affects the diffusion of signalling molecules and may contribute to osteochondroma initiation. For this reason, the possibility of sampling error could account for the inconsistencies in the data from patient samples in which no LOH was identified [32]. The detection of a second hit in osteochondromas may depend on the ratio of wild-type versus mutated chondrocytes present.

In addition to mouse models, studies have been conducted to determine the localisation of proliferative cells in osteochondromas. In normal growth plates hypertrophic chondrocytes do not proliferate but instead undergo apoptosis. Interestingly, the marker for proliferative cells proliferating cell nuclear antigen (PCNA) was detected in prehypertrophic and hypertrophic chondrocytes of osteochondromas while absent in hypertrophic chondrocytes of normal growth plates [1]. Nestin is a type VI intermediate filament protein expressed in proliferative neural cells during development. It is noteworthy that Nestin was detected in prehypertrophic and hypertrophic chondrocytes of osteochondromas while absent in normal growth plates [9]. Nestin protein levels were observed to be higher in osteochondromas of young patients and proteins levels decreased significantly with increasing patient age [9]. These studies support the indication that prehypertrophic and hypertrophic chondrocytes in osteochondromas proliferate constantly and fail to terminally differentiate. The presence of neural marker nestin in proliferating osteochondromas is intriguing.

Challenges and opportunities

Significant progress has been made in the quest to understand the cell biology of osteochondroma and its associated diseases, offering several avenues of opportunities for further exploration. Based on current findings from animal models, opportunities to re-examine LOH in human osteochondromas should be considered. The resemblance

of osteochondromas in FOP patients to those in MHE patients is fascinating. With the use of the animal models, further analysis of the BMP signalling pathway may bring more insight into the molecular mechanism of MHE pathogenesis. Although remarkable steps have been made in understanding the cell biology of osteochondroma formation, several challenges and questions still remain. While animal models have expanded our insight into the development of osteochondromas, it is possible that animal and human chondrocytes do not respond in the same way to heterozygous levels of *EXT*. Mouse chondrocytes may have ways to tolerate or even compensate for an inactive *EXT* allele whereas human chondrocytes do not have a way to do so. Larger numbers of patients need to be studied to confirm any findings and be statistically significant, but due to the rareness of these conditions, accomplishing this is a challenge. What determines the severity of disease and how can it be minimised? What drives malignant transformation in osteochondroma and can this be prevented? What is the role of BMP signalling, BMP receptors and interactions with HS? These are some of the central challenges for future studies that will explore novel approaches to better understand these important bone disorders.

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Conflict of interest The authors declare that they have no conflict of interest.

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