The pathogenic roles of heparan sulfate deficiency in Hereditary Multiple Exostoses

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Abstract

Heparan sulfate (HS) is an essential component of cell surface and matrix proteoglycans (HS-PGs) that include syndecans and perlecan. Because of their unique structural features, the HS chains are able to specifically interact with signaling proteins–including bone morphogenetic proteins (BMPs)-via their HS-binding domain, regulating protein availability, distribution and action on target cells. Hereditary Multiple Exostoses (HME) is a rare pediatric disorder linked to germline heterozygous loss-of-function mutations in *EXT1* or *EXT2* that encode Golgi-resident glycosyltransferases responsible for HS synthesis, resulting in a systemic HS deficiency. HME is characterized by cartilaginous/bony tumors-called osteochondromas or exostoses- that form within perichondrium in long bones, ribs and other elements. This review examines most recent studies in HME, framing them in the context of classic studies. New findings show that the spectrum of *EXT* mutations is larger than previously realized and the clinical complications of HME extend beyond the skeleton. Osteochondroma development requires a somatic "second hit" that would complement the germline *EXT* mutation to further decrease HS production and/levels at perichondrial sites of osteochondroma induction. Cellular studies have shown that the steep decreases in local HS levels: derange the normal homeostatic signaling pathways keeping perichondrium mesenchymal; cause excessive BMP signaling; and provoke ectopic chondrogenesis and osteochondroma formation. Data from HME mouse models have revealed that systemic treatment with a BMP signaling antagonist markedly reduces osteochondroma formation. In sum, recent studies have provided major new insights into the molecular and cellular pathogenesis of HME and the roles played by HS deficiency. These new insights have led to the first ever proof-of-principle demonstration that osteochondroma formation is a druggable process, paving the way toward the creation of a clinically-relevant treatment.

Keywords

Heparan Sulfate; Heparan sulfate proteoglycans; Hereditary Multiple Exostoses; EXT1; EXT2; Signaling proteins and pathways; Drug treatment

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Introduction

Heparan sulfate (HS) is a versatile and variable glycosaminoglycan and is a critical component of cell surface and matrix proteoglycans (HSPGs) that include syndecans, glypicans and perlecan [1–4]. HS owes its complexity and character to a biosynthetic pathway that is carried out in the endoplasmic reticulum and Golgi and involves a series of multiple and sequential steps coordinated with synthesis and routing of different proteoglycan core proteins. HS synthesis initiates with the attachment of xylose to prescribed serine residues specific for each proteoglycan core protein and is followed by attachment of two galactose residues and glucuronic acid to form the tetrasaccharide linkage region [1–3]. Chain polymerization starts with the addition of a N-acetyl-D-glucosamine (GlcNAc) residue to the linkage region by the enzyme EXTL2 or EXTL3 ( ) and proceeds with the sequential and systematic addition of glucuronic acid (GlcA) and GlcNAc residues by a Golgi-resident enzyme complex composed of EXT1 and EXT2, eliciting repetitive disaccharide units and eventually producing chains with an overall size of 20 to 25 kDa. While these steps are ongoing, the growing chains are subjected to a series of concurrent structural modifications that start with elimination of certain acetyl groups from GlcNAc clusters and their replacement with sulfate by the action of members of the N-deacetylase-N-sulfotransferase family. The modifications continue with epimerization of some D-glucuronic acid residues to L-iduronic acid and sulfation at positions C2, C6 or C3 around glucosamine and glucuronic/iduronic rings by O-sulfotransferase family members. Such multiple serial biosynthetic and modification steps result in chains with diverse and unique sulfation and sugar modification patterns within 6–12 sugar residue-long segments flanked by segments of largely unmodified and un-sulfated sugars [3]. Additional processing of HS chains can occur at the cell surface by the action of the endosulfatases SULF1 and SULF2 that remove 6-O sulfate groups [5, 6]. The HS chains can also be trimmed and digested to various extents by the activity of extracellular heparanase [7, 8]. Because the intracellular and cell surface biosynthetic and modifying enzymes are expressed in tissue- and developmental-specific manners as are the proteoglycan core proteins, the overall output and structural characteristics of HS and HSPGs significantly differ in different tissues and cell types and are modulated at distinct prenatal and postnatal stages and processes [3, 4, 9, 10].

Members of the bone morphogenetic protein (BMP), hedgehog, fibroblast growth factor (FGF) and Wnt signaling protein families are essential regulators of multiple and critical developmental and postnatal processes that include progenitor cell recruitment, cell differentiation, tissue morphogenesis and homeostasis, and organogenesis [11–13]. A common and telling feature of most of these proteins is that they possess a specific domain recognizing and interacting with HS [14, 15]. The HS-binding domains contain several basic residues (Arg and/or Lys) spaced by hydropathic amino acids, have an average pI > 11, and vary greatly in length, organization and structure in different proteins [16, 17], though these features appear to be strictly conserved and may thus be important for specificity of HS-protein interactions [14]. Reciprocally, the 6–12 sugar residue-long sulfated and modified segments along the HS chains may have preferential protein-selective binding characteristics [3]. The resulting protein-HS interactions have many functions, but a key one is to regulate, limit and delimit protein distribution, diffusion, gradient formation, turn-over, bioavailability

and action on target cells and tissues [9, 10]. This was originally and prominently demonstrated by the analysis of *Drosophila* embryos bearing loss-of-function mutations in *tout-velu*, the homologue of vertebrate *Ext1* [18, 19]. The mutant embryos exhibited an abnormal and broad distribution of hedgehog protein and ectopic expression of its receptor *Patched* and were affected by developmental abnormalities, including defective segmentation polarity. Chondrocytes in zebrafish mutants lacking *dackel* (the homologue of vertebrate *Ext2*) exhibited abnormal growth, lost polarity and invaded surrounding tissues, thus behaving as cells in osteochondromas [20]. A subsequent study in mice bearing a hypomorphic *Ext1* mutation showed that tissues including the growth plates in prenatal developing skeletal elements contained about 10% of normal HS levels and displayed a broader and unruly distribution of Indian hedgehog (Ihh), associated with abnormalities in growth plate function and skeletal growth and elongation [21]. Loss-of-function mutations in *EXT1* and *EXT2* have also been detected in various forms of human cancers, likely causing abnormal cell behavior and proliferation via greater availability of growth factors and cytokines [22, 23]. Mutations in *Glypican-6* core protein were found to cause recessive Omodysplasia and impairment of endochondral ossification [24]. Recently, homozygous missense mutations in *EXTL3* were identified in patients within unrelated families with a neuro-immuno-skeletal dysplasia syndrome characterized by multiple defects, including lack of T cells, kyphosis, neural deficiencies, brachydactyly and/or craniofacial malformations [25–27]. In sum, a normal complement and physiologic expression and function of HS and HSPGs are clearly required for embryonic development, postnatal growth, tissue homeostasis and organ function, and abnormalities in those components, expression patterns and mechanisms can lead to disease.

Hereditary Multiple Exostoses and its diverse clinical complications

A disease most relevant to the present review article is Hereditary Multiple Exostoses (HME) that is associated with heterozygous loss-of-function mutations in *EXT1* or *EXT2* [28–31]. HME, also known as Multiple Osteochondromas (MO), is a rare orphan pediatric disorder with an incidence of about 1:50,000 individuals worldwide, without apparent geographic preference [32, 33]. The most obvious trait of the disease (from which its name) is the presence of benign cartilage-capped bony tumors called exostoses or osteochondromas that emerge within the perichondrium flanking the growth plates of long bones, vertebrae, ribs and cranial base in young and adolescent patients [34–37]. Many of the patients bear a large number of osteochondromas throughout their skeleton by the time their growth plates close at the end of puberty. Because of their large number, multiple anatomical locations and considerable size, the osteochondromas can, and do, cause a number of health problems, including skeletal deformities and bowing, joint ankylosis, skeletal growth disparities, and impingement of surrounding muscles, nerves and blood vessels. Fig. 1 provides vivid examples of osteochondroma burden and skeletal defects in HME patients, as revealed by computed tomography (CT) and X-ray imaging. Fig. 1A depicts the rib cage of a 37 yr-old HME patient exhibiting numerous osteochondromas along multiple ribs (Fig. 1A, arrow), infringing on mechanical function and neighboring tissues. A large mass made of soft tissues and calcified tissue was present on the upper right area near the clavicle (Fig. 1A, double arrow) that turned out to be a Grade 1 chondrosarcoma at pathology and was eventually

resected by surgery. The pelvis of a 23 yr-old patient exhibited multiple osteochondromas located along the upper and lower iliac crest, pubic ramus and femurs (Fig. 1B). The pubic symphysis was likely fused by the osteochondromas, and the functioning of the left hip joint was compromised. Fig. 1C is from an 8 yr-old patient and reveals the presence of two large osteochondromas in posterior medial portion of the humerus (Fig. 1C, arrow), a small osteochondroma in distal radius, and bending of radius toward ulna. Fig. 1D illustrates the right arm from a 20 yr-old patient exhibiting significant bending of radius and ulna. The severe shortening of distal ulna created an ulna-minus wrist. The bulkiest osteochondromas were located in the distal shaft of radius and ulna and were present also in scapula and humerus.

Currently, surgery is the most common treatment for HME patients by which the most symptomatic, problematic and accessible osteochondromas are resected in order to ameliorate or correct major skeletal defects. However, osteochondromas may be difficult to reach or be located in potentially dangerous and delicate locations; as a consequence, many are often left in place, leading to long lasting problems and concerns [38]. in addition, osteochondromas undergo transformation in about 2–3% of patients and turn into chondrosarcomas (Fig. 1A) [39, 40]. Though rarely, these malignant cancers can become life threatening because of their typical resistance to chemo- or irradiation therapy, and their frequency appears to correlate with the number of benign osteochondromas left in place long term [39, 40].

Osetochondromas forming on the intracanal surface of the vertebrae are particularly dangerous and worrisome since they often impinge on, and affect the function of, the spinal cord and can cause nerve damage, physical weakness and progressive impediment of motion [41, 42]. Intracanal osetochondromas are seen in a significant number of HME patients and given their propensity to cause such problems, studies have recommended that all HME children be given routine MRI or CT screening and evaluation of their spine at presentation and consider surgical intervention [42]. A recent study focused on an additional possible complication of the spine in HME patients –scoliosis- that is not often included in assessment and natural history analyses of HME clinical symptoms [43]. The authors recruited 50 female and male HME patients with a mean age of 28.1 years (range 2–77 years) and clinically assessed them at presentation. Based on a disease severity classification that took into consideration deformities, functional limitation and number of osetochondroma-containing anatomical sites, the authors subdivided the patients into class I (least affected) to class III (most affected) groups. Clinically observable scoliosis was detected in about 70% of all patients exhibiting a mean primary curve of 15.3° and a mean minor curve of 10.6°, and spine severity encompassed King type I to type IV classification, but no type V [44]. Statistical calculations and analysis indicated that moderate scoliosis was significantly linked to disease severity and prevalent in class III patients, but was not related to overall number of osetochondromas, gender or limb deformities. Thus, mild to moderate scoliosis may be common among HME patients, but the data need to be verified in other and larger cohorts of patients before reaching conclusions about clinical significance and implications.

The multiple skeletal problems and physical difficulties associated with HME, including those described above, have long been known and appreciated, but more recent studies have focused attention to non-skeletal and often overlooked aspects of HME pathology. In a particularly attentive and revealing study [45], the authors contacted all the over 300 known patients with HME in the Netherlands and offered participation in an age-specific survey and questionnaire of: their daily function; school and professional activities; physical symptoms including pain; and overall well-being. Most adults and about 30% of the children completed the questionnaire, and data were assessed statistically by various methods. The majority of adult patients were employed, but a significant fraction of them reported to have changed jobs in connection to their own physical difficulties or a need to adapt to challenging work environments. A small number of patients were unable to hold jobs having been found to be medically unfit to work. Based on standard life quality assessment survey [46], the patients had low scores in several categories compared to control groups. About 50% of the children with HME described difficulties in school that affected and limited their participation in certain social activities and sports. Not surprisingly, both young and adult patients reported experiencing persistent pain. Data and insights in this study correlate well with those in a subsequent study of about 100 HME patients that included 57 adults and 42 children [47]. Together, these and other studies underline the pervasive and intrusive nature of HME with its negative effects on quality of life, social and personal well-being and activities, and even self-esteem. The studies call for closer and attentive clinical evaluation of HME patients, special attention to their physical and non-physical difficulties, health assessment over time, and specific remedies for diverse needs including early intervention.

EXT mutations and HME pathogenesis

As indicated above, the EXT1 and EXT2 proteins form Golgi-resident supermolecular complexes in which EXT1 exerts enzymatic function and EXT2 has a structural and scaffold role, with both proteins needed for efficient HS polymerization [48–50]. As a consequence, heterozygous mutations in either gene in HME patients lead to a systemic HS deficiency of about 50% [51]. This partial loss of HS has been found to be in itself detrimental to diverse physiologic mechanisms, including postprandial lipid clearance and metabolism and pancreatic beta-cell reserve functioning [52, 53]. However, a partial HS loss is not sufficient to cause multiple osteochondroma formation [54, 55]. As prescribed by Kundson Tumorigenesis Hypothesis [56], This process would require a somatic "second hit", leading to further local decreases in HS synthesis and/or levels and causing resident cells to misbehave and turn tumorigenic [22]. Lines of evidence generated by several groups including ours have provided insights into these questions and have shed light on the spectrum of *EXT* mutations, nature of possible second hits, and consequences on osteochondroma formation and HME pathogenesis [57].

Genetic analyses conducted after those originally linking HME with *EXT* mutations [28, 30] have demonstrated that the spectrum of *EXT* mutations in this disease is quite broad and includes nonsense, missense, frame shift and splice-site mutations in *EXT1* or *EXT2* [31, 58, 59]. Mutations registered in the Multiple Osteochondroma Mutation Database (URL: http://medgen.uantwerpen.be/LOVDv.2.0/) exceeds 650 at present [58], but no definitive system is available to classify disease severity amongst HME patients and establish reliable

genotype-phenotype correlations [60, 61]. This situation likely reflects the fact that clinically, HME is variable and can affect patients to divergent degrees, even within family members with the same *EXT* mutation and amongst unrelated patients with the same mutation [61]. In general however, patients with *EXT1* mutations usually have a more severe clinical phenotype broadly and variously defined in terms of overall number of osteochondromas, skeletal deformations, anatomical sites affected, and physical difficulties and limitations [62]. The more severe phenotypes associated with *EXT1* mutations possibly reflect its enzymatic function in HS synthesis compared to the structural roles played by EXT2.

HME does not have preferential geographical occurrence or different frequency around the world as pointed out above, and recent *EXT* mutation surveys in local populations in Japan, Poland, Italy and Spain have confirmed these basic important notions [63–66]. Not surprisingly, these studies have identified additional novel mutations in *EXT1* or *EXT2*, broadening the mutation spectrum in HME to over 700. These studies raise anew long-standing questions regarding: how each mutation –and particularly the several distinct missense mutations- would interfere with EXT protein structure and function; whether and which of the mutations could lead to a dominant-negative effect on EXT complex function and HS synthesis; and which types of "second hits" could occur in HME patients and how they would relate to, and cooperate with, the germline heterozygous EXT mutations, eventually leading to steeper local HS loss and osteochondroma formation. To date, the best documented types of second hits observed in surgical retrieval human osteochondromas from HME patients are loss-of-heterozygosity (LOH), aneuploidy or other large genomic changes, all rendering local resident cells *EXT1*- or *EXT2*-null [54, 67–69]. To directly evaluate the significance of such genetic changes and their relevance to HME pathogenesis, several groups conducted mouse studies. Single heterozygous $Ext1^{+/-}$ or $Ext2^{+/-}$ mutant mice were found to be largely normal [50, 70], clearly showing that a partial decrease in *Ext* expression and HS synthesis is well tolerated and largely compatible with normal development and postnatal life. However, conditional loss of both *Ext1* alleles in growth plate and/or perichondrium cells (accompanied by a steep decrease in HS levels) caused formation of multiple osteochondromas in long bones, ribs and vertebrae, thus mimicking the human HME phenotype and affirming the need for severe Ext/HS loss in disease initiation and progression [71–73]. In this regard, it is interesting to note that a few HME patients have been found to bear germline compound heterozygous mutations in both *EXT1* and *EXT2* [58, 59, 64]. It had remained unclear whether those patients would still require an additional second hit to develop HME or whether those inborn compound mutations would be sufficient. While this question is still pending, our own studies in mice have shown that compound heterozygous $Ext1^{+/-};Ext2^{+/-}$ mutant mice did develop multiple osteochondromas by 2 months of age [70], while companion single heterozygous mice did not as also shown previously [50]. Because both Ext1 and Ext2 are needed for HS synthesis, the compound heterozygous mice were expected to produce about 25% of HS compared to wild type mice, and our direct analyses of HS levels and distribution in mutant versus wild type growth plates confirmed that prediction. It is possible then that patients with germline compound heterozygous mutations in *EXT1* and *EXT2* may not require an additional second hit to develop a HME phenotype. Taken together, human and mouse studies carried out so

far have established that single heterozygous mutations in *EXT1* or *EXT2* (and the resulting 50% reduction in systemic HS levels) are not sufficient to trigger a full-fledged HME phenotype. Rather, additional genetic changes are required that would cause a steep drop in HS levels, transform the phenotype of local resident cells, and provoke osteochondroma formation.

In this regard, it is of interest to further consider the distinct disease phenotype of patients with homozygous *EXTL3* missense mutations identified recently [25–27]. As pointed out above, these patients suffer from various immune, neural and skeletal health problems and display a partial systemic HS deficiency (evaluated in serum and urine), but do not have typical symptoms of HME. Indeed, mutations in *EXTL3* or *EXTL2* have not been reported in HME patients [58]. It is likely then that the HS deficiency elicited by the homozygous missense *EXTL3* mutations is moderate and insufficient to trigger HME and could also be compensated by expression of *EXTL2* within growth plates and perichondrium where the development of osteochondromas occurs.

Mechanisms of osteochondroma development

The above studies have been quite important and illuminating regarding a need for additional somatic genetic changes, but did not experimentally test whether stochastic events such as LOH would indeed be sufficient to trigger HME. Also, the studies did not analyze how a severe drop in local HS levels would alter the phenotype of resident cells and trigger osteochondroma formation. The authors of a recent important study tackled the first question by creating transgenic mouse lines that contain head-to-head *loxP* sites flanking exon 2 in *Ext1* (termed *Ext1^{e2fl/e2fl}*) [71]. This genetic design was expected to yield a stochastic distribution of forward and inverted flanked segments after transient expression of *Cre* recombinase [74], leading to the generation of chimeric tissues that contained a low prevalence of mutant cells following inversion of both alleles. *Cre* expression was induced by including doxycycline in drinking water of compound *Ext1^{e2fl/e2fl};Col2-rtTA-Cre* mice from postnatal day 8 to day 15 (P8 to P15). Previous studies showed that *Cre* expression driven by *Col2* regulatory sequences targets cells in both growth plate and neighboring perichondrium [75]. Numerous and large osteochondromas emerged near the knees and along the rib cage over the following 4 to 8 weeks in doxycycline-treated *Ext1^{e2fl/e2fl};Col2-rtTA-Cre* mice, but not in heterozygous *Ext1^{e2fl/+};Col2-rtTA-Cre* mice or companions not given doxycycline. Loss of *Ext1* enzymatic activity and HS synthesis in homozygous *exon2*-inverted tissues was confirmed by immunohistochemistry. Interestingly, *Ext1^{e2fl/e2fl};Osterix-CreER^T* mice expressing *CreER^T* in hypertrophic chondrocytes and bone cells [76] did not develop osteochondromas following tamoxifen treatment. This important study revealed that: stochastic loss of both *Ext1* alleles is sufficient for, and does lead to, a HME-like phenotype; loss of one allele is not sufficient and is tolerated; and osteochondroma formation is ascribable to cells targeted by *Col2*- but not *Osterix*-driven Cre, pointing to specificity of cells involved in tumor formation.

These observations were extended in a concurrent and equally important study [72]. The authors relied on standard floxed *Ext1* mice and mated them with *Col2-CreER^T* mice [77], the latter previously assumed to express *Cre* recombinase only after tamoxifen treatment.

However, the authors had observed a low level of leakiness by the *Cre* transgene without tamoxifen treatment, thus causing a random stochastic deletion of both *Ext1* alleles in a small number of *Col2*-expressing cells. As pointed out above, the targeted cells included growth plate chondrocytes and adjacent perichondrial cells [75]. Numerous osteochondromas formed in long bone epiphyses and along the ribs in *Ext1^(f/f);Col2-CreER^T* mice by one month of age, but not in heterozygous *Ext1^(f/+);Col2-CreER^T* mice or those lacking the *Cre* transgene. Close histological inspection and analysis of each osteochondroma showed that the cells were a mixture of mutant and wild type cells, affirming the notion that mutant cells can recruit surrounding healthy cells into tumor formation and growth [67, 69]. Taken together, the above studies provided the most direct evidence to date that stochastic inactivation of both *Ext* alleles in a small number of cells is sufficient to elicit osteochondroma formation and leads to the development of a HME-like phenotype over time.

While providing important new information on HME molecular pathogenesis, the above studies did not examine aspects of HME cellular pathogenesis, and in particular the origin and nature of cells responsible for osteochondroma formation and what cellular mechanisms would be deranged as a consequence of local HS loss. At this regard, it is important to point out that osteochondromas were originally thought to derive from mutant growth plate chondrocytes (reviewed in [34]). However, we and other groups suggested that instead, osteochondromas originated from mesenchymal cells located in perichondrium [40, 55, 78] known to contain progenitors normally engaged in lateral appositional expansion of skeletal elements as well as tissue repair [79]. To directly test our hypothesis, we selectively targeted cells within perichondrium and asked whether conditional ablation of *Ext1* alleles in those cells would be sufficient to provoke osteochondroma formation. We also asked what cellular mechanisms may be involved. We used *Gdf5Cre* mice that strongly express *Cre* recombinase in perichondrium flanking the epiphyseal portion of growth plates [78], being unique in that regard. Multiple osteochondroma-like masses did develop in *Ext1^(f/f);Gdf5Cre* mice over time that displayed a typical growth plate-like cartilaginous organization. A similar induction of osteochondroma formation occurred when long bone rudiments from E18.5 *Ext1^(f/f)* mouse embryos reared in organ culture were treated with either adeno-Cre (to ablate *Ext1*) or Surfen, a HS antagonist drug [80]. These experiments revealed also that the onset of osteochondroma formation in vivo and in vitro was preceded by ectopic and excess BMP signaling (a major pro-chondrogenic pathway) within perichondrium as indicated by phosphorylated SMAD1/5/8 levels. In good correlation, we found that treatment of micromass cultures of *Ext1^(f/f)* mouse embryo limb bud mesenchymal cells with adeno-Cre or Surfen caused: excessive chondrogenesis and cartilage nodule formation; overexpression of chondrogenic master and matrix genes; and cellular over-responsiveness to exogenous BMP proteins. Surface plasmon resonance assays indicated that interference with HS function by Surfen treatment reduced the physical association kinetics and interactions of recombinant BMP2 with immobilized HS chains. Taken together, our data strongly suggested that: osteochondromas originate from perichondrial progenitor cells; and loss of HS or interference with HS function provoke ectopic excessive BMP signaling in perichondrium, covert the phenotype of local progenitors from mesenchymal to chondrogenic, and induce osteochondroma formation. An important implication of these findings is that under

physiologic circumstances, normal levels of HS and *Ext* expression would maintain the innate mesenchymal phenotype of perichondrial cells and prevent signaling by locally-expressed BMPs [81, 82]. It is important to note that while HS appears to normally restrain BMP signaling [83, 84], HS has the opposite effect on, and promotes, FGF signaling which is a major anti-chondrogenic pathway expressed in several non-cartilaginous tissues including perichondrium [85–87]. Thus, while boosting BMP signaling, the HS deficiency in HME could dampen FGF signaling, and this combination would alter the phenotype of perichondrial progenitors, induce chondrogenic differentiation and induce osteochondroma development (see model in Fig. 2).

Therapeutic targets

In addition to their basic value and interest, the above data provided tantalizing clues and ideas about possible therapeutic targets. Given that ectopic BMP signaling in perichondrium precedes osteochondroma formation, we tested the possibility that systemic treatment with a BMP signaling antagonist could inhibit osteochondroma formation or growth [35]. To mimic HME pathogenic course more closely, we used an inducible mouse model of HME in which osteochondroma formation was first induced in juvenile mice during maximal skeletal growth. Thus, *Ext1^{f/f};AggrecanCreER* mice were injected with tamoxifen once at 5 weeks of age. On day 1 after tamoxifen injection, half of the mice were treated with the potent BMP signaling antagonist LDN-193189 (3 mg/kg) by daily IP injection [88], and the remaining half received daily injections of vehicle. After 6 weeks, the vehicle-treated mice exhibited multiple and large osteochondromas at every skeletal site examined, including long bones, ribs and cranial base. The tumors initially exhibited a typical cartilaginous growth plate-like organization and with time, acquired a bony stem in their proximal region linking them to the adjacent skeletal element. In mice treated with LDN-193189, however, osteochondroma formation was markedly reduced. Image analysis and quantification by micro-computed tomography and serial histological inspection showed that the decrease amounted to over 65% compared to vehicle-treated mice. No appreciable side effects of drug treatment were observed. Interestingly, when we examined early time points, we found that osteochondroma formation in vehicle-treated mice was preceded by ectopic excessive BMP signaling (as expected from our earlier studies) and a reciprocal decrease in phosphorylated ERK1/2 levels. The latter are major downstream effectors of FGF signaling [85, 89], providing evidence that osteochondroma formation is indeed preceded –and promoted-by opposite changes in BMP versus FGF signaling (see Fig. 2). Our data were the first to provide proof-of-principle evidence that osteochondorma formation is amenable to –and can be inhibited by- drug treatment [35]. Similar observations have just been reported by another group [90].

Studies have indicated that there is an inverse relationship between *EXT* and *heparanase* expression in several types of cancer cells [91–93]. On the surface, these observations appear paradoxical and counter-intuitive by suggesting that when HS production decreases as a result of decreased *EXT* expression or *EXT* mutations, the cells would up-regulate expression of heparanase to decrease the residual HS levels even further. In agreement with those cancer studies, osteochondromas resected from HME patients were originally shown to contain higher amounts of heparanase protein and RNA compared to normal cartilage from healthy donors [94]. We used immunohistochemistry on similar surgical retrieval

specimens collected from HME patients at our Hospital and confirmed that heparanase was abundant and widespread in all osteochondroma chondrocytes, but was much lower in control growth plate chondrocytes (obtained from non-HME patients undergoing surgery for polydactyly). To test for mechanistic implications, we used recombinant human heparanase and found that it boosted chondrogenesis and BMP signaling in micromass cultures of mouse embryo limb bud mesenchymal cells. Notably, treatment of the cultures with bacterial heparitinase, Surfen or rhBMP2 up-regulated endogenous heparanase gene expression, pointing to positive regulatory loops between heparanase expression and response to decreasing HS levels or availability of HS-binding ligands. Notably also, we found that the potent heparanase inhibitor SST0001 [95] strongly inhibited chondrogenesis in the micromass cultures. Together, the data provide further evidence that heparanase is a potential important culprit in HME pathogenesis and thus, a potential therapeutic target. The data suggest also that an increase in heparanase expression could represent one of the "second hits" in HME pathogenesis.

Conclusions and perspectives

The above synopsis of current human and mouse HME literature demonstrates that there have been considerable advances in this biomedical research field over the last few years. Though the most obvious clinical trait of the disease are the osteochondromas and associated skeletal problems, HME can provoke additional difficulties and health problems for the patients that make the disease a syndrome. Regarding the molecular and cellular pathogenesis of osteochondroma formation, it is now well established that tumor formation requires steep decreases in *EXT* expression and/or HS levels. The former would result from LOH or other genetic changes, while the latter would be directly ascribable to: decreased *EXT* expression; compound heterozygous *EXT1* and *EXT2* mutations; or increases in heparanase expression. At the cellular levels, the steep local decrease in HS levels is likely to derange the fine balances amongst different resident signaling pathways within perichondrium; in particular, the HS loss would enhance BMP signaling and would decrease FGF signaling. As a result, the phenotype and lineage assignment of progenitors located in perichondrium would shift from mesenchymal to chondrogenic, promoting ectopic chondrogenesis and osteochondroma formation. As pointed out above, most members of key signaling protein families possess a specific HS-binding domain and under normal circumstances, the resulting interactions with HS and HSPGs regulate protein distribution and action. Hence, the steep local HS deficiency in HME could alter other signaling pathways in addition to BMP and FGF. Indeed, we showed a few years ago that mouse mutants expressing low HSPG family members in cartilage exhibited ectopic hedgehog signaling in perichondrium, followed by osteochondroma-like tissue formation [96]. Formation of these tumors would thus be the end result of a multiplicity of concurrent and opposing changes in signaling pathways (Fig. 2). These realizations and insights are not only important on their own, but have allowed the identification of plausible and possible therapeutic targets. Our direct demonstration that a drug-based inhibition with BMP signaling significantly decreases osteochondroma formation in HME model mice provides definitive proof-of-principle that HME is amenable to pharmacologic intervention [35]. Interference with BMP signaling by direct means –via drugs such as LDN-193189- or by

indirect means -via drugs such as palovarotene [97]- should thus be considered as a possible treatment for HME deserving of clinical consideration and testing.

Acknowledgments

Abbreviations used

HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
GlcNAc	N-acetyl-D-glucosamine
GlcA	glucuronic acid
HME	Hereditary Multiple Exostoses
BMP	bone morphogenetic protein
FGF	fibroblast growth factor
LOH	loss of heterozygosity

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Highlights

Heparan sulfate is a component of cell surface and matrix proteoglycans and regulates many developmental processes, including signaling protein distribution and action

Hereditary Multiple Exostoses is a rare disease caused by *EXT* mutations and heparan sulfate deficiency and is characterized by multiple growth plate-associated osteochondromas

This review assesses recent studies in the genetics of HME and the molecular and cellular pathogenesis of osteochondroma formation

Recent research advances have provided major new mechanistic insights and are paving the way toward a pharmacological treatment for osteochondroma prevention
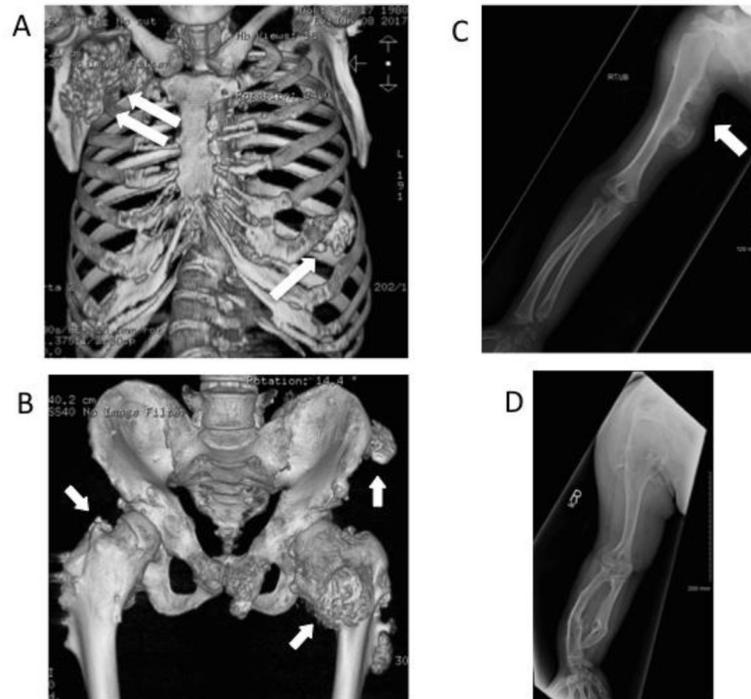
Fig. 1.
Diagnostic CT and X-ray images obtained from HME patients. (A) This CT scan depicts the rib cage of a 37 yr-old HME patient exhibiting multiple osteochondromas along the ribs (arrow) and a large malignant chondrosarcoma Grade 1 on the upper right area near the clavicle (double arrow). The osteochondromas likely impinged on mechanical rib function, and the malignant tumor was eventually resected by surgery. (B) This CT scan shows the pelvis of a 23 yr-old patient exhibiting multiple osteochondromas along the upper and lower iliac crest (arrows), pubic ramus and femurs. The osteochondromas appear to fuse the pubic symphysis and their aggressive presence and size affected the left hip joint (arrow). (C) This X-ray image is from an 8 yr-old patient and reveals two large osteochondromas in posterior medial portion of humerus (arrow), a small osteochondroma in distal radius, and bending of radius toward ulna. (D) This X-ray image illustrates the right arm from a 20 yr-old patient displaying a marked degree of bending of radius and ulna. The severe shortening of distal ulna created an ulna-minus wrist. The bulkiest osteochondromas are located in the distal shaft of radius and ulna and are present also in scapula and humerus. All images were generously provided by the MHE Center at the Paley Orthopedic & Spine Institute, West Palm Beach, Florida.
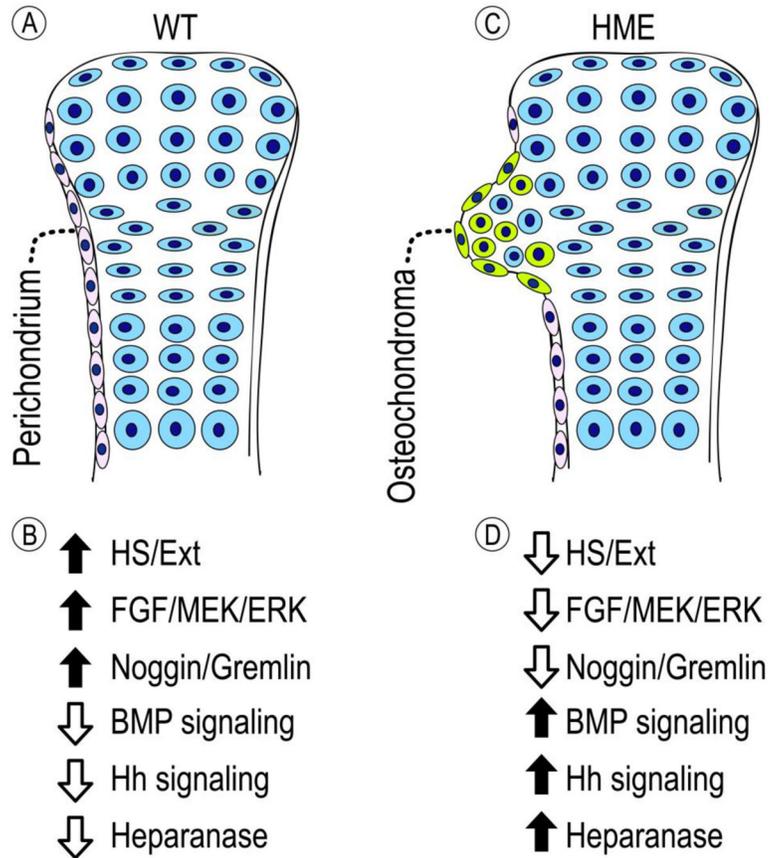
Fig. 2.
Schematic illustrating possible changes in regulatory steps that underlie osteochondroma formation. (**A**) In healthy wild-type (WT) circumstances, perichondrium (in off-white) would define the boundary of growth plate (in blue) in skeletal elements such as long bones. Resident perichondrial cells would be characterized by a normal mesenchymal phenotype and express traits including: a flat cell morphology; normal *EXT* expression and HS levels; strong anti-chondrogenic mechanisms, including FGF and ERK/MEK signaling and *Noggin* and *Gremlin* expression [98]; and low activity/expression of pro-chondrogenic mechanisms including BMP and hedgehog signaling and heparanase. (**B**) Up and down arrows depict the high and low levels of respective traits in normal perichondrium. (**C**) During the course of HME, LOH or other "second hits" would cause a steep and nearly complete loss of *EXT* expression and/or HS levels in local cells (in green) within perichondrium bordering the heterozygous *EXT* mutant growth plate. This would cause concerted decreases in anti-chondrogenic pathways and reciprocal increases in pro-chondrogenic pathways and heparanase expression in mutant cells (summarized in **D**), thus altering homeostatic signaling mechanisms and triggering differentiation of perichondria progenitors into round-shaped chondrocytes (in green). The growing osteochondromas would contain a mixture of mutant (green) and heterozygous (blue) cells, the latter being recruited into the osteochondroma forming process by mutant cells. The changes occurring in BMP, hedgehog

and FGF signaling pathways and in expression of heparanase could each offer plausible therapeutic targets to inhibit osteochondroma inception and/or growth.