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RARE BONE DISEASES (C LANGMAN AND E SHORE, SECTION EDITORS)

Hereditary Multiple Exostoses: New Insights into Pathogenesis, Clinical Complications, and Potential Treatments

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Abstract
Purpose of Review Hereditary multiple exostoses (HME) is a complex musculoskeletal pediatric disorder characterized by osteochondromas that form next to the growth plates of many skeletal elements, including long bones, ribs, and vertebrae. Due to its intricacies and unresolved issues, HME continues to pose major challenges to both clinicians and biomedical researchers. The purpose of this review is to describe and analyze recent advances in this field and point to possible targets and strategies for future biologically based therapeutic intervention.
Recent Findings Most HME cases are linked to loss-of-function mutations in *EXT1* or *EXT2* that encode glycosyltransferases responsible for heparan sulfate (HS) synthesis, leading to HS deficiency. Recent genomic inquiries have extended those findings but have yet to provide a definitive genotype-phenotype correlation. Clinical studies emphasize that in addition to the well-known skeletal problems caused by osteochondromas, HME patients can experience, and suffer from, other symptoms and health complications such as chronic pain and nerve impingement. Laboratory work has produced novel insights into alterations in cellular and molecular mechanisms instigated by HS deficiency and subtending onset and growth of osteochondroma and how such changes could be targeted toward therapeutic ends.
Summary HME is a rare and orphan disease and, as such, is being studied only by a handful of clinical and basic investigators. Despite this limitation, significant advances have been made in the last few years, and the future bodes well for deciphering more thoroughly its pathogenesis and, in turn, identifying the most effective treatment for osteochondroma prevention.

Keywords Hereditary multiple exostoses · Multiple osteochondromas · EXT1 · EXT2 · Heparan sulfate · Genotype-phenotype correlations · Signaling proteins · Drug treatment

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Introduction

Hereditary multiple exostoses (HME), also known as multiple osteochondromas (MO), is a rare orphan autosomal dominant pediatric disorder that has an incidence of about 1:50,000 [1, 2]. HME is characterized by nonmalignant cartilage-capped bony tumors—called osteochondromas or exostoses—that form within the perichondrium flanking the growth plates of long bones, ribs, hip, and vertebrae in very young and adolescent patients [3]. Patients often display a significant number of osteochondromas throughout their skeleton by the time the growth plates close at the end of puberty. Because of their location, sizes, and considerable number, osteochondromas can, and do, cause a number of health problems [1–5]. Surgery is currently the common treatment for HME patients and is used to resect the most symptomatic and problematic osteochondromas and ameliorate associated skeletal defects, such as skeletal element bowing, growth disparities, or nerve impingement [4, 6, 7]. In about 2% of the patients, osteochondromas can undergo malignant transformation, turning into chondrosarcomas or osteosarcomas that can be

life threatening because of their typical resistance to chemo- or irradiation therapy [8, 9].

Over 90% of HME cases are found to be associated with heterozygous loss-of-function mutations in *EXT1* or *EXT2* [10–13], genes that encode Golgi-resident glycosyltransferases responsible for the synthesis and assembly of heparan sulfate (HS) chains onto the core protein of syndecans, glypicans, and other HS-rich proteoglycans [14–16]. Because the EXT1 and EXT2 proteins are organized as a complex and are both required for HS synthesis, heterozygous mutations of either *EXT* gene result in a systemic HS deficiency of about 50% in HME patients [17]. Such partial deficiency can affect diverse physiologic processes and mechanisms including lipid metabolism and clearance in patients [18••] but appears to be insufficient to trigger osteochondroma formation [7, 19]. In line with the Knudson hypothesis of tumorigenesis [20], osteochondroma formation in HME requires a "second hit" that would further lower the HS levels, render the affected local cells unable to retain a normal phenotype, and turn them into tumorigenic cells [7, 19]. Genetic studies and single-cell analyses have indicated that loss-of-heterozygosity (LOH), aneuploidy, or other changes can occur in human osteochondromas, rendering resident cells *EXT1*- or *EXT2*-null [21–24]. Concurrent studies by others have described patients with alternative genetic changes, including compound heterozygous *EXT1* and *EXT2* mutations [25–27]. Such divergent genetic scenarios and possible ensuing pathogenic mechanisms in patients correlate well with mouse studies we and others conducted over the last few years. Single heterozygous $Ext1^{+/-}$ or $Ext2^{+/-}$ mutant mice were found to be largely normal [28, 29••] confirming the notion that a single heterozygous *Ext* mutation is not sufficient to trigger full-fledged HME. However, compound heterozygous $Ext1^{+/-};Ext2^{+/-}$ mutant mice and conditional *Ext1*-null mice (both producing much less HS than single heterozygous mice in affected tissues) did display multiple osteochondromas and mimicked the human HME phenotype in several respects [29••, 30••, 31••, 32]. Thus, osteochondroma formation requires a second genetic event and a major decrease in—but not necessarily a complete loss of—HS.

Unlike glycosaminoglycans such as hyaluronate, the HS chains are exceedingly complex macromolecules [14, 15, 33]. During the EXT1/EXT2-mediated HS chain polymerization onto serine residues along the proteoglycan core protein, the chains undergo a concurrent series of modifications and reactions carried out by a large array of enzymes, resulting in N-acetylglucosamine deacetylation and N-sulfation, epimerization of glucuronic acid into iduronic acid, and O-sulfation at position C2, C3, or C6 in the glucosamine residues [14, 15, 33]. These concerted processes produce chains with unique patterns of sulfation and sugar modifications in 6–12 sugar residue-long segments flanked by segments of largely unmodified and unsulfated sugars [33], thus eliciting a significant degree of structural heterogeneity and uniqueness among HS chains. Further processing of the chains can occur at the cell surface where the endosulfatases SUF1 and SULF2 can remove specific sulfate groups [34, 35]. Because the intracellular and cell surface-modifying enzymes are expressed in tissue-specific manners, the overall output and structures of HS chains can differ in different tissues and cells [33]. Studies have shown that the HS chains—and the proteoglycans of which they are part—exert a large number of developmental and physiologic functions [14, 33]. Prominent and essential is their ability to interact with key signaling proteins, including members of the fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and hedgehog families, and regulate protein distribution, diffusion, turnover, bioavailability, and action on target cells and tissues [36–38]. Signaling proteins all have a specific HS-binding domain [36, 38]. Notably, HS deficiency was originally shown to cause a broad distribution of, and unruly and widespread action by, Indian hedgehog (Ihh) within mouse long bone growth plates [39]. More recent studies by our group with a mouse model of HME indicated that conditional *Ext1* ablation resulted in broad and ectopic canonical BMP signaling in the perichondrium along long bone growth plates that was followed by ectopic chondrogenesis and osteochondroma-like development, thus linking excess BMP signaling to osteochondroma formation [40].

The above synopsis makes it clear that the last few decades of dedicated work on HME and HS biology and chemistry have provided fundamental insights into a variety of important processes and mechanisms relevant to disease pathophysiology and osteochondroma development. The present review aims to delineate and analyze examples of recent clinical and laboratory studies of the last few years that have contributed important new information and insights into the spectrum of HME complications as well as insights into plausible paths toward biologically based therapies for HME.

Quality of Life and Health Complications

The multiple osteochondromas (exostoses) from which HME takes its name are for the most part benign outgrowths along the skeleton. However, because they are physically associated to the growth plates, they have the potential and propensity to interfere with skeletal elongation and function, causing growth retardation, malformations, and bending of neighboring skeletal elements such as ulna and radius [1, 4, 41, 42]. Because of their significant size and number, the mature osteochondromas can also cause impingement of surrounding tissues and structures including muscles, tendons, vessels, skin, and spinal cord, creating additional and potentially severe physical issues and even cosmetic concerns. These and other symptoms and complications of HME are well established and widely appreciated (Table 1), but recent

Table 1 Spectrum of clinical symptoms and complications among HME patients

Nature and location	References
Skeletal alterations in limbs, chest, and spine	[2, 4–7]
Coxa valga	[2, 43, 44]
Limb length discrepancies	[4]
Spinal stenosis	[45]
Scoliosis	[46•, 47]
Short stature	[1, 41]
Nerve, tendon, and vessel impingement	[2–4]
Wound-healing abnormalities	[48]
Social and quality of life difficulties	[49••, 50, 51]

The above list is not comprehensive and is meant to convey the encompassing pathological nature of HME. Note that symptoms and complications can vary significantly in incidence and severity from patient to patient. The cited studies are examples of studies carried out in each area

clinical studies have contributed important insights and an awareness of additional potential health concerns as well as social difficulties for the patients.

In a particularly thorough study [49••], Goud and collaborators reached out to all 322 known HME patients in the Netherlands and asked them to participate in an age-specific questionnaire, focusing on their well-being, daily activities, school and professional engagements, and physical symptoms including pain. The majority of adults and about a third of the children completed the survey, and data were closely assessed and tested for statistical significance using a variety of methods. While the majority of adult HME patients were employed, an appreciable number of them had changed jobs because of physical difficulties or the need to adjust to specific—and at times challenging—work environments. A small, but significant, number of the patients were medically unfit to work. Overall quality of life assessment using RAND-36 scales [52] indicated that the patients had lower scores in several categories compared to control groups. Nearly half of the HME children had difficulties in school, limiting participation in sports and other activities. A common problem among all young and adult patients was persistent pain. This study points to the pervasive and intrusive nature of HME that can negatively affect quality of life, social and personal well-being and activities, and even self-esteem. The observations and insights correlate quite well with those in a subsequent study of about 100 HME patients (57 adults and 42 children) conducted by Chhina et al. [50]. Using health-related quality of life (HRQL) assessment methods adjusted for age, the authors found that HME patients had lower scores in all domains compared to relevant Canadian and US control populations, with the exception of emotional role limitations. These and other data provide an appreciation not only for the encompassing nature of HME and its effects at multiple health levels (Table 1) [51] but also a need for a closer clinical attention, continuous monitoring of patients over time, and appropriate remedies for diverse needs including early intervention.

The above studies make clear that the musculoskeletal problems of HME can affect patients at multiple levels, and recent studies have further analyzed how insidious those physical difficulties can actually be. One common skeletal complication of HME is coxa valga [43], a deformity of the hip characterized by an increase in the angle between the femoral neck and shaft. Over time, this deformity can have increasingly negative effects on skeletal function, including hip joint malfunction and subluxation, and quality of life. A recent interesting study of young HME patients aimed to follow the developmental patterns and changes of the hip over time in relation to initial physical assessment and evaluation [44]. The patients (mean age 6.0) were divided into two groups according to Hilgenreiner epiphyseal angle (HEA) at presentation and were reassessed over time using HEA, neck-shaft angle (NSA), acetabular index (AI), center-edge angle (CEA), and other criteria via systematic physical and radiographic evaluation. The data indicated that there was a significant correlation between HEA at presentation and NSA at skeletal maturity, indicating that initial developmental and pronounced abnormalities can have ever more deleterious and lasting consequences over time. The study reaffirms a need for, and importance of, careful clinical assessment at presentation and early intervention.

Osteochondromas developing along the intracanal surface of the vertebrae can be particularly worrisome since they may impinge on, and compromise the function of, the spinal cord and lead to nerve damage, physical weakness, and progressive impediment of motion [4, 45, 53]. Because of the relatively high incidence of intracanal osteochondromas in HME patients and potential long-term problems [53], a previous study recommended that all HME children be subjected to routine MRI or CT screening of the spine at presentation [45]. A recent and rather unique study [46•] has focused on another possible spine complication in HME patients: scoliosis. This condition is not usually included in natural history analyses of common HME clinical symptoms [1, 2, 4], and a previous study associated scoliosis as secondary to limb skeletal defects [54]. In the new study, Matsumoto et al. clinically examined 50 HME patients (20 females and 30 males) with a mean age of 28.1 years (range 2–77 years). Based on their own disease severity classification that took into consideration deformities, functional limitation, and number of osteochondroma-containing anatomical sites, the patients were subdivided into class I (least affected) to class III (most affected) groups. Interestingly, scoliosis was observed in about 70% of all patients who exhibited a mean primary curve of 15.3° and a mean minor curve of 10.6°. Scoliosis severity spanned King type I to type IV, but no type V [55], and statistical analyses

suggested that moderate scoliosis was significantly related to disease severity and more prevalent in class III patients but was not related to sex, number of osteochondromas, or limb deformity. The authors concluded that mild to moderate scoliosis is actually common among HME patients and proposed that defective FGF signaling—consequent to HS deficiency—may underline or contribute to scoliosis. Given previous natural history studies that did not emphasize scoliosis as a major disease complication [1, 2, 4], the data in this potentially very important study would need to be verified and extended to other patient cohorts. Also, the proposed mechanistic basis of scoliosis, as being due to defective FGF signaling, remains speculative at this point.

Spectrum and Roles of *EXT* Mutations

As indicated above, studies dating back over 20 years have established that the majority of HME cases are associated with heterozygous loss-of-function mutations in *EXT1* or *EXT2*, with most of the mutations being familial and about 10% sporadic [10–13]. On the basis of those findings, HME is normally defined as an autosomal dominant disorder; yet, formation of its most conspicuous skeletal trait—the osteochondromas—appears to require a second hit as summarized above. Recent studies from several groups including ours have shed additional light on the spectrum of *EXT* mutations in specific patient cohorts and their functional ramification and significance in pathogenesis.

Following the original studies that linked *EXT* genes to HME [10, 12] and to HS synthesis [56, 57], subsequent studies reinforced those findings and identified numerous nonsense, frame shift, missense, and splice-site mutations in *EXT1* and *EXT2* in HME patients [13, 26, 27], currently numbering over 650 registered in the Multiple Osteochondroma Mutation Database (MOdb) [26] (url: http://medgen.uantwerpen.be/LOVDv.2.0/). Invariably, mutations in *EXT1* have been found to associate with a more severe HME musculoskeletal phenotype as defined by the number of osteochondromas, anatomical sites affected, deformities, and physical limitations [26, 27, 42]. The more severe consequences of *EXT1* mutations compared to *EXT2* mutations likely reflect the fact that the EXT1 protein has a major catalytic function in HS polymerization, while EXT2—though required—may have structural or supportive roles within the Golgi-associated EXT1/EXT2 complexes [58]. We should note that despite several attempts, there is still no widely accepted system to evaluate and classify disease severity among HME patients [8, 59], and similarly, there is no clear and reliable genotype-phenotype correlation [60, 61]. Such difficulties may be due to the fact that clinically, HME is highly variable even within members of a family sharing a common mutation and among unrelated patients with the same mutation [61], making it difficult to critically assess and establish severity and disease course and raising the possibility that genetic background and the nature of "second hits" have a major influence in HME.

Recent studies have provided further insights into the population genetics of HME by examining cohorts of patients in different regions of the world. Ishimaru et al. analyzed 112 patients in 71 HME families in Japan and carried out PCR genomic analysis of DNA samples of peripheral blood [62]. The authors identified mutations in *EXT1* or *EXT2* in 47 families only (about 65% of total), while the remainder did not exhibit coding mutations in either gene, at least with the methodology used. *EXT1* mutations were more prevalent than *EXT2* mutations, and 22 out of a total of 52 mutations were novel. Of interest was the fact that a few families (3/71) had heterozygous mutations in both *EXT1* and *EXT2*, confirming the occurrence of this relatively rare genotype first reported in previous studies [26, 27]. The most common mutations observed were inactivating—frame shift, nonsense, and splice site—while about 40% were missense mutations.

Composition and familial distribution of *EXT* mutations in this study differ from those observed in other national cohorts of HME patients. In the first mutational analysis of HME patients in Spain, Sarrion et al. analyzed 39 unrelated HME patients [63]. They detected a heterozygous mutation in *EXT1* or *EXT2* in 95% of them, with 18 mutations being novel, and the vast majority of the mutations were in *EXT1* (74%). Five of the *EXT1* mutations were deletions that were identified by multiplex ligation-dependent probe amplification (MLPA) assays. Two patients had concurrent changes in both *EXT1* and *EXT2*. Interestingly, patients with missense mutations exhibited a lower number of osteochondromas at presentation compared to patients with other types of mutations, including nonsense and frame shift, hinting to the possibility that missense mutations are more tolerated and less pathogenic. An additional interesting finding was that patients with *EXT2* mutations were more affected than those with *EXT1* mutations based on osteochondroma number as an index of disease severity. The latter finding is at variance with what has been consistently observed in previous studies, and the authors do point out that this difference was likely due to the small number of patients examined.

In a first analysis of HME patients in Poland, Jamsheer et al. investigated 33 unrelated HME patients [64]. Using Sanger sequencing and MLPA, they detected a heterozygous mutation in the coding exons of *EXT1* or *EXT2* in about 55 and 30% of patients, respectively, with 15 mutations being novel. The incidence of mutations in this cohort is thus similar to that observed in larger population studies previously [26]. Lastly, Ciavarella et al. studied a large cohort of HME patients in the southern part of Italy, and using a combination of exon sequencing, MLPA, and array CGH analyses, they identified 66 mutations and one large *EXT2* deletion, amounting to an

overall incidence of about 75% [65]. Twenty mutations as well as the *EXT2* deletion were novel. In line with previous studies, the *EXT1* mutations were found to be located all along the protein backbone, while *EXT2* mutations were more centered toward the N-terminus. Computer-assisted 3D modeling and protein modeling algorithms were used to predict the impact of the *EXT1* missense mutations on protein folding and enzymatic function.

When considered together, the above studies reaffirm the notion that the majority of HME cases are linked to heterozygous *EXT* mutations and most often *EXT1* mutations, regardless of the specific region of the world considered. They underline the still puzzling fact that rather than being concentrated in hot spots, the *EXT1* mutations are scattered all along the coding region and continue to grow in number, bringing to fore lingering questions about how each mutation—and particularly missense mutations—would interfere with protein structure and function, whether and which of the mutations could lead to a dominant-negative effect, and how different cell types and tissue would respond to each mutation. What the above studies and previous analogous studies did not address is what may be the cause of HME in the sizable number of patients without apparent *EXT1* or *EXT2* coding mutations. Likewise, the studies did not tackle the issue of how each *EXT* mutation—be congenital or de novo—would interact and cooperate with a "second hit" needed to trigger cell transformation of skeletogenic cells and osteochondroma formation. Depending on the nature of the second hit, the primary *EXT* mutation could conceivably elicit different outcomes in terms of extent of osteochondroma formation, course and severity of HME, and even progression to malignancy. To date, the best documented type of second hit in human osteochondromas is LOH [21–24], but mouse studies have shown that in addition to conditional *Ext*-null mice, double heterozygous $Ext1^{+/-};Ext2^{+/-}$ mice also develop osteochondromas and a HME-like phenotype [29]. Thus, it is pertinent to ask: Are compound heterozygous *EXT1* and *EXT2* mutations sufficient to drive osteochondroma formation in patients or is there still a need for an additional hit such as LOH? Do all osteochondromas in a given patient share the same genetic changes or not? It is important to point out here that in general, LOH has a fairly high incidence in mitotically active tissues and is of course random [66, 67]. Could cells in tissues and organs other than growth plate and perichondrium undergo LOH or another second hit in HME patients? Could these mutant cells misbehave and cause other symptoms and disease manifestations such as defective skin healing or local pain [48]? Notably, Yamaguchi and coworkers did show that homozygous *Ext1* ablation in postnatal neurons causes symptoms of autism in mice [68••].

With a few rare examples [16], the biological consequences of most *EXT* mutations on protein function have not been tested directly but are usually predicted and inferred based on structural considerations. While nonsense and frame shift mutations can more readily be envisioned as being markedly detrimental to protein function, missense mutations are more challenging. As reiterated by the recent study by Ciavarella et al. [65], computer-assisted analyses were able to predict detrimental effects for some, but not all, *EXT1* missense mutations. The uncertainties of the current situation raise the pertinent question as to whether all the previously reported *EXT* mutations are in fact pathogenic or whether some may actually be variants with minimal to no detrimental effect. One way to test that question would be to determine in silico whether such "mutations" occur in otherwise healthy individuals. In a very recent study, we did carry out such analysis [69••]. We used the Exome Aggregation Consortium (ExAC) [70], a data bank that has amassed genomic sequences from the protein-coding portions of the genome from over 60,000 individuals without severe pediatric disease (specifically screened out) and representing diverse populations of European, Asian, African, and Latino ancestries. Using this resource along with the HME MOdb [26], we extracted all the amino acid-altering missense variants and nonsense, frame shift, or splice-site variants of *EXT1* and *EXT2* from both the ExAC and MOdb and identified coding variants that were present in both. We found that six *EXT1* and four *EXT2* missense mutations previously described in HME patients and listed in the MOdb were also present in the ExAC, suggesting that these variants have either been misclassified as pathogenic mutations or are not fully penetrant.

Osteochondroma Genesis and Therapeutic Targets

By being the most overt symptom and common health complication of HME, osteochondromas have been the focus of much research attention. Their onset initiates with the formation of ectopic growth plate-like cartilage oriented approximately at a 90° angle with respect to the adjacent endogenous growth plate. The ectopic cartilaginous masses continue to elongate over time, undergo ossification in their proximal portion, and maintain a cartilaginous cap distally, even when overall growth ceases. It was originally thought that osteochondromas derive from growth plate chondrocytes located along the border with perichondrium, as reviewed previously [7]. We and other groups suggested that instead, osteochondromas originate from progenitor cells located within the inner layer of the perichondrium [9, 40, 71], in line with many studies showing that such layer is a repository of progenitor cells normally engaged in lateral appositional growth or tissue repair [72]. Whatever the exact source of cells producing the osteochondromas, what could be the underlying mechanisms of regulation and could such mechanisms represent plausible therapeutic targets?

Two previous important studies made use of advanced transgenic mouse approaches to tackle these questions. To directly

test whether somatic LOH is able to drive osteochondroma formation, Jones et al. [30] created new transgenic mouse lines containing head-to-head *loxP* sites flanking exon 2 in *Ext1* (termed $Ext1^{e2fl/e2fl}$). Transient exposure to *Cre* recombinase was expected to yield a stochastic distribution of forward and inverted flanked segments [73] and generate chimeric tissues containing a low prevalence of inversion of both alleles in some cells. The authors induced *Cre* expression by including doxycycline in the drinking water of compound $Ext1^{e2fl/e2fl}$;*Col2-rtTA-Cre* mice from postnatal day 8 to day 15 (P8 to P15). *Cre* expression driven by *Col2* regulatory sequences targets both growth plate and neighboring perichondrial cells [74]. Multiple osteochondromas did form in the knees and rib cage over a 4- to 8-week period 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 verified by immunohistochemistry. Interestingly, when the authors examined $Ext1^{e2fl/e2fl}$;*Osterix-CreER*^{T} mice that express *CreER*^{T} in hypertrophic chondrocytes and bone cells [75], tamoxifen treatment did not elicit the formation of osteochondromas.

In a concurrent study, Matsumoto et al. [31] used standard floxed *Ext1* mice and mated them with *Col2-CreER*^{T} mice [76] expected to express *Cre* recombinase only after tamoxifen treatment. However, the authors detected a low-level leakiness of the transgene in the absence of tamoxifen exposure that caused a random deletion of *Ext1* in a small number of *Col2*-expressing cells, likely growth plate chondrocytes as well as adjacent perichondrial cells as pointed out above [74]. They found that numerous osteochondromas had formed in long bones and ribs of compound $Ext1^{f/f}$;*Col2-CreER*^{T} mice by 1 month of age, but not in heterozygous $Ext1^{f/+}$;*Col2-CreER*^{T} mice or those lacking the transgene. In line with other studies [23, 24], cells comprising the osteochondromas turned out to be a mixture of mutant and wild-type cells, affirming the notion that mutant cells can recruit surrounding healthy cells into tumor formation and growth. Together, the above important studies by Jones et al. and Matsumoto et al. have provided the most direct evidence to date that stochastic loss of both *Ext* alleles can trigger osteochondroma development and that occurrence of such event in a small number of cells is sufficient for tumor formation.

Though highly revelatory and original, the above studies did not provide detailed information into possible specific cells and mechanisms that underlie the genesis and growth of osteochondromas. To tackle this key issue, we carried out a series of studies in which we specifically targeted cells within the perichondrium flanking the growth plate in developing long bones [19, 40]. We asked whether conditional ablation of *Ext1* in those cells was sufficient to provoke osteochondroma formation and what cellular mechanisms may be involved. As *Cre* deleter, we used *Gdf5Cre* mice that strongly and constitutively express *Cre* recombinase in the perichondrium flanking the epiphyseal portion of growth plates [40], being unique in that regard. We found that multiple osteochondromas did develop in $Ext1^{f/f}$;*Gdf5Cre* mice over time and initially had a typical growth plate-like cartilaginous organization. A similar induction of osteochondroma formation occurred when long bone anlage explants from E18.5 $Ext1^{f/f}$ mouse embryos were locally treated with either adeno-Cre (to ablate *Ext1*) or Surfen, a small compound HS antagonist [77]. Interestingly, osteochondroma initiation in vivo or in vitro was preceded by ectopic and excess BMP signaling within the perichondrium as indicated by phosphorylated SMAD1/5/8 levels. Treatment with adeno-Cre or Surfen also triggered excess chondrogenesis and cartilage nodule formation and overexpression of chondrogenic master and matrix genes in $Ext1^{f/f}$ limb bud mesenchymal cells in micromass culture. Surface plasmon resonance assays showed that interference with HS function by Surfen reduced the physical association and interactions of recombinant BMP2 with HS and increased cell responsiveness to endogenous and exogenous BMP proteins. Together, our studies strongly suggested that osteochondromas originate from perichondrial cells and that, normally, a main function of Ext1/HS in the perichondrium is to restrain and limit BMP signaling, a major prochondrogenic pathway [78]. Thus, a similar excessive BMP signaling could occur in HME as a consequence of major local drops in *EXT* expression and HS levels. Notably, HS normally promotes—and is required for—FGF signaling, a major antichondrogenic pathway expressed in several noncartilaginous tissues including the perichondrium [79–81]. Thus, while boosting BMP signaling, the HS deficiency in HME could dampen FGF signaling, and such combination could change the phenotype of perichondrial cells, induce ectopic chondrogenesis, and lead to osteochondroma development (see model in Fig. 1).

The cancer literature has previously shown that there is an inverse relationship between *EXT* and *heparanase* expression in several types of cancer cells [82–84]. This is somewhat paradoxical and counterintuitive and indicates that when the ability of cells to produce HS decreases as a result of decreased *EXT* expression, the cells upregulate the expression of heparanase to decrease the HS levels even further. In line with those cancer studies, human osteochondromas were shown to contain higher levels of heparanase protein and RNA compared to growth plate cartilage from healthy donors [85••]. To verify these important findings, we used immunohistochemistry and did in fact find that heparanase was widespread and readily detectable in all chondrocytes within osteochondroma specimens from HME patients but was much lower and

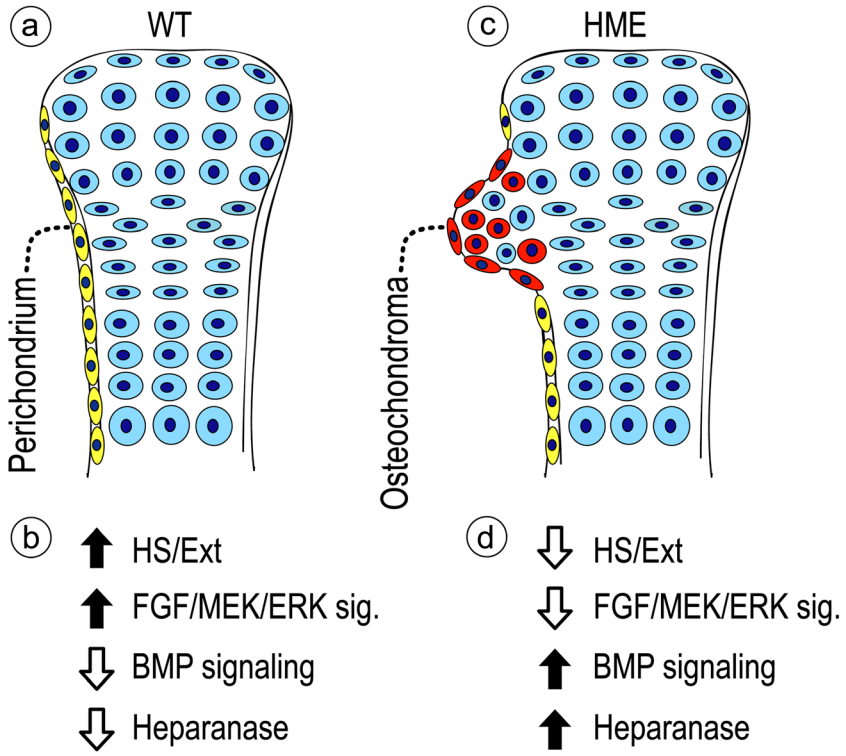

Fig. 1 The schematic illustrates a series of regulatory steps that could cause inception and promotion of osteochondroma formation and growth in HME patients. (**a**) In healthy wild-type (*WT*) circumstances, the perichondrium (in *yellow*) would delineate the boundary with, and closely flank, the growth plate (in *blue*) of skeletal elements such as long bones. The perichondrial cells would be characterized by typical mesenchymal and fibroblastic traits including the following: a flat cell morphology, normal EXT expression and HS levels, strong antichondrogenic mechanisms including FGF expression and ERK/MEK signaling, and low activity/expression of prochondrogenic mechanisms including BMP signaling and heparanase. (**b**) *Up and down arrows* depict the high and low levels of phenotypic traits in normal perichondrium. (**c**) During the course of HME, LOH or another second hit would cause a steep and nearly complete loss of EXT expression and/or HS levels in local cells (in *red*) along the perichondrial border with the heterozygous EXT mutant growth plate. This would result in steep decreases in antichondrogenic pathways and reciprocal increases in prochondrogenic pathways and heparanase expression in the mutant cells (depicted in **d**), thus altering their homeostatic mechanisms and triggering differentiation into round-shaped chondrocytes (in *red*). The growing osteochondromas would contain a mixture of mutant (*red*) and heterozygous (*blue*) cells, the latter being recruited into the osteochondroma forming process by the mutant cells. The changes occurring in the activity of the BMP and FGF signaling pathways and in the expression of heparanase could each offer a plausible therapeutic target and strategy to block osteochondroma inception and/or growth

restricted to hypertrophic chondrocytes in normal growth plate [86••]. To uncover possible mechanistic implications and significance, we used recombinant human heparanase and found that it markedly stimulated chondrogenesis and BMP signaling in limb bud mesenchymal cell micromass cultures. It also stimulated cell migration and proliferation. Strikingly, treatment of the cultures with either bacterial heparitinase or Surfen upregulated endogenous heparanase gene expression. Thus, we tested the potent heparanase inhibitor SST0001 [87] and found that it strongly inhibited chondrogenesis in vitro. Taken together, our data and those in a previous study [85••] suggest that heparanase is a potential important culprit in HME. Its broad expression in osteochondromas and its ability to stimulate chondrogenesis, BMP signaling, cell migration, and cell proliferation could be causally linked to osteochondroma genesis and growth (see model in Fig. 1). The data also suggest that heparanase could represent a "second hit" in HME pathogenesis as well as a therapeutic target.

Conclusions and Perspectives

As underlined at the outset of this review, the intricacies and unresolved issues of HME continue to pose major challenges for both clinicians and biomedical researchers. However, there is little doubt that significant progress has been made over the last few years. It is now clear and increasingly appreciated that HME is not simply a musculoskeletal disorder. Its pervasive, progressive, and aggressive nature and its effects on a number of tissues and processes can have more global and systemic consequences on the health, well-being, social interactions, and personal perception in patients (Table 1), requiring greater sensitivity and attention by caregivers and possible early intervention on certain symptoms before they worsen. The

mutational spectrum affecting the *EXT* genes, and *EXT1* in particular, continues to grow, making it clear that these genes are broadly vulnerable. The data provide further impetus and urgency to a need to clarify how each mutation contributes to HME pathogenesis and in which manner each interacts with a second hit to elicit osteochondroma formation. Our recently published population study [69••], however, raises a note of caution in this regard and indicates that some clinically identified missense variants implicated in HME may have been misclassified as pathogenic mutations; thus, each variant will indeed need to be tested directly to provide conclusive evidence of pathogenic relevance and potency. Mouse models of HME have now provided fairly unequivocal evidence that loss of both *EXT* alleles—as it would occur in patients via LOH—is one powerful and sufficient mechanism by which skeletogenic cells can turn tumorigenic and produce osteochondromas. It will be interesting and important to extend those analyses to patients and determine whether LOH is a common feature of osteochondromas, whether other types of second hits occur, and how consistent such changes are from osteochondroma to osteochondroma in the same patient. LOH was detected in about 60% of the osteochondromas (6/8) analyzed by Reijnders et al. [23], and the authors suggested that the cellular heterogeneity of the tumors may have prevented LOH detection in 100% of the samples. This is certainly possible, but it is also plausible that other second hits could occur in HME [24]. Relevant data and insights in this area could help to understand in greater details and depth: how exactly such genetic changes and ensuing drop in HS levels derange the phenotype of skeletogenic cells; which protein signaling pathways are affected and in what manner; what additional mechanisms may be involved; why the affected HS-deficient cells lose their normal functions and differentiation potentials; and how they turn into tumorigenic cells able to even recruit normal surrounding cells into tumor growth. Lastly but importantly, it is still unknown whether osteochondroma formation can be inhibited, prevented, or even reversed and whether the HS deficiency could be corrected. Some hope comes from the recent mouse studies described above [40, 86••, 88] indicating that pharmacologic interference with either BMP signaling or heparanase action markedly inhibited chondrogenesis. Given that chondrogenesis is the very first step in the formation of osteochondromas, such interventions could in turn inhibit osteochondroma growth altogether (Fig. 1). Other signaling pathways, including the hedgehog or FGF pathway, could offer additional strategies [23, 89, 90]. Formal proof-of-principle evidence of effectiveness of such putative treatments in HME animal models is still lacking, but given the pace of current research, such evidence is surely within reach, making it possible to begin to envision clinical trials.

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Compliance with Ethical Standards

Conflict of Interest M. Pacifici declares that he is part of a patent application on heparanase as a putative therapeutic target in HME.

Human and Animal Rights and Informed Consent All studies involving laboratory animals or human subjects were performed after approval by the appropriate institutional review boards. When required, written informed consent was obtained from all participants.

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