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# Multiple Hereditary Exostosis, EXT Genes, and Skeletal Development

By Linda J. Sandell, PhD

**M**ultiple hereditary exostosis is an autosomal dominant inherited disease in which osteochondral growths occur on the periphery of bones. These growths are comprised of bone surrounded by a cap of cartilage. A small number of these exostoses proceed to a low-grade chondrosarcoma<sup>1,2</sup>. Although the disease can occur spontaneously, it has been estimated that 80% of affected individuals have a positive family history<sup>2</sup>.

Research on the genetics of multiple hereditary exostosis over the past thirty years has been productive. Advances in the understanding of the disease have paralleled the methodological advances that have occurred in the field of molecular genetics. Initially, it was recognized that multiple hereditary exostosis is often inherited and that large families were available for genetic mapping. As techniques for gene mapping improved, regions of the chromosomes involved were identified, localized, and eventually subjected to DNA sequencing. The genes identified, the exostosins, were found to encode known enzymes whose function within the disease could be reasonably predicted. Mouse models were created, and the hypothesized function of these genes was verified. Many surprises were encountered along the way, which served to uncover important biological principles. The understanding of human multiple hereditary exostosis is a paradigm for the power of combining modern molecular biology, genetics, and clinical science.

## The Genetics of Multiple Hereditary Exostosis

**I**n the early 1990s, the clearly autosomal dominant inheritance of multiple hereditary exostosis was recognized by clinicians and DNA techniques became available to localize the inheritance patterns in the DNA. By genetic linkage analysis, Hecht et al. and Le Merrer et al. were able to localize the inheritance patterns of these families to three chromosomal locations: 8q24.1, 11p11-13, and 19p<sup>2,3</sup>. These genes were identified as tumor-suppressor genes, and loss of heterozygosity in these regions was associated with transformation into chondrosarcoma<sup>2,4</sup>.

The genes that cause multiple hereditary exostosis were called exostosins and named EXT1, EXT2, and EXT3. EXT1 and EXT2, corresponding to the chromosomal localizations on

chromosomes 8 and 11, respectively, were identified by positional cloning<sup>5,6</sup>, whereas EXT3 has not yet been identified and its linkage to patients with multiple hereditary exostosis has been questioned<sup>7</sup>. Based on DNA sequence homology, genome screens have uncovered additional members of the gene family, three EXT-like (EXTL) genes, bringing the total number of similar genes to six. However, only EXT1 and EXT2 have been associated with both familial multiple hereditary exostosis and spontaneous multiple hereditary exostosis, and more than 80% of unrelated patients who have been tested have a mutation in one of these two genes<sup>2,4</sup>.

## Function of EXT Genes

**T**he EXT1 and EXT2 genes encode two glycosyltransferase subunits of the heparan sulfate (HS)-synthesizing system that elongates HS chains to specific proteins belonging to a class called proteoglycans. The HS chain is a linear glycosaminoglycan made up of alternating D-glucuronic acid (GlcAc) and N-acetyl-D-glucosamine (GlcNAc) subunits. The synthesis of HS chains is a very complex post-translational event, initiated with a protein linkage through serine. The HS chains are elongated with the alternating GlcAc and GlcNAc residues via a complex of enzymes that includes the EXT1 and EXT2 glycosyltransferases. The length of the chains can vary and appears to be cell specific. Mutation in the glycosyltransferase genes, usually by causing a frame-shift in protein elongation or missense in amino acid code, creates truncated forms of the enzymes that those genes encode<sup>6,8</sup>, leading to lower enzyme activity and less HS chain synthesis. In fact, chondrocytes isolated from persons with multiple hereditary exostosis contain less enzyme<sup>9</sup>.

The connection between HS-synthesizing glycosyltransferases and the multiple hereditary exostosis genes was made by two independent studies. EXT1 was demonstrated to encode for a protein that could rescue HS biosynthesis in an HS-deficient mutant cell line<sup>10</sup>. EXT2 was identified as an HS co-polymerase purified from bovine serum<sup>11</sup>. Mutations in EXT1 and EXT2 result in the formation of clinically indistinguishable exostoses. While both enzymes are able to transfer GlcNAc and GlcAc, they are not functionally redundant

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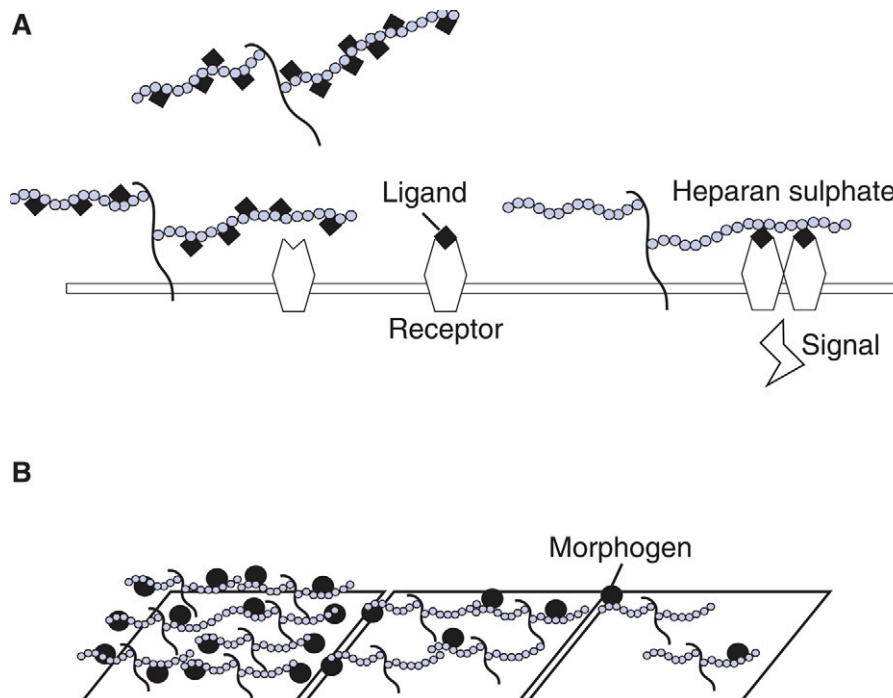


Fig. 1  
Function of HS proteoglycans. Syndecans are transmembrane or extracellular matrix proteoglycans made up of alternating GlcAc and GlcNAc carbohydrate residues attached to a protein backbone. A: Binding of growth factor ligands (black squares) to HS chains (circles). B: Distribution of morphogen (black circle) attached to HS chains (white circles) of proteoglycan<sup>22</sup>. (Reprinted, with permission, from: Nadanaka S, Kitagawa H. Heparan sulphate biosynthesis and disease. *J Biochem.* 2008; 144:8.)

in vivo; instead, EXT1 and EXT2 seem to be a complementary pair that forms a stable enzyme complex in vivo. The EXT1/EXT2 complexes have considerably higher glycosyltransferase activity than either EXT1 or EXT2 alone<sup>10</sup>.

The products of the EXTL genes are able to transfer GlcNAc<sup>12,13</sup> and are thought to be involved in transferring the first GlcNAc residue to the linkage region to initiate HS synthesis. While the roles of these enzymes have not been completely determined in vivo, at least EXTL2 and EXTL3 may have additional biological functions in the regulation of HS synthesis and the determination of chain length but are not necessary for initiation and elongation of HS chains.

### Function of Heparan Sulfate

The HS chains of these proteoglycans are responsible for a variety of functions primarily involving carbohydrate-protein interactions. Heparan sulfate chains are found on a variety of proteoglycans, including the large proteoglycan versican, found primarily in blood vessels; perlecan, the major proteoglycan in basement membranes and many other tissues, including the developing limb and chondrocytes; and smaller proteoglycans called syndecans. Syndecans are found in most tissues and occur in a family of four proteins (Fig. 1, A)<sup>14</sup>; they are found at the cell surface bound into the membrane as receptors or in the extracellular matrix.

At the cell surface or in the extracellular matrix, the proteoglycans act as ligands or co-receptors where the HS chains are necessary for receptor recognition and binding<sup>15</sup>. HS proteoglycans are known to be necessary for signaling of fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF- $\beta$ ) and are involved in the gradient formation of morphogens such as hedgehog or bone morphogenetic proteins (BMPs) (Fig. 1, B). HS proteoglycans also influence the formation of amyloid fibrils in the brain and help to incorporate lipoproteins into cells in the liver. The distribution and function of proteins that contain HS chains are extensive, and not all have been well characterized. Since these proteoglycans occur throughout the body, it is unclear why mutations in the genes coding for HS chains cause multiple hereditary exostosis, a phenotype apparently restricted to bone.

### Lessons from Developmental Biology: Drosophila and Mice

Most of the insight into the function of HS proteoglycans and the role of the EXT genes has come from studies in *Drosophila* and mice, as a result of genetic manipulations. In *Drosophila*, there are three orthologs of mammalian EXT genes, EXT1 (tout-velu), EXT2 (sister of tout-velu), and EXTL3 (brother of tout-velu). The phenotypes resulting from muta-

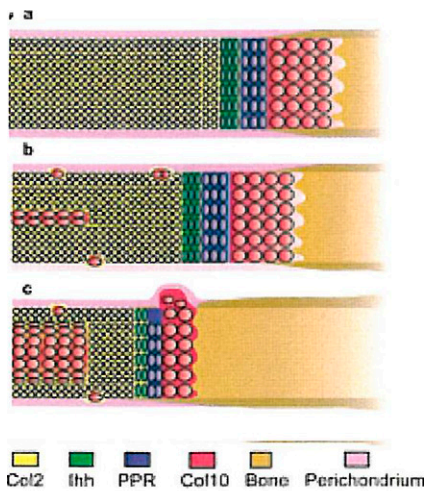
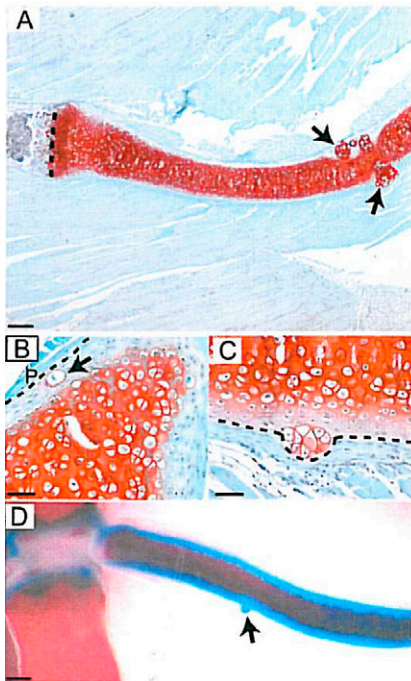


Fig. 2

Hypothesis for creation of exostoses from dysregulated chondrocytes. Chondrocyte nests in the  $Ext2^{+/-}$  heterozygote. **Top panel:** a: Normal growth occurs until embryonic day 14.5 of long-bone development. b: Due to dysregulated growth-factor signaling, isolated chondrocytes in the periphery of the developing cartilage anlagen undergo terminal differentiation by proliferation and hypertrophy. c: When exposed to the vasculature from the passing growth plate, cells undergo endochondral bone development. **Bottom panel:** A: Safranin O stained longitudinal section of a rib of the  $Ext2^{+/-}$  mouse, showing nodules (arrows) that are forcing the perichondrium to bulge out. B: Safranin O stained transverse section through the rib, showing a single displaced chondrocyte (arrow) near the perichondrium (P). C: Higher magnification of safranin O stained longitudinal section, showing a nodule. D: Low magnification of alcian blue (cartilage) and alizarin red (bone) staining of a rib of an  $Ext2^{+/-}$  mouse, showing a large nodule (arrow). Scale bars: 200  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in B and C; and 300  $\mu\text{m}$  in D. Reproduced and adapted with permission from: Stickens D, Zak BM, Rougier N, Esko JD, Werb Z. Mice deficient in *Ext2* lack heparan sulfate and develop exostoses. *Development*. 2005;132:5062, 5066. Reprinted with permission.



tions in these genes provided insight into the primary functions of HS proteoglycans. In all of these mutant phenotypes, the morphogenetic gradients of the *Drosophila* proteins Hedgehog (mammalian homolog *hg*), Wingless (mammalian homolog *Wnt*), and Decapentaplegic (mammalian homolog *BMP*) were interrupted, causing these proteins to accumulate in front of the mutant cells<sup>16</sup>. All three protein pathways are involved in both early embryonic development and mammalian skeletal development. Targeted deletion of the *Ext1* gene in mice (mouse genes are indicated with lower case letters) abolishes the synthesis of HS<sup>17</sup> and disrupts signaling of FGFs, TGF- $\beta$ , Wnts, and BMPs and results in early embryonic lethality around the time of gastrulation. Mice that are heterozygous for the *Ext1*

deletion show somewhat increased chondrocyte proliferation and delayed hypertrophic differentiation, probably due to increased Indian Hedgehog (*Ihh*)<sup>18</sup>. Mice that are hypomorphic for the *Ext1* allele (which means they have a mutation with a reduced level of gene activity) survive to a later embryonic stage, with expanded growth plates and an expanded range of Indian Hedgehog signaling. These results indicate that, in mice, one of the critical roles of HS is to establish a gradient of Indian Hedgehog signaling to induce the proper differentiation of chondrocytes in the growth plate.

Deletion of the *Ext2* gene was expected to result in the same phenotype as deletion of the *Ext1* gene, because human mutations in *EXT1* and *EXT2* result in the same phenotype.

However, as Stickens et al. showed, this is not the case<sup>19</sup>. Inactivation of the *Ext2* gene results in early embryonic lethality similar to the *Ext1* deletion, but compared with Indian Hedgehog-null mice, which die mid-gestation, the *Ext2*-null mice die earlier with defects in the extra-embryonic structures and gastrulation. This early demise could imply that fibroblast growth factor (fgf) signaling is involved; however, while *fgfr1* and *fgf8* cannot be ruled out, *fgf4* and *fgf2* mutant mice die shortly after implantation, earlier than the time at which *Ext*-null mice die. The *Ext*-null phenotype also does not coincide with the phenotype of members of the BMP or Wnt pathway families. Consequently, it is possible that multiple pathways, or an as yet undiscovered pathway, are modulated by the *Ext* genes. Additional interesting models used by developmental biologists to study *Ext* genes include *Caenorhabditis elegans* (a nematode<sup>20</sup>) and zebrafish<sup>21</sup>.

### Alternate Hypotheses for Initiation of Exostoses

The hypothesis that haploinsufficiency of an *EXT* gene (i.e., one allele inactivated by a mutation, with the resulting reduced gene product not sufficient for a normal phenotype) reduces enzymatic activity of the glycosyltransferases, causing formation of exostoses, is not the only plausible explanation for this condition. An alternate hypothesis for the initiation of exostoses has been presented by Stickens et al.<sup>19</sup>. This hypothesis involves the interaction of the FGF and BMP-TGF- $\beta$  pathways. As noted above, FGF signaling pathways depend on HS. In long-bone growth plates, FGF signaling shortens proliferative cell columns, both by decreasing chondrocyte proliferation directly and by suppressing *Ihh* expression. BMPs antagonize the effects of FGF signaling and are necessary for chondrocyte differentiation. Therefore, mutations in the *EXT1* and *EXT2* genes decrease HS synthesis, which reduces FGF signaling and leads to defects in chondrocyte differentiation. Whether the premature differentiation of chondrocytes leads to the formation of exostoses or whether the processes are distinct is not known. However, a model that combines the two possibilities into a unifying hypothesis is presented. If, due to a slightly deranged signaling system, some chondrocytes near the perichondrium differentiate into hypertrophic chondro-

cytes (characterized by type-X collagen synthesis), as the growth plate grows past this nest of hypertrophic chondrocytes, vascular elements of the growth plate are exposed to the hypertrophic chondrocyte nest and bone formation is initiated (Fig. 2, upper panel; the nested chondrocytes from the *Ext2*<sup>+/-</sup> mice are shown in Fig. 2, lower panel). This could result in growth at a 90° angle from the normal bone growth as seen in an exostosis, and this hypothesis could explain the low penetrance of the phenotype and the variable distribution of exostoses.

### Conclusions

While the exact mechanism by which underproduction of HS proteoglycan chains causes exostosis formation is not fully understood, the synergism of molecular biology, genetics, and biochemistry combined with the power of animal models and the observations of astute clinicians has produced a superior understanding of the inheritance and biology of multiple hereditary exostosis. The pursuit of knowledge about this disease has in turn stimulated the development of molecular biology, impacting the understanding of both normal development and other disease processes. The efforts focused on understanding this particular disease have provided an outstanding paradigm for the roles of HS chains in regulating biological processes, the delineation of the underlying causes of other cartilage and bone diseases, and some of the factors that enable early embryo survival. Finally, because the *EXT* genes have been shown to be tumor-suppressor genes and HS proteoglycans are intimately involved in angiogenesis and cancer, study of multiple hereditary exostosis could even shed light on the mechanisms of cancer development. ■

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