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EXT1 regulates chondrocyte proliferation and differentiation during endochondral bone development

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Abstract

Multiple Hereditary Exostoses (MHE) is an autosomal dominant skeletal disorder most frequently caused by mutations in the *EXT1* gene. MHE affects proper development of endochondral bones, such that all affected individuals present with exostoses adjacent to the growth plate of long bones, while some individuals exhibit additional bone deformities. EXT1 functions as a heparan sulfate (HS) co-polymerase, and when defective causes improper elongation of glycosaminoglycan side chains on core proteins of HS proteoglycans. Although analysis of heterozygous *EXT1*-deficient mice has failed to reveal any significant gross morphological variations in skeletal development, significant alterations in molecular signaling occur in the developing long bones. Our results indicate that defects in EXT1 and the resulting reduction in HS lead to enhanced Indian Hedgehog diffusion causing an increase in chondrocyte proliferation and delayed hypertrophic differentiation.

Keywords: Chondrocyte; Endochondral bone; Multiple Hereditary Exostoses

Introduction

Multiple Hereditary Exostoses (MHE) is an autosomal dominant skeletal disorder that is caused by mutations at one of at least two distinct loci: 8q24.1 (*EXT1*) [1] and 11p11–12 (*EXT2*) [2]. Most cases of MHE, about 70%, are caused by mutations in the *EXT1* gene. Individuals with MHE present with exostoses, or cartilaginous benign bone tumors, located at the growth plates of endochondral bones, and additionally can display bone deformities, joint fusions, and short stature (OMIM 133700). EXT1 is a member of the EXT-related gene family that is composed of *EXT1* [3], *EXT2* [4], *EXTL1* [5], *EXTL2* [6,7], and *EXTL3* [7,8]. The *EXT1* gene encodes a type II transmembrane protein with glycosyltransferase activity, and specifically functions as a heparan sulfate (HS) co-polymerase with EXT2 in a hetero-oligomeric complex [9,10]. The EXT1/EXT2 complex elongates glycosaminoglycan (GAG) side chains on core proteins of heparan sulfate proteoglycans (HSPGs).

Cell surface HS and HSPGs are known to play critical roles in the extracellular matrix as co-receptors for signaling molecules, modulators of protein transport, stabilizers for cell–cell adhesion, and participate in many other aspects of cellular communication [11]. For example, HS and HSPGs are known to be important modulators of FGF/FGF receptor interaction and specificity [12,13], the organization of Wnt proteins at the cell surface [14], the interactions between BMP dimers and their receptors [15,16], the interaction between Noggin and the cell surface [17], and in maintaining the proper morphogen gradient and diffusion of Hedgehog (Hh) during development [18].

Since a haploinsufficiency of EXT1, and the correlated reduction of HS, are implicated in the bone disorder of MHE, impairments in one or more of the aforementioned

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signaling pathways could be responsible for some or all of the phenotypes associated with MHE. The process of endochondral ossification is tightly regulated by many of these signaling pathways. Indian hedgehog (Ihh) is a master regulator of chondrocyte proliferation and differentiation during endochondral bone development. It is expressed early near the center of skeletal elements, and later becomes restricted to prehypertrophic and hypertrophic chondrocytes [19–21]. Ihh signals regulate the onset of hypertrophic differentiation via an indirect regulation of parathyroid hormone-related peptide, PTHrP, expressed in the periarticular region. High concentrations of PTHrP maintain immature chondrocytes in a proliferative state. When PTHrP concentrations fall below a threshold level, chondrocytes initiate hypertrophic differentiation and begin to express *Ihh*. This indirect regulation controls the distance from the periarticular region at which hypertrophic differentiation occurs [21]. Ihh has also been shown to positively regulate chondrocyte proliferation independent of its role in controlling differentiation by acting directly through proliferating chondrocytes [22].

In order to understand the molecular details of MHE, Lin et al. [23] disrupted the primary locus for MHE, *EXT1*, through homologous recombination in mouse embryonic stem (ES) cells. Since the *EXT1* null (*EXT1* −/−) mice are embryonic lethal at E8.5, skeletal analysis could not be performed. However, initial observations by Lin et al. [23] demonstrated that heterozygous *EXT1* mice (*EXT1*+/−) live to adulthood and display no gross skeletal phenotype during post-natal development. Subsequent analyses of these adult heterozygous mice have demonstrated mild reductions in humerus and femur bone density as compared to wild-type bones (unpublished data). In addition, cytological examination of the growth plates of adult *EXT1*+/− long bones show an increase in chondrocyte numbers within the columnar growth plate, although the overall length of long bones are comparable to those of wild-type mice (unpublished data). Given the mild skeletal phenotype in the adult *EXT1*+/− mice, we examined embryonic bone development for significant phenotypic changes, as well as changes in the molecular mechanisms regulating endochondral ossification. To examine the role EXT1 plays during endochondral bone development, we have analyzed bone length and morphology, chondrocyte proliferation, the expression of specific markers of chondrocyte differentiation, as well as the involvement and interaction of signaling pathways, specifically the Ihh/PTHrP negative feedback loop.

Materials and methods

Animal husbandry

EXT1 mouse strains were previously described [23]. Mice were used in accordance to guidelines established by the animal care facilities at the University of Houston.

Skeletal preparations

Skeletal preparations were performed on E14.5 and E18.5 mouse embryos according to a modified protocol based on McLeod [24]. Briefly, embryos were fixed in 95% ethanol, treated with acetone, stained with an alazarin red/alcian blue mixture (1 volume 0.3% Alcian Blue 8GS; 1 volume 0.1% Alizarin Red S type; 1 volume glacial acetic acid; 17 volumes 70% ethanol), cleared in 0.5% or 1% potassium hydroxide, and transferred through 20%, 50%, and 80% glycerol solutions in 0.5% or 1% potassium hydroxide.

In situ hybridization

Paraffin-embedded tissue sections (6 μm) were prepared from wild type and *EXT1*+/− embryonic day 16.5 (E16.5) mouse limbs after overnight fixation in 4% paraformaldehyde at 4°C. Antisense digoxygenin-labeled riboprobes were generated using the MaxiScript In Vitro Transcription kit (Ambion) with a digoxigenin RNA labeling mix (Roche). Plasmid constructs used for probe preparation were as follows: *collagen 2a1* [25], *collagen 10a1* [26], *indian hedgehog* [19], and *patched* [27]. Prior to hybridization, sections were deparaffinized in xylene, taken through an ethanol rehydration series, postfixed in 4% paraformaldehyde, rinsed in PBS, and treated with 10 μg/ml Proteinase K and 0.1 M triethanolamine with acetic anhydride for 10 min each followed by PBS rinses. Slides were prehybridized at 65°C [50% formamide, 5× SSC, 2% SDS, 2% Boehringer Blocking Reagent (BBR), 250 μg/ml tRNA, and 100 μg/ml heparin] for 3 h and hybridization was then carried out at 65°C for at least 14 h. Following washes and a 1-h blocking step in 20% heat-inactivated sheep serum and 2% BBR, slides were treated with 1:2500 dilution of anti-digoxigenin-AP fab fragments (Roche) overnight at 4°C. Slides were washed and finally incubated in a NBT/BCIP solution (125 μg/ml BCIP and 250 μg/ml NBT in NTM) for 3–5 h at room temperature in order to detect a color reaction.

Immunohistochemistry and BrdU analysis

Slides containing comparable sections from E16.5 mouse limbs were used in the immunohistochemistry procedures according to the Zymed HistoMouse-SP Kit (AEC) and Zymed BrdU staining protocols with minor modifications. Prior to peroxidase quenching, slides used for antibody staining of Ihh were incubated with 1 mg/ml hyaluronidase at 37°C for 15 min. On the day of harvesting E16.5 embryos for BrdU analysis, the mother was given an intraperitoneal injection of 0.1 ml per 10 g of body weight of a 10 mg/ml bromodeoxyuridine (BrdU) (Sigma): 1.2 mg/ml fluorodeoxyuridine (FdU) (Sigma) stock solution. At least three different sections were counted for four wild type and four *EXT1*+/− E16.5 mouse tibias in order to calculate BrdU indices.