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Epiphyseal abnormalities, trabecular bone loss and articular chondrocyte hypertrophy develop in the long bones of postnatal *Ext1*-deficient mice☆

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ABSTRACT

Long bones are integral components of the limb skeleton. Recent studies have indicated that embryonic long bone development is altered by mutations in *Ext* genes and consequent heparan sulfate (HS) deficiency, possibly due to changes in activity and distribution of HS-binding/growth plate-associated signaling proteins. Here we asked whether *Ext* function is continuously required after birth to sustain growth plate function and long bone growth and organization. Compound transgenic *Ext1*$^{f/f}$;*Col2CreERT* mice were injected with tamoxifen at postnatal day 5 (P5) to ablate *Ext1* in cartilage and monitored over time. The *Ext1*-deficient mice exhibited growth retardation already by 2 weeks post-injection, as did their long bones. Mutant growth plates displayed a severe disorganization of chondrocyte columnar organization, a shortened hypertrophic zone with low expression of collagen X and MMP-13, and reduced primary spongiosa accompanied, however, by increased numbers of TRAP-positive osteoclasts at the chondro–osseous border. The mutant epiphyses were abnormal as well. Formation of a secondary ossification center was significantly delayed but interestingly, hypertrophic-like chondrocytes emerged within articular cartilage, similar to those often seen in osteoarthritic joints. Indeed, the cells displayed a large size and round shape, expressed collagen X and MMP-13 and were surrounded by an abundant Perlecan-rich pericellular matrix not seen in control articular chondrocytes. In addition, ectopic cartilaginous outgrowths developed on the lateral side of mutant growth plates over time that resembled exostotic characteristic of children with Hereditary Multiple Exostoses, a syndrome caused by *Ext* mutations and HS deficiency. In sum, the data do show that *Ext1* is continuously required for postnatal growth and organization of long bones as well as their adjacent joints. *Ext1* deficiency elicits defects that can occur in human skeletal conditions including trabecular bone loss, osteoarthritis and HME.

Introduction

Long bones are integral components of the limb skeleton where they provide anatomical definition and structural support and permit movement via their epiphyseal diarthrodial synovial joints. Their development and growth initiate during embryogenesis with the formation of mesenchymal cell condensations that undergo chondrogenesis and assemble the initial cartilaginous anatomical blueprint of each limb element, be it a femur or a metacarpal. Growth and elongation of the long bones continue postnatally and end at puberty when the growth plates located at each epiphyseal end close [1]. In addition to elongation, the growing long bones need to be sculpted into anatomically complex and functional structures in which various portions and regions acquire distinct shapes and organization, an example of which being the proximal femoral head and the distal knee condyles. The elongated and relatively narrow diaphysis is the first to undergo endochondral ossification with ingression of blood vessels and formation of a primary ossification center, while the wider epiphyses ossify starting late in embryogenesis and then postnatally with the formation of the secondary ossification center [2]. Spared the endochondral ossification process are the most epiphyseal chondrocytes that become permanent

Abbreviations: HS, heparan sulfate; BMP, bone morphogenetic protein; FGF, fibroblast growth factor; HSPGs, heparan sulfate proteoglycans; Ihh, Indian hedgehog; HME, Hereditary Multiple Exostoses; MMP-13, matrix metalloprotease 13; VEGFs, vascular endothelial growth factors; TRAP, tartrate-resistant acid phosphatase.

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articular chondrocytes and sustain movement of the joints through life via their abundant and resilient extracellular matrix and production of synovial fluid components including hyaluronate and lubricin [3,4]. Given the multiplicity of processes and steps required for the genesis, growth and morphogenesis of long bones and joints, it is not at all surprising that the regulation of their development and growth is equally complex [5]. Much has been uncovered in these areas over the last several years, particularly with regard to the roles of members of the hedgehog, bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt signaling protein families and members of the Sox, Runx and Ets transcription factor families [6]. However, relatively little continues to be understood about several other pertinent processes including how the epiphyses and diaphysis undergo differential morphogenetic processes, how articular chondrocytes acquire their permanent phenotype, and how the hypertrophic zone of growth plate interacts and communicates with the underlying marrow and vascular progenitor cell population to sustain trabecular bone and primary spongiosa formation.

Ext1 is a member of the exostosin protein family that includes the *Ext2*, *Ext3* and *Extl* genes [7]. *Ext1* and *Ext2* form heteromeric glycosyltransferase protein complexes in the Golgi complex and are responsible for the synthesis of heparan sulfate (HS) [7]. This glycosaminoglycan is an important component of cell surface and matrix-associated proteoglycans that include Syndecans, Glypicans and Perlecan. The heparan sulfate proteoglycans (HSPGs) are expressed in unique patterns in different tissues and organs [8] and can influence a variety of developmental and physiologic processes that include cell determination and differentiation, cell–matrix interactions, receptor recycling and organogenesis [9,10]. Importantly, their HS chains serve as binding partners for key signaling proteins such as hedgehogs, restrict and limit their distribution, activity and target selection, and can thus influence their roles in development and growth [11–13]. For instance, mouse embryos carrying a hypomorphic gene trap mutation in *Ext1* that results in a severe reduction in *Ext1* protein levels and HS production – survive until E14.5–E16.5, but display skeletal growth retardation and deformities, delays in chondrocyte hypertrophy, and a much broader and abnormal distribution of Indian hedgehog (Ihh) within the long bone growth plate [11]. Ablation of *Ext1* in early embryonic limb buds causes severe impairment of mesenchymal prechondrogenic cell condensation and cartilaginous primordium formation [12], and *Ext1*- and HS-deficiency in synovial joint-forming cells leads to defects in joint formation and even joint fusion [13,14]. Furthermore, heterozygous *Ext1*-null mouse embryos exhibit changes in growth plate chondrocyte proliferation and hypertrophic differentiation [15]. There is also evidence that *Ext* deficiency occurring postnatally can cause skeletal abnormalities as well [16,17]. In a recent study Jones and collaborators created an innovative floxed *Ext1* mouse line with head-to-head *loxP* sites that elicits stochastic generation of *Ext1*-null or wild type chondrocytes in cartilage after mating with *Col2rtTACre* mice. The authors found that the resulting mutant mice displayed postnatal skeletal aberrations and in particular, developed several large cartilaginous tumor-like outgrowths near the epiphyses of their long bones [18]. The outgrowths resembled the cartilaginous exostoses that form near the growth plates of children with Hereditary Multiple Exostoses (HME), a syndrome caused by dominant loss-of-function mutations in *Ext1* or *Ext2* and consequent HS deficiency [19,20].

The present study was carried out to further test and clarify the roles of *Ext* genes in postnatal long bone growth, organization and structure. To do so, we created conditional compound mouse mutants bearing a standard floxed *Ext1* gene ($Ext1^{f/f}$) [22] and the *Col2CreERT* transgene that directs *CreERT* expression in chondrocytes [22]. We induced $Ext1^{f/f}$ ablation by tamoxifen injection at early postnatal time points and examined the developmental and structural consequences on long bones over time. We found that conditional loss of *Ext1* expression postnatally is incompatible with normal skeletal growth and results in a number of structural and functional deficiencies in both the long bones and their joints that also resemble human skeletal pathologies.

Materials and methods

Transgenic mice

All studies were conducted with approval by the IACUC. Creation of the *loxP*-modified *Ext1* allele and establishment of the $Ext1^{f/f}$ mouse line were described previously [21]. *Col2CreERT* transgenic mice that encode a *Cre* recombinase linked to a modified estrogen ligand binding domain under the control of *collagen 2a1* promoter sequences were generated and provided by Dr. S. Mackem, NCI [22]. $Ext1^{f/f}$ mice were mated with *Col2CreERT* mice, and the resulting $Ext1^{f/f}$;*Col2CreERT* mice and appropriate controls ($Ext1^{f/f}$ or $Ext1^{f/+}$) received a single intraperitoneal injection of tamoxifen (100 μg/10 μl/mouse) at postnatal day 5 (P5). Genotyping was performed using tail DNA and amplified by PCR. Mice were sacrificed 2, 4, and 8 weeks from the time of tamoxifen injection. The total numbers of mice studied in $Ext1^{f/f}$ and $Ext1^{f/f}$;*Col2CreERT* groups were: 4 each at 2 weeks; 2 each at 4 weeks; and 3 each at 8 weeks time points. We also created $Ext1^{f/f}$ and $Ext1^{f/f}$;*Col2CreERT* in *RosaR26R* background to monitor *CreERT* expression and activity by LacZ reporter staining after tamoxifen injection.

Skeletal analyses

Whole skeletons were analyzed by soft x-ray imaging using Bioptics piXarray100 (Kenmore, WA) in automatic exposure control mode. Total mouse body lengths were measured from nose to sacral tip, excluding the tail length. Lengths of long bones were determined from x-ray images using Image J software.

Chondrocyte cultures, DNA isolation and PCR

Cartilage was isolated from epiphyses of tibia, femur and humerus and radius of the P12 $Ext1^{f/f}$ and $Ext1^{f/f}$;*Col2CreERT* mice that had received a single tamoxifen injection (100 μg/10 μl/mouse) at P5 as above. Epiphyseal cartilage pieces isolated from individual animals were digested with 0.05% trypsin in HBSS for 1 h at 37 °C, and the liberated cells consisting mostly of contaminating fibroblasts were discarded. The remaining fragments were further incubated with 86 U/ml collagenase Type I (Worthington Biochemical Corporation, Lakewood, NJ) in serum-free DMEM overnight. Dissociated cells were filtered and plated on 60 mm agarose coated petri dishes (BD Biosciences, Franklin Lakes, NJ) and cultured in DMEM containing 10% FBS (Gemini Bio-Products, West Sacramento, CA) in suspension for 1 day to eliminate the few additional non-chondrogenic cells. The surviving cells – mostly chondrocytes – were finally collected and used to prepare DNA samples.

DNA was isolated using the Qiagen Mini Kit (Qiagen), according to the manufacturer instructions, quantified with BioMate 3 Spectrophotometer, Thermo Spectronic (Thermo Fisher Scientific Inc., Waltham, MA), and PCR amplified with specific primer pairs for detection of intact or recombined floxed allele as described [16]. Bands were visualized on 2% agarose gels, and GAPDH was used as internal control.

To further confirm the efficacy of *Cre* recombinase activity, qPCR was performed on genomic DNAs to specifically detect intact *Ext1* allele. DNA samples above were combined with SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) and primers for non-recombined intact allele or GAPDH as internal control. Reaction was performed using an ABI 7900 HT machine (Applied Biosystem, Foster City, CA) according to the manufacturer's protocols.

Histological, histochemical and immunohistochemical analyses

Knee and wrist joints were dissected and fixed with 4% (v/v) paraformaldehyde, decalcified with EDTA for 7–10 days and embedded in paraffin. Serial longitudinal 5 μm-thick sections of tibiae, femurs and the distal portions of ulnae and radii were processed for staining with