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Delayed hypertrophic differentiation of epiphyseal chondrocytes contributes to failed secondary ossification in mucopolysaccharidosis VII dogs

Sun H. Peck, Ph.D.^{a,b}, Philip J.M. O'Donnell, B.A.^{a,b}, Jennifer L. Kang^{a,b}, Neil R. Malhotra, M.D.^a, George R. Dodge, Ph.D.^b, Maurizio Pacifici, Ph.D.^c, Eileen M. Shore, Ph.D.^{b,d}, Mark E. Haskins, V.M.D., Ph.D.^e, and Lachlan J. Smith, Ph.D.^{a,b,*}

^aDepartment of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

^bDepartment of Orthopaedic Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

^cDivision of Orthopedic Surgery, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

^dDepartment of Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

^eDepartment of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

Abstract

Mucopolysaccharidosis (MPS) VII is a lysosomal storage disorder characterized by deficient β -glucuronidase activity, which leads to the accumulation of incompletely degraded glycosaminoglycans (GAGs). MPS VII patients present with severe skeletal abnormalities, which are particularly prevalent in the spine. Incomplete cartilage-to-bone conversion in MPS VII vertebrae during postnatal development is associated with progressive spinal deformity and spinal cord compression. The objectives of this study were to determine the earliest postnatal developmental stage at which vertebral bone disease manifests in MPS VII and to identify the underlying cellular basis of impaired cartilage-to-bone conversion, using the naturally-occurring canine model.

Control and MPS VII dogs were euthanized at 9 and 14 days-of-age, and vertebral secondary ossification centers analyzed using micro-computed tomography, histology, qPCR, and protein immunoblotting. Imaging studies and mRNA analysis of bone formation markers established that secondary ossification commences between 9 and 14 days in control animals, but not in MPS VII animals. mRNA analysis of differentiation markers revealed that MPS VII epiphyseal

* **Correspondence** Lachlan J. Smith, Ph.D., Assistant Professor, Department of Neurosurgery, University of Pennsylvania, 424 Stemmler Hall, 3450 Hamilton Walk, Philadelphia, PA 19104, USA, Ph. 215 746 2169, Fax. 215 573 2133, lachlans@mail.med.upenn.edu.

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chondrocytes are unable to successfully transition from proliferation to hypertrophy during this critical developmental window. Immunoblotting demonstrated abnormal persistence of Sox9 protein in MPS VII cells between 9 and 14 days-of-age, and biochemical assays revealed abnormally high intra and extracellular GAG content in MPS VII epiphyseal cartilage at as early as 9 days-of-age. In contrast, assessment of vertebral growth plates and primary ossification centers revealed no significant abnormalities at either age.

The results of this study establish that failed vertebral bone formation in MPS VII can be traced to the failure of epiphyseal chondrocytes to undergo hypertrophic differentiation at the appropriate developmental stage, and suggest that aberrant processing of Sox9 protein may contribute to this cellular dysfunction. These results also highlight the importance of early diagnosis and therapeutic intervention to prevent the progression of debilitating skeletal disease in MPS patients.

Keywords

Lysosomal Storage Disorder; Vertebra; Long Bones; Endochondral Ossification; Chondrocyte Differentiation; Glycosaminoglycan

1. Introduction

The mucopolysaccharidoses (MPS) are a family of genetic, lysosomal storage diseases that are characterized by deficiencies in the activity of one of the 11 acid hydrolases responsible for degradation of glycosaminoglycans (GAGs). Each of the MPS subtypes is characterized by mutations in a gene encoding for one of these lysosomal enzymes, each of which results in the incomplete degradation and subsequent accumulation of GAG fragments [1]. Mucopolysaccharidosis VII (Sly Syndrome), one of the rarest subtypes affecting approximately 1:250,000 live births world-wide [2], is characterized by deficient β -glucuronidase (GUSB) activity, leading to the aberrant accumulation of incompletely degraded fragments from three different types of GAGs – chondroitin, dermatan, and heparan sulfates [3, 4]. While disease manifestations are present in multiple organ systems, MPS VII patients exhibit particularly severe skeletal abnormalities [5-7]. In the spine, vertebral body dysplasia contributes to progressive kyphoscoliosis and spinal cord compression, diminishing patient life expectancy and quality of life [8]. The molecular mechanisms linking aberrant GAG accumulation to progressive skeletal disease in MPS VII are poorly understood, impeding development of effective treatments.

In previous work, using the naturally-occurring canine model [9], we described the presence of radiolucent, cartilaginous lesions in peripheral regions of vertebral bodies of MPS VII dogs and described similar lesions in the vertebrae of a 19-year-old human MPS VII patient who had severe kyphoscoliotic deformity at the time of death [7, 10]. Through biomechanical experiments, we demonstrated that these lesions result in reduced stiffness and increased range of motion of each intervertebral joint and are a likely basis of progressive spinal deformity [10]. Subsequent longitudinal, radiographic studies of MPS VII dog spines suggested that the persistence of these lesions is a consequence of delayed, and ultimately failed, cartilage-to-bone conversion [7]. During postnatal development, bone formation in vertebrae occurs through a process of endochondral ossification similarly to

long bones, in which cartilaginous rudiments, populated by chondrocytes of mesenchymal lineage, initially form a template for future ossification. These chondrocytes mature through proliferative, pre-hypertrophic, and hypertrophic stages, and ultimately reach terminal differentiation, which is followed by apoptosis of cells and subsequent invasion by osteoblasts to initiate osteogenesis [11-13]. Chondrocyte maturation precedes bone formation initially in primary centers of ossification, and subsequently in secondary centers of ossification and adjacent growth plates, enabling longitudinal bone growth [14]. The timing and rate of chondrocyte maturation is regulated by a highly orchestrated pattern of secreted growth factors, including bone morphogenetic proteins, Indian hedgehog, fibroblast growth factors, and Wnts, and many of these growth factors exhibit dependence on GAGs for their extracellular distribution and cellular signal transduction [15-18].

The objectives of this study were to determine the earliest postnatal developmental stage at which failed bone formation manifests in MPS VII and to identify the associated underlying cellular basis of impaired cartilage-to-bone conversion. We hypothesized that aberrant GAG accumulation in MPS VII vertebral epiphyseal tissue disrupts the timing and rate of chondrocyte maturation, which in turn contributes to delayed and, ultimately, failed vertebral bone formation.

2. Materials and Methods

2.1. Animals and Tissue Collection

For this study, we used the naturally-occurring canine model of MPS VII. MPS VII dogs have a missense mutation (R166H) in the GUSB gene and exhibit a similar skeletal phenotype to human patients [9, 10, 19-21]. Animals were raised at the University of Pennsylvania School of Veterinary Medicine under NIH and USDA guidelines for the care and use of animals in research, and all studies were carried out with IACUC approval. Animals were housed in a 12-hour on-off light cycle, temperature and humidity controlled (68-78 °F, 40-70% humidity) barrier facility with grated run housing, and with the mother and littermates housed together until euthanasia. Mothers were given ad lib access to food and water, and pups were monitored with daily weight measurements and provided food supplementation as needed. All animals were verified by vet staff to have no health complications (other than genetic disease in the case of MPS VII animals) at the time of euthanasia.

Litter-matched control (heterozygous) and MPS VII (homozygous) dogs were identified via gene sequencing analysis at birth. Animals were euthanized at 9 or 14 days-of-age, defined as number of postnatal days, using 80 mg/kg of sodium pentobarbital in accordance with American Veterinary Medical Association guidelines (n=5 for both control and MPS VII animals at each time point). Time points of 9 and 14 days-of-age were selected following evaluation of lumbar spine plain radiographs from our previous longitudinal study [7]. These radiographs indicated that bone formation in the secondary ossification centers of the vertebrae commenced sometime between 7 and 30 days-of-age. Gestational periods were carefully monitored to ensure reproducibility of postnatal ages between different litters, which were found to be accurate to within one day. There were no significant differences in size (nose to rump length or weight) between MPS VII and control animals at either of these

early ages. Any size differences present between individual animals were likely attributable to natural variations within and between litters. The thoracic and lumbar spines, and hind legs, were dissected out immediately following euthanasia and individual vertebrae, proximal femurs, and knees (containing the distal femur, proximal tibia, and joint capsule) were isolated for further analyses as outlined below.

2.2. mRNA Expression of Bone Formation and Chondrocyte Differentiation Markers

For each animal, epiphyseal tissue from both the cranial and caudal sides of the T12 vertebrae was excised adjacent to the growth plate, combined and flash frozen in liquid nitrogen. Adjoining intervertebral disc tissue was carefully removed prior to excision of cartilage. Tissue was pulverized using a mortar and pestle, and RNA was extracted using serial TRIzol (Ambion; Austin, TX)-chloroform extractions [22], with 100% isopropanol and a solution of 1.2 M NaCl and 0.8 M sodium citrate (Sigma-Aldrich; St. Louis, MO) in ddH₂O used in a 1:1 mixture to separate contaminating GAGs from the RNA pellet at the precipitation step. Following precipitation, extracted RNA was in-column treated with RNase-free DNase on miRNeasy columns (Qiagen; Valencia, CA) and eluted following the manufacturer's protocols.

cDNA was synthesized from isolated RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, CA). qPCR reactions were carried out on 10 ng of each cDNA sample per well using Fast SYBR Green Master Mix (Applied Biosystems; Foster City, CA) on a StepOnePlus Real-Time PCR system (Life Technologies; Carlsbad, CA). Primers were designed against NCBI *canis familiaris* mRNA sequences (Supplementary Table 1).

The presence or absence of bone formation in vertebral epiphyseal tissue at each age was established by measuring mRNA expression of osteoblast markers, alkaline phosphatase (ALPL) and osteocalcin (BGLAP). The mean differentiation stage of the resident chondrocyte population in vertebral epiphyseal tissue at each age was established by measuring mRNA levels of differentiation stage markers as follows: Resting/proliferative stage: SRY (sex determining region Y)-box 9 (SOX9); Pre-hypertrophic stage: runt-related transcription factor 2 (RUNX2) and parathyroid hormone receptor 1 (PTH1R). Hypertrophic stage: myocyte-specific enhancer factor 2C (MEF2C) and collagen type X, alpha-1 (COL10A1). Expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and are presented as % GAPDH ($100 \times [2^{-(C_T \text{ target gene} - C_T \text{ GAPDH})}]$).

2.3. Sox9 Protein Expression

Epiphyseal tissue from both the cranial and caudal sides of T11 vertebrae were excised, combined, and flash frozen in liquid nitrogen. Tissue was pulverized with a mortar and pestle, and protein was extracted, first with cytoplasmic extraction buffer (10 mM HEPES pH 7.6, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO), 3.5 µL 10% NP40 per 300 µL buffer) followed by nuclear extraction buffer (50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA,

protease inhibitor cocktail, 10% glycerol). Total protein concentration was measured, and 30 µg protein loaded per sample.

Western blotting was performed using Mini-Protean TGX 4-15% gels (Biorad; Hercules, CA) in a Tris-Glycine/SDS buffer system. Gels were transferred on to polyvinylidene fluoride (PVDF) membranes using the iBlot 2 transfer stack (Invitrogen; Carlsbad, CA). Blots were blocked in 5% milk in TBS-T, incubated in rabbit anti-Sox9 (Thermo Fisher Scientific; Rockford, IL) and mouse anti-β-actin (Abcam; Cambridge, MA) primary antibodies, washed in TBS-T, then incubated in goat anti-rabbit IRDye 680 and goat anti-mouse IRDye 800CW secondary antibodies (LI-COR Biosciences; Lincoln, NE). Blots were then visualized and quantified using densitometry with an Odyssey Infrared Imaging System (LI-COR Biosciences; Lincoln, NE). Sox9 protein levels in both fractions were normalized to total cell β-actin levels.

2.4. Intracellular and Extracellular Sulfated Glycosaminoglycan Content

To evaluate differences in GAG accumulation in vertebral secondary ossification centers between control and MPS VII animals at 9 days-of-age, total sulfated GAG content in epiphyseal tissue was measured using the dimethylmethylene blue (DMMB) assay [23]. Cranial and caudal epiphyseal tissue from L1 vertebrae were excised, combined, and incubated in 1 mg/mL collagenase in Dulbecco's modified Eagle's medium (DMEM) for ~8 hours at 37 °C until completely digested and cells fully released from the extracellular matrix. Digested samples were strained through a 50 µm mesh filter and total number of cells determined. Digests were then centrifuged at 600g for 5 minutes to separate the supernatant (extracellular fraction) from the cell pellet (intracellular fraction). Cells were washed in 1X phosphate buffered saline (PBS), then both the extracellular and intracellular fractions were further digested with 1 mg/mL pronase overnight at 37 °C. Total sulfated GAG in each fractions was normalized to total cell count.

2.5. Micro-computed Tomography (µCT)

Whole T7 vertebral bodies were excised and fixed in 1:10 buffered formalin at 4 °C for one week, then scanned using high-resolution micro-computed tomography (µCT) (VivaCT40; Scanco Medical AG, Brüttisellen, Switzerland). Sequential axial images through the entire vertebra were obtained with an isotropic voxel size of 19 µm, an integration time of 380 ms, peak tube voltage of 70 kV, current of 0.114 mA, and an acquisition of 1000 projections per 180°. A three-dimensional Gaussian filter of 1.2 with a limited, finite filter support of 2 was used for noise suppression, and mineralized tissue was segmented from air or soft tissue using a threshold of 158. Bone formation in vertebral secondary ossification centers (epiphyses) was visualized through 3D µCT reconstructed images of the cartilaginous regions on the cranial and caudal ends of the primary ossification centers. To quantify bone formation in the primary ossification centers, standard 3D morphometric analyses were performed using Scanco software to calculate bone volume/total volume (BV/TV) and apparent mineral density (bone mineral density, BMD) calibrated against hydroxyapatite (HA) standards (0-784 mg HA/cm³).

2.6. Histology

Formalin-fixed T7 vertebral bodies (following μ CT imaging), femoral heads and knee joints from each animal were decalcified (Formical 2000, Decal Chemical Corporation; Tallman, NY). A 3 mm-thick mid-coronal segment was cut from each vertebral body, and 5 mm-thick midsagittal segments were cut from each femoral head and knee joint, and processed into paraffin. To confirm the presence or absence of bone formation in secondary ossification centers, sections 8 μ m-thick were double-stained with Alcian blue and picosirius red (ABPR) for GAG and collagen, respectively, then imaged under bright field light microscopy (Eclipse 90i; Nikon; Tokyo, Japan). To analyze growth plate morphology, adjacent sections were double-stained with hematoxylin and eosin (H&E) and imaged using differential interference contrast (DIC) microscopy. Total numbers of proliferative and hypertrophic chondrocytes were counted manually in a standardized 1 mm-wide region in the center of the growth plate and normalized to total area using NIS-Elements software (Nikon; Tokyo, Japan). The mean height of the proliferative and hypertrophic zones were calculated from the same standardized region.

2.7. Statistical analyses

We used a biological sample size of $n=5$ for both control and MPS VII animals at each time point based on our previous therapeutic and pathogenesis studies in dogs [7, 10, 24-26] where $n=5$ yielded a power > 0.90 for all outcome measures. For most quantitative outcome measures, (mRNA, protein, BV/TV, BMD, and growth plate morphology) significant differences were established using 2-way analyses of variance (age and disease state as independent variables) and post-hoc Tukey's tests (Systat; Systat Software Inc., San Jose, CA). For GAG measurements, significant differences between control and MPS VII at 9 days-of-age were established using unpaired t-tests. Values of $p < 0.05$ were considered significant, with trends defined as $p < 0.1$. Exact p-values were calculated to three decimal places and presented, except for where $p < 0.001$, which are reported as $p < 0.001$. All results are expressed as mean $\pm$ standard deviation.

3. Results

3.1. Delayed bone formation in the secondary ossification centers of MPS VII vertebrae

Analysis of vertebral secondary ossification centers using μ CT showed no evidence of bone formation in either control or MPS VII animals at 9 days-of-age (Figure 1A). At 14 days-of-age, however, there was evidence of initial bone formation in the secondary ossification centers of control animals (red boxes, Figure 1A), while none was apparent in MPS VII animals. The presence or absence of secondary ossification in vertebral bodies at both ages was confirmed with mid-coronal histological sections (Figure 1B), with results supporting μ CT data. Additional confirmation of the presence or absence of secondary ossification was undertaken by measuring mRNA expression of bone formation markers in vertebral epiphyseal tissue (Figure 1C). ALPL and BGLAP expression levels were 4.9 and 3.4-fold higher respectively at 14 days-of-age compared to 9 days-of-age in control animals ($p < 0.001$, Figure 1D). There was no difference in expression levels of ALPL or BGLAP in MPS VII animals between 9 and 14 days-of-age.

3.2. Failed hypertrophic differentiation of MPS VII vertebral epiphyseal chondrocytes

To establish the differentiation stage of resident chondrocytes in vertebral epiphyseal cartilage, mRNA levels of chondrocyte differentiation markers were measured using qPCR. SOX9 mRNA expression was significantly lower at 14 days compared to 9 days for both control and MPS VII animals (1.5 and 1.9% of 9 days, respectively, both $p < 0.001$, Figure 2). For controls, mRNA expression levels of all pre-hypertrophic and hypertrophic stage markers were significantly higher (all $p < 0.001$) at 14 days compared to 9 days and significantly higher than MPS VII animals at both ages (all $p < 0.001$). For MPS VII animals, there were no significant differences in the expression levels of any pre-hypertrophic or hypertrophic markers between 9 and 14 days. At 9 days, RUNX2 mRNA expression trended higher for control animals compared to 9-day MPS VII animals ($p = 0.068$) and was significantly higher compared to 14-day MPS VII animals (2-fold, $p = 0.013$). At 9 days, both PTH1R and MEF2C mRNA expression levels for control animals were significantly higher than 9-day MPS VII animals (1.9-fold, $p = 0.009$ and 2.7-fold, $p < 0.001$, respectively) and significantly higher than 14-day MPS VII animals (2.4-fold, $p < 0.001$ and 1.7-fold, $p = 0.016$, respectively).

3.3. Persistent Sox9 protein expression in MPS VII vertebral epiphyseal tissue

In order to investigate the role of Sox9 protein in the juncture between proliferative and hypertrophic stages in chondrocyte differentiation, cytoplasmic, nuclear, and total Sox9 protein expression levels in vertebral epiphyseal tissue were measured and normalized to total cell β -actin. Representative Western blots for cytoplasmic and nuclear fractions are shown in Figures 3A and B, respectively. Cytoplasmic Sox9 protein expression was significantly lower at 14 days compared to 9 days for control animals (39.5% of 9 days, $p = 0.022$) but was not significantly different for MPS VII animals (Figure 3C). Cytoplasmic Sox9 protein in MPS VII animals trended towards lower expression at 9 and 14 days compared to 9-day controls ($p = 0.091$ and 0.082 , respectively, Figure 3C). Nuclear Sox9 protein expression for control animals was lower at 14 days compared to 9 days (15.2% of 9 days, $p = 0.026$, Figure 3D). Nuclear Sox9 protein in 9 and 14-day MPS VII animals trended towards lower expression compared to 9-day controls ($p = 0.061$ and 0.074 respectively, Figure 3D). Total Sox9 protein expression levels were calculated by the addition of expression for cytoplasmic and nuclear fractions and normalized to whole cell β -actin protein expression (Figure 3E). Total Sox9 protein expression was significantly lower for 14-day control animals compared to 9-days controls (25.9%, $p = 0.026$), and both 9-day and 14-day MPS VII animals (31.9%, $p = 0.029$ and 37.2%, $p = 0.029$, respectively).

3.4. Aberrant glycosaminoglycan accumulation in MPS VII vertebral epiphyseal cartilage

To investigate aberrant accumulation of GAGs in MPS VII vertebral epiphyseal cartilage, extracellular and intracellular sulfated GAG content of vertebral epiphyseal tissue from 9-day animals were measured. Glycosaminoglycan content was significantly higher for both extracellular (1.5-fold, $p = 0.048$) and intracellular fractions (2.3-fold, $p < 0.001$) for MPS VII animals compared to controls (Figure 4).

3.5. Delayed bone formation in the secondary ossification centers of MPS VII long bones

To confirm that delayed commencement of secondary ossification also occurred at other skeletal sites, long bones (proximal femurs and knee joints) were examined histologically (Figure 5). At 9 days, evidence of initial bone formation was observed in the proximal femurs of control animals (arrow, Figure 5A), but not MPS VII animals. At 14 days, there was evidence of bone formation in the proximal femurs of both control and MPS VII animals (arrows, Figure 5A). In the knee joints (distal femurs and proximal tibia), evidence of bone formation was only observed for 14-day control animals (arrows, Figure 5B).

3.6. Vertebral growth plate morphology and primary ossification centers

Differences in vertebral growth plate morphology were assessed histologically. Representative images from each of the four study groups are shown in Figure 6A. There were no significant differences in the proliferative or hypertrophic zone heights, or cellularity between any of the study groups at either age (Figures 6B and C, respectively). Analyses of vertebral body primary ossification centers using μ CT revealed no significant differences between control and MPS VII animals in BV/TV or BMD at either 9 or 14 days-of-age (Figure 6D and E).

4. Discussion

In this study, we sought to determine the earliest postnatal developmental window within which abnormal bone formation becomes apparent, and to identify the nature of the associated cellular dysfunction in the naturally-occurring MPS VII canine model. Despite many years of research, the underlying cellular basis of skeletal disease in MPS disorders remains poorly understood. While most subtypes of MPS present with substantial skeletal abnormalities [27, 28], poor understanding of the underlying molecular mechanisms currently impedes development of targeted therapies. In the spine, manifestations include odontoid hypoplasia, vertebral body dysplasia and collapse, dural thickening, kyphoscoliosis, and intervertebral disc degeneration, which are collectively associated with significant neurological complications [29-32]. While there are currently no clinically approved treatment strategies for MPS VII, a number of strategies are in use for other subtypes, although their efficacy for improving spinal manifestations is limited. These strategies include, predominantly, hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) [33-35]. ERT for MPS VII patients using recombinant human GUSB is currently undergoing clinical trials, and while early results are encouraging and suggest an overall positive clinical effect [36], efficacy for correcting spine and bone disease in the patients has not been established. ERT for correction of bone disease in MPS VII presents with additional challenges, including the large size of the GUSB enzyme making diffusion into poorly-vascularized tissue more difficult, and the overall greater severity of the disease to be corrected. In a prior study, we showed that neonatal gene therapy, which produced large amounts of circulating GUSB enzyme, did not significantly improve vertebral secondary ossification in MPS VII dogs [26]. Studies in MPS VII mice suggest that HSCT, ERT, and gene therapy have partial efficacy for normalizing bone formation, with osteoblasts and marrow cells appearing to be more responsive than chondrocytes [37-42]. The results of these studies suggest that it is likely that MPS VII

patients will require adjuvant therapies, delivered in combination with systemic treatments such as ERT or HSCT, that specifically target skeletal disease manifestations.

In the current study, we identified the developmental window within which failed vertebral bone formation first manifests in MPS VII dogs. This window is the ideal developmental stage within which to examine the underlying cellular basis of the disease. Examination of vertebral bodies at 9 and 14 days-of-age through μ CT imaging, histology, and mRNA analyses demonstrated conclusively that vertebral epiphyseal bone formation commences in control animals across this window but does not in MPS VII animals. Our studies have focused primarily on the vertebrae due to the associated progressive and clinically significant spinal deformity observed in MPS VII patients; however, since long bones also form through endochondral ossification, we examined bone formation in the secondary ossification centers in the hips and knee joints. Histological staining showed similar delayed onset of secondary ossification in the proximal and distal femur, and proximal tibia, confirming that delayed bone formation in MPS VII was not limited to the vertebrae and is most likely attributable to similar molecular mechanisms in other skeletal sites. The extent of epiphyseal bone formation in the long bones of control animals at 9 and 14 days appeared more advanced than in the vertebrae, suggesting that the timing of commencement of secondary ossification is site-specific and that this developmental window, while appropriate for mechanistic studies of bone formation in the vertebrae, may need to be further refined for other tissues.

Chondrocyte differentiation during endochondral ossification progresses through tightly regulated stages, each of which is delineated by distinct and well-established gene expression patterns [43]. The transcription factor, SOX9, is considered to be a master regulator of chondrocyte differentiation. During early condensation of cartilaginous rudiments, SOX9 expression is required to direct MSCs into the chondrogenic lineage [44]. Later, SOX9 contributes to maintaining chondrocytes in the proliferative state, and downregulation of SOX9 expression permits chondrocytes to move from proliferation into pre-hypertrophy by allowing subsequent upregulation of RUNX2 and PTH1R. The chondrocytes then mature into hypertrophy with MEF2C upregulation and ultimately COL10A1 expression [45, 46]. MEF2C is an activating transcription factor for COL10A1 expression [47] and, thus, controls hypertrophic differentiation in a direct manner. Measurement of mRNA expression levels of these stage-specific markers indicated that epiphyseal chondrocytes from control animals progress through normal phases of differentiation as expected across the 9 to 14 days window, exiting proliferation (decreased SOX9), undergoing hypertrophy (increased RUNX2, PTH1R, MEF2C, COL10A1), and paving the way for bone formation by 14 days (increased ALPL BGLAP). In controls, as expected from mRNA expression levels of SOX9, both nuclear and cytoplasmic Sox9 protein expression also decreased from 9 to 14 days. In contrast, epiphyseal chondrocytes from MPS VII animals, while also exhibiting decreased SOX9 mRNA expression from 9 to 14 days, failed to proceed to hypertrophy, and thus failed to initiate bone formation within this same time frame. Interestingly, Sox9 protein levels in MPS VII chondrocytes remained unchanged between 9 and 14 days, despite a significant decrease in SOX9 mRNA expression levels within the same developmental window. It is possible that this aberrant persistence of Sox9 protein in MPS VII contributes to the failure of chondrocytes to

progress from proliferation to hypertrophy, which ultimately leads to delayed bone formation. There is evidence that Sox9 protein may directly antagonize the binding of MEF2C to the COL10A1 promoter [45] and can also disrupt or repress the expression or activity of other regulatory elements that initiate and sustain chondrocyte hypertrophic differentiation, including β -catenin, RUNX2, and extracellular matrix molecules [48-50]. Further investigations are underway to determine the molecular mechanism through which persistent Sox9 protein expression may be contributing to failed chondrocyte hypertrophic differentiation.

Previous studies using large animal models of MPS have reported low bone content and mineral density at older ages, and clinical studies suggest that human MPS patients may present with an osteoporosis-like phenotype [24, 51-53]. In the current study, which focused on a much earlier stage of postnatal growth than previous studies, delayed commencement of secondary ossification in MPS VII dogs contrasted with no detectable abnormalities in the growth plates or primary ossification centers. This suggests that the earliest affected developmental pathways are those that are required for activation of secondary ossification. Epiphyseal cartilage contains high quantities of GAGs, and during the transition from cartilage to bone, this GAG-rich epiphyseal tissue is being actively remodeled and resident cells are metabolically active. The associated increase in GAG turnover may accentuate the pathological consequences of GUSB inactivity. Vertebral epiphyseal tissue from MPS VII animals exhibited higher levels of both intra and extracellular sulfated GAGs compared to controls at 9 days-of-age, the developmental stage immediately preceding commencement of secondary ossification. While the exact molecular mechanisms are yet to be elucidated, it is likely that this abnormal GAG accumulation is contributing to cellular dysfunction. Glycosaminoglycans are well-established regulators of the biological activity of signaling molecules that regulate the timing and rate of chondrocyte hypertrophic differentiation during endochondral ossification [15-18]. We hypothesize that abnormal extracellular accumulation of GAGs in MPS VII results in aberrant growth factor distribution and availability. The affinity of GAGs for specific growth factors is a function of their fine structure including sulfation, and ongoing work is investigating whether the GAGs accumulating in MPS VII exhibit structural abnormalities that may affect their biological function. Abnormal intracellular GAG accumulation likely contributes to cellular dysfunction by increasing cellular stress. Elucidating these extracellular and intracellular mechanisms of cellular dysfunction will be crucial for future development of therapies that are able to specifically target bone disease manifestations in MPS patients.

In summary, in this study, we have pinpointed the developmental window when bone disease first manifests in MPS VII dogs. We have shown that delayed secondary ossification can be traced to a failure of resident chondrocytes to transition from proliferation to hypertrophic differentiation at the appropriate developmental stage, and that abnormal extracellular and intracellular GAG accumulation and aberrant Sox9 protein regulation may be contributing to cellular dysfunction. We have shown that delayed secondary ossification occurs in vertebrae and long bones and precedes pathological changes in growth plates and primary ossification centers, which likely manifest at later stages of postnatal development. The results of this study lay the foundation for future mechanistic studies of bone disease, not just in MPS VII but other MPS subtypes. Finally, these results highlight the importance

of early diagnosis and therapeutic intervention to prevent the progression of debilitating skeletal disease in MPS patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Authors' roles: SHP contributed to the conceptual design, performed experiments, and drafted the manuscript. PJMO and JLK performed experiments. NRM, GRD, MP, and EMS contributed to conceptual design and critically revised the manuscript. MEH contributed to conceptual design, supervised raising of the animals, and critically revised the manuscript. LJS contributed to the conceptual design and drafted the manuscript. All authors approved the final version of the manuscript prior to submission.

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Highlights

- Developmental stage when bone disease first manifests in MPS VII dogs identified.
- Delayed secondary ossification occurs in both vertebrae and long bones.
- Chondrocytes fail to transition from proliferation to hypertrophic differentiation.
- Aberrant Sox9 protein regulation may be contributing to chondrocyte dysfunction.
- Primary ossification centers and growth plates normal at early postnatal ages.

Figure 1. Bone formation in vertebral secondary ossification centers of control and MPS VII animals at 9 and 14 days-of-age

A. Representative, mid-coronal μ CT cross-sections of T7 vertebrae. Red boxes indicate bone formation in the secondary ossification centers of 14-day control animals. **B.** Representative mid-coronal histological sections of T7 vertebrae (Alcian blue/picrosirius red stain). Inset shows evidence of initial bone formation in the secondary ossification centers of 14-day control animals. **C.** Schematic of vertebral epiphyseal tissue excision. **D.** Messenger RNA expression levels of bone formation markers (ALPL: alkaline phosphatase; BGLAP: osteocalcin). N = 5 for all groups. S: Secondary ossification centers (epiphyses); P: Primary ossification centers; scale bar = 1 mm. [#]p < 0.001 versus all other groups.

Figure 2. Messenger RNA expression levels of proliferation and hypertrophic differentiation markers of vertebral epiphyseal chondrocytes from control and MPS VII animals at 9 and 14 days-of-age

Both control and MPS VII chondrocytes exit the proliferative stage between 9 and 14 days-of-age, but only control chondrocytes progress to hypertrophy. N=5 for all groups. +p < 0.1; *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.001 versus all other groups.

Figure 3. Sox9 protein expression in control and MPS VII vertebral epiphyseal chondrocytes at 9 and 14 days-of-age

A. Representative images (biological n=2 per group) of cytoplasmic protein fraction immunoblots for control and MPS VII vertebral epiphyseal chondrocytes at 9 and 14 days.

B. Representative images (biological n=2 per group) of nuclear protein fraction immunoblots for control and MPS VII vertebral epiphyseal chondrocytes at 9 and 14 days.

C. Quantification of cytoplasmic Sox9 protein expression levels. **D.** Quantification of nuclear Sox9 protein expression levels. **E.** Quantification of total Sox9 protein expression levels. N=5 for all groups, normalized to total cell β-actin. +p < 0.1; *p < 0.05.

Figure 4. Sulfated glycosaminoglycan content of control and MPS VII vertebral epiphyseal cartilage at 9 days-of-age

A. Extracellular GAG. **B.** Intracellular GAG. N=5 for all groups, normalized to total cell count. *p < 0.05; ***p < 0.001.

Figure 5. Bone formation in long bone secondary ossification centers of control and MPS VII animals at 9 and 14 days-of-age

A. Proximal femurs (PF). **B.** Knees (distal femur: DF and proximal tibia: PT). Arrows: initial bone formation in secondary ossification centers; scale bar = 2 mm. Midsagittal, Alcian blue/picrosirius red stained sections.

Figure 6. Growth plate morphology and primary ossification center bone content of control and MPS VII vertebrae at 9 and 14 days-of-age

A. Representative images of vertebral growth plates for each group. PZ: proliferative zone; HZ: hypertrophic zone; S: Secondary ossification centers (vertebral epiphyseal tissue); P: Primary ossification centers scale bar = 100 μm. **B.** Quantification of growth plate proliferative and hypertrophic zone heights. **C.** Quantification of cell density in growth plate proliferative and hypertrophic zones, normalized to area. **D.** Bone volume fraction (BV/TV) of vertebral primary ossification centers. **E.** Bone mineral density of vertebral primary ossification centers. N=5 for all groups. No significant differences in growth plate morphological parameters or primary ossification center bone content were found between control and MPS VII at either age.