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# Delayed Chondrocyte Differentiation and Altered Indian Hedgehog Signaling Contribute to Failed Vertebral Bone Formation in Mucopolysaccharidosis VII

**Chelsea M. del Alcazar, BS**
**Joseph A. Chiaro, BS**
**Eileen M. Shore, PhD**
**Mark E. Haskins, VMD, PhD**
**Lachlan J. Smith, PhD**

University of Pennsylvania, Philadelphia, PA, USA.

## Introduction

Mucopolysaccharidosis VII (MPS VII) is a lysosomal storage disorder characterized by deficient activity of the enzyme β-glucuronidase, resulting in accumulation of poorly-degraded chondroitin, heparan, and dermatan sulfate glycosaminoglycans (GAGs).[1,2] While MPS VII results in multi-systemic disease manifestations, skeletal abnormalities are particularly prevalent and significantly impact patient quality of life.[1,2] In the spine, manifestations include accelerated disc degeneration, and malformed and misaligned vertebral bodies, leading to kyphoscoliotic deformity and spinal cord compression. There are currently no therapies that effectively treat spine disease in MPS VII. In previous studies, we demonstrated the presence of radiolucent, cartilaginous lesions at the vertebral epiphyses, which significantly compromise the structural and mechanical integrity of the intervertebral joint.[3,4] We hypothesize that these lesions represent failed cartilage-to-bone conversion during post-natal development; the underlying pathological mechanisms responsible, however, remain unknown. During endochondral ossification, chondrocytes undergo distinct stages of differentiation, a process that is tightly regulated by a complex array of secreted growth factors.[5] One such growth factor, Indian Hedgehog (IHH), performs crucial roles to regulate chondrocyte hypertrophic differentiation.[5] The stability, distribution, and binding of IHH is regulated in part by GAGs, particularly heparan and chondroitin sulfates.[6,7] The objective of this study was to investigate the mechanisms responsible for failed cartilage-to-bone conversion in MPS VII, and specifically, to examine associations between abnormal GAG accumulation, abnormal chondrocyte differentiation, and altered IHH signaling in developing MPS VII vertebrae, using the naturally occurring canine model.

## Methods

All animal studies were performed with IACUC approval. Normal and MPS VII affected dogs were euthanized at either 2 weeks of age (each n=3) or 6 weeks of age (each n=2). These ages represent developmental stages immediately before and after commencement of secondary ossification in the vertebral bodies of normal animals. MPS VII dogs were identified at birth by DNA mutation genotyping. Following euthanasia with 80mg/kg of intravenous barbiturate, thoracic and lumbar vertebral bodies were isolated. For histological analysis (both 2 and 6 week old animals), whole vertebral bodies were fixed in 4% paraformaldehyde and processed into paraffin. For analysis of GAG accumulation, sections were either stained with Alcian blue, or immunostained for chondroitin sulfate. For analysis of growth plate morphology, sections were stained with hematoxylin and eosin. The mean height of the proliferative zone and the total number of proliferating chondrocytes were calculated from a standardized 2mm-wide region in the center of the growth plate. To examine cells responding to IHH, sections were immunostained for the IHH receptor patched-1 (PTC1), which is upregulated downstream of IHH pathway activation. All slides were imaged and analyzed under bright field microscopy. For mRNA analysis (2 week old animals only), cartilage was isolated from vertebral epiphyses and RNA isolated. Expression of hypertrophy markers (COL10A1 and RUNX2) and hedgehog pathway genes (IHH and PTC1) was determined using real time RT-PCR. All target genes were normalized to β-actin, and analyzed via the comparative CT method. Differences in measured parameters between normal and MPS VII at each age were established using Student's t-tests (significance = $p<0.05$).

## Results

Alcian blue and chondroitin sulfate staining demonstrated significant abnormal increased GAG accumulation in the vertebral epiphyses of MPS VII animals, even at 2 weeks of age (Figure 1). In MPS VII dogs, the height of the growth plate proliferative zone was lower for MPS VII veterbrae compared to normals (69% of normal at 2 weeks of age ($p<0.05$), and 57% of normal at 6 weeks-of-age ($p=0.07$)), as was the total number of growth plate proliferating chondrocytes (63% of normal at 2 weeks of age ($p<0.05$), and 56% of normal at 6 weeks of age ($p=0.06$)). At 6 weeks of age, decreased and aberrant PTC1 staining

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chelsd@vet.upenn.edu

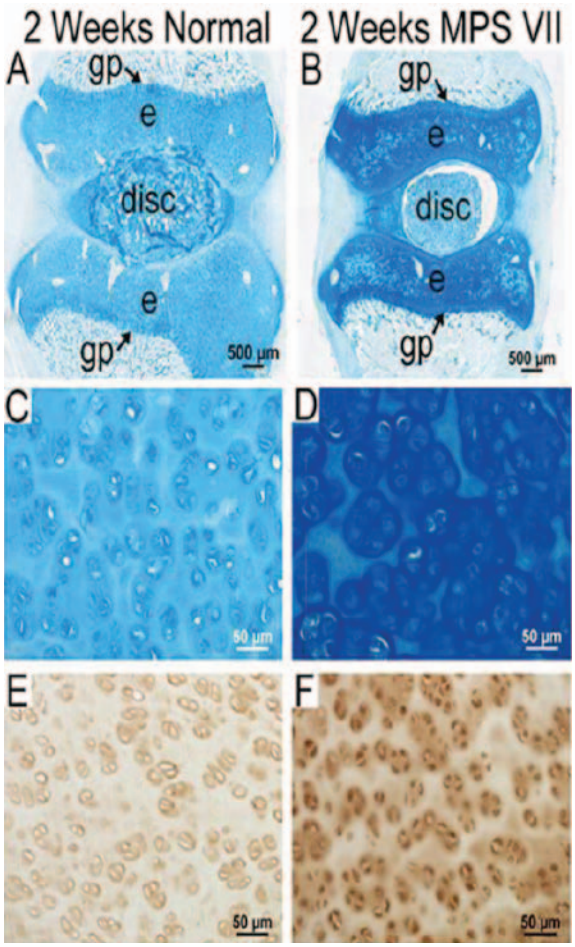


**Figure 1.** Abnormal GAG accumulation in the vertebral epiphyseal cartilage of MPS VII dogs compared to normals is striking even at 2 weeks-of-age. A-D) Alcian blue stain for total GAG. E-F) Chondroitin sulfate; e = epiphysis, gp = growth plate.

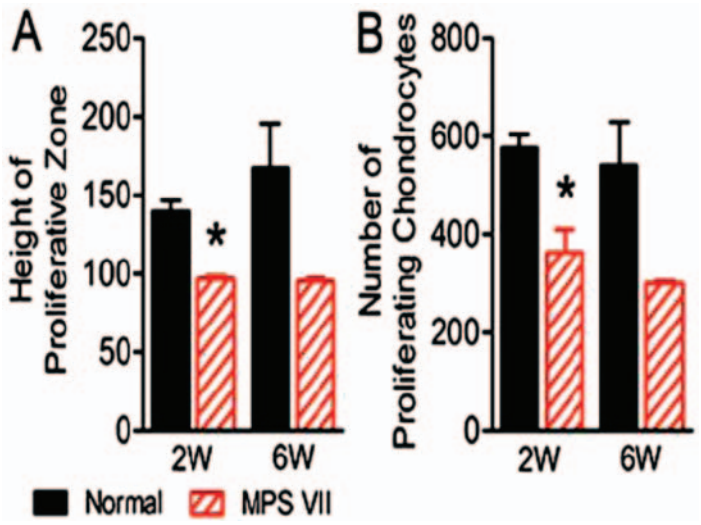


**Figure 2.** Differences in (A) Proliferative zone height and (B) Number of proliferating chondrocytes between normal and MPS VII vertebral growth plates at 2 and 6 weeks of age. *p<0.05 vs normal.

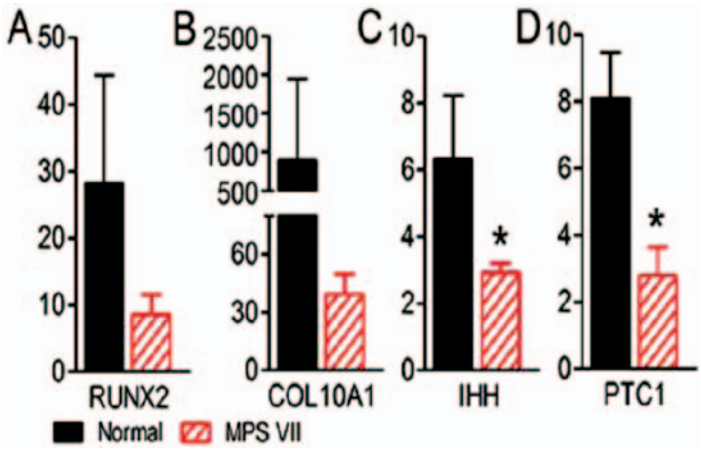


**Figure 3.** mRNA expression of hypertrophic markers and IHH pathway genes at 2 weeks-of-age. A. RUNX2. B. COL10A1. C. IHH. D. PTC1. *p<0.05 vs normal.

was detected in MPS VII vertebral epiphyses compared to normals (Figure 2). The mRNA expression levels of IHH and PTC1 were significantly lower for MPS VII animals (46% and 34% of normal respectively, both $p<0.05$, Figure 3). Expression of hypertrophy markers COL10A1 and RUNX2 was also lower, although not significantly (4% and 30% of normal respectively, Figure 3).

## Discussion

In this study, we investigated mechanisms of failed cartilage-to-bone conversion in MPS VII vertebrae. Key findings include striking, abnormal GAG accumulation within the epiphyseal cartilage of MPS VII vertebrae as early as 2 weeks of age, morphological abnormalities of the growth plate at 2 and 6 weeks of age, and decreased mRNA expression of hypertrophic markers at 2 weeks of age, all indicative of delayed chondrocyte differentiation. The IHH signaling pathway plays a critical role in endochondral ossification, both through direct induction of chondrocyte differentiation and indirectly through activation of other signaling pathways, such as parathyroid hormone-related peptide signaling.[8,9] Here we demonstrate significantly decreased mRNA expression of both IHH and PTC1 in MPS VII vertebral epiphyseal cartilage at 2 weeks of age, and decreased and aberrant protein expression of the IHH receptor Patched-1 at 6 weeks of age. Our data support that the IHH pathway is a promising therapeutic target for normalizing bone formation in MPS VII. These results also highlight the importance of early therapeutic intervention to prevent progression of bone disease in MPS VII.

## Significance

MPS VII is associated with severe spine disease, which significantly impacts patient quality of life, and for which there are currently no effective treatments. This work elucidates mechanisms of failed bone formation in MPS VII vertebrae and implicates the IHH pathway as a potential therapeutic target.

## Acknowledgments

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