

# Impaired Wnt Signaling Contributes to Delayed Chondrocyte Differentiation in Mucopolysaccharidosis VII Dogs

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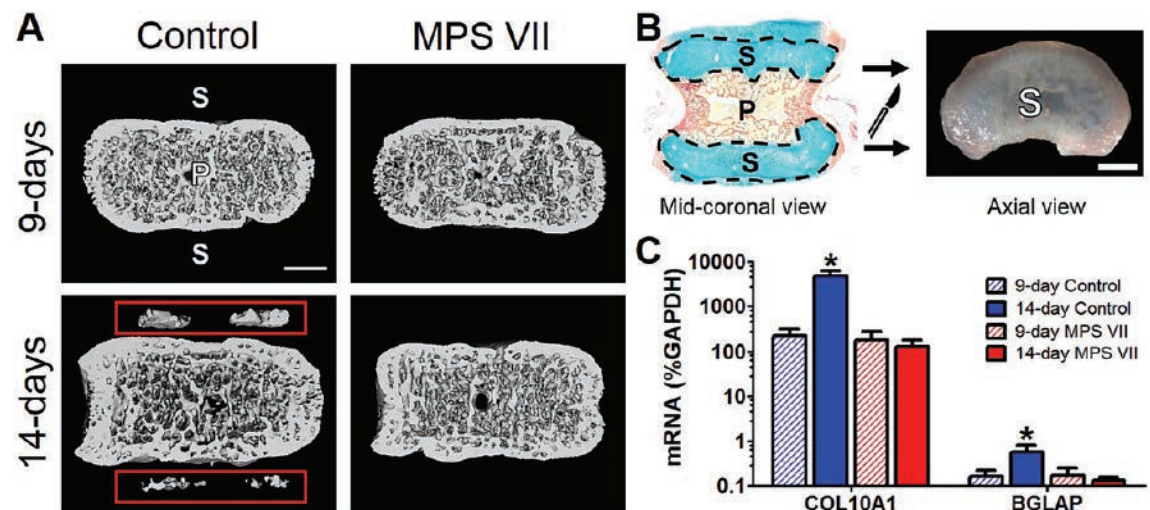
## Introduction

Mucopolysaccharidosis (MPS) VII is a lysosomal storage disorder characterized by mutations in the  $\beta$ -glucuronidase (GUSB) gene. Impaired GUSB enzyme activity leads to the incomplete digestion and progressive accumulation of heparan, chondroitin, and dermatan sulfate glycosaminoglycan (GAG) byproducts.<sup>1</sup> MPS VII presents with severe skeletal manifestations, which are particularly prevalent in the spine. Vertebral dysplasia due to failed cartilage-to-bone conversion during postnatal development leads to kyphoscoliosis and spinal cord compression, significantly reducing patient quality of life and life expectancy.<sup>2,3</sup> Using the naturally-occurring canine model, we previously identified the developmental stage (between 9 and 14 days-of-age) when failed vertebral bone formation first manifests in MPS VII<sup>4</sup> and subsequently found that resident chondrocytes in the vertebral epiphyseal cartilage fail to undergo hypertrophic differentiation (Figure 1). GAGs perform crucial roles in the distribution and availability of many secreted signaling molecules that regulate chondrocyte differentiation.<sup>5,6</sup> We hypothesized that aberrant GAG accumulation in MPS VII epiphyseal cartilage disrupts these signaling

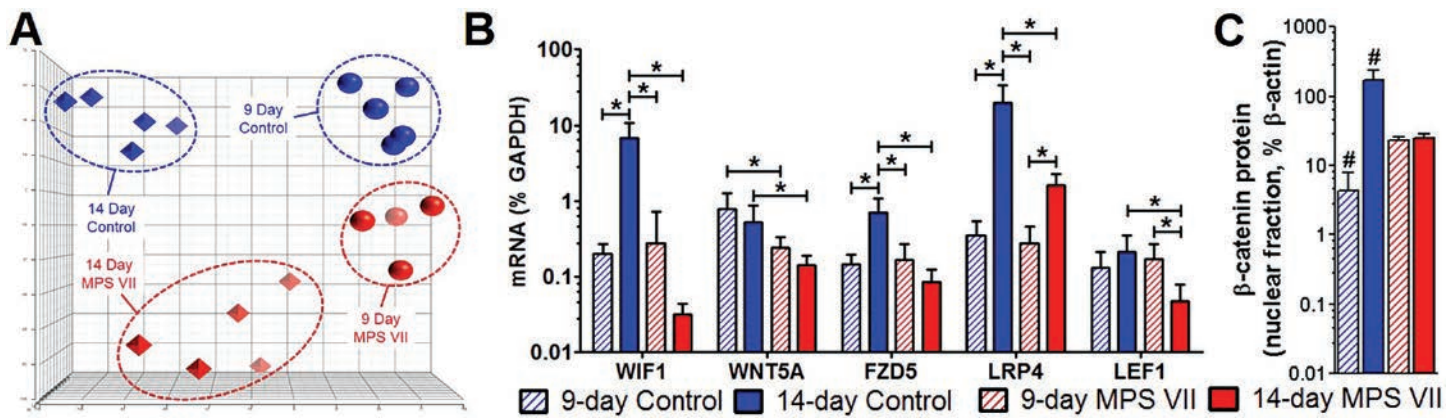
pathways, preventing initiation of chondrocyte differentiation at the appropriate developmental stage. Our objectives in this study were to 1) establish pathways that fail to activate in MPS VII epiphyseal cartilage using whole-transcriptome sequencing (RNA-Seq) and 2) examine cellular responses to related secreted growth factors using a cartilage explant model.

## Methods:

With IACUC approval, vertebral epiphyseal cartilage from unaffected control and MPS VII dogs was collected postmortem at 9 and 14 days (n = 5 all groups, schematic, Figure 1B). High quality total RNA (RIN > 7) was extracted from each sample, and RNA-Seq libraries were prepared using the TruSeq mRNA stranded kit (Illumina; San Diego, CA). Paired-end, 100-base pair sequencing was performed (Illumina HiSeq 2500) and results mapped to the canine genome. Differential gene expression between all groups was determined with DESeq2,<sup>7</sup> with litter as a covariate and adjusted for false discovery rate (significance, p < 0.05). Differential mRNA expression was confirmed using qPCR, and nuclear  $\beta$ -catenin protein levels were measured via Western blots and densitometry. For explant culture studies, vertebral epiphyseal cartilage explants from 9 day control (n = 4) and MPS VII (n = 2) animals were cultured for 1, 3, or 7



**Figure 1.** Representative mid-coronal microCT images of T7 vertebrae. Red boxes: bone formation in secondary ossification centers in 14-day control animals. (B) Schematic of vertebral epiphyseal cartilage excision (mid-coronal ABPR-stained histological section). (C) mRNA levels of COL10A1 (hypertrophic marker) and BGLAP (bone marker) in vertebral epiphyseal cartilage at 9 and 14 days-of-age. N = 5; scale = 1mm; \*p < 0.05 vs all. S: Secondary and P: Primary ossification center.



**Figure 2.** (A) RNA-Seq PCA plot. (B) mRNA levels of Wnt/ $\beta$ -catenin signaling pathway genes measured by qPCR. (C) Nuclear  $\beta$ -catenin protein expression in control and MPS VII vertebral epiphyseal chondrocytes. N = 5; \*p < 0.05; #p < 0.05 vs all.

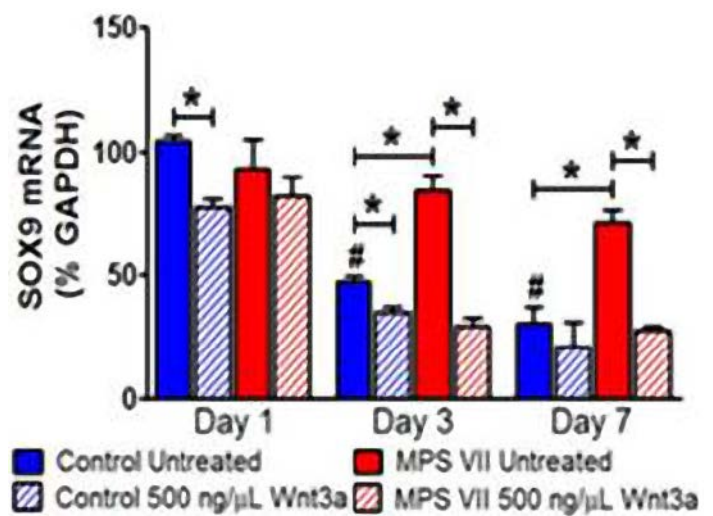
days in serum-free medium ( $\alpha$ -mem, 0.1% BSA, 1% PSF) in the presence or absence of 500 ng/mL Wnt3a (R&D, Minneapolis, MN). Following culture, RNA was isolated from explants and SOX9 mRNA expression was determined using qPCR. Significance ( $p < 0.05$ ) was established with 2-way analyses of variance and post-hoc Tukey's tests (RNA-Seq confirmatory mRNA and protein expression) or unpaired t-tests (cell culture mRNA expression).

## Results

Principal component analysis (PCA) of global gene expression from RNA-Seq showed distinct clustering of each sample group (Figure 2A), indicating clear effects of both age and disease state between all groups. A total of 411 and 1104 genes were significantly differentially expressed with a fold-change greater than 2 between control and MPS VII at 9 and 14 days, respectively. The Wnt/ $\beta$ -catenin pathway was identified as the top dysregulated bone formation pathway at both ages with 14 and 54 pathway-associated genes differentially expressed at 9 and 14 days, respectively. Specifically, there was significantly lower expression of both key inhibitory elements and activating molecules in MPS VII compared to controls, verified by qPCR (Figure 2B). Immunoblots showed significantly higher levels of nuclear  $\beta$ -catenin protein at 14 compared to 9 days in controls but no change in MPS VII (Figure 2C). Control explants treated with Wnt3a exhibited a significant decrease in SOX9 mRNA after 1 and 3 days. After 3 and 7 days, untreated control explants also exhibited a significant decrease in SOX9 expression (Figure 3). In contrast, MPS VII explants exhibited no significant response to Wnt3a at day 1, but did exhibit significant decreases in SOX9 mRNA after 3 and 7 days of treatment. Unlike untreated controls, untreated MPS VII explants did not show decreased SOX9 expression after 3 or 7 days.

## Discussion

Wnt/ $\beta$ -catenin signaling regulates both the timing and rate of chondrocyte differentiation during endochondral ossification.<sup>8,9</sup> Our results demonstrate that in MPS VII epiphyseal cartilage, Wnt/ $\beta$ -catenin signaling does not



**Figure 3.** SOX9 mRNA for control (n = 4) and MPS VII (n = 2) explants with Wnt3a treatment after 1, 3 and 7 days. \*p < 0.05, #p < 0.05 vs day 1.

activate at the appropriate developmental stage to initiate and sustain chondrocyte differentiation. Lower expression of both inhibitory and activating molecules in the Wnt pathway suggests that MPS VII chondrocytes experience decreased Wnt signaling but are unable to upregulate compensatory responses. Sustained low levels of nuclear  $\beta$ -catenin protein expression from 9 to 14 days in MPS VII is consistent with this mechanism. Wnt3a is the prototypical activator of Wnt/ $\beta$ -catenin signaling, potentiating chondrocyte maturation and subsequent bone formation.<sup>10</sup> Downregulation of SOX9 expression is necessary for chondrocytes to proceed from proliferation to hypertrophy.<sup>11</sup> Control explants (with healthy chondrocytes) treated with Wnt3a exhibited an immediate decrease in SOX9; further, with increasing culture time, untreated control explants exhibited decreased SOX9, suggesting an intrinsic propensity of healthy cells to mature towards hypertrophy even in the absence of exogenous signals. In contrast, MPS VII explants exhibited a delayed response to Wnt3a treatment, and in the absence of Wnt3a

continued to express high levels of SOX9 after 3 and 7 days, suggesting an intrinsic inability of diseased cells to both respond to exogenous signals and transition from proliferation to hypertrophy. These results provide the basis for further mechanistic investigations of skeletal disease in MPS VII and identify the Wnt/ $\beta$ -catenin pathway as a potential therapeutic target.

### Significance

MPS VII is associated with severe skeletal disease for which there are currently no effective treatments. This study identifies the Wnt/ $\beta$ -catenin pathway as a promising target for therapeutic intervention in MPS VII.

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