Temporal Dysregulation of Chondrocyte Maturation and Extracellular Matrix Modification Following Neonatal Type III Collagen Loss in Murine Femoral Head Cartilage

Tiffany C. Wu[1-3], Daniel C. Stewart[1], William Yen[1], Yuchen Liu[5], Sidney Wong[1,4], Lin Han[5], Robert L. Mauck[2,3,6], and Susan W. Volk[1]
[1]Dept. of Clinical Studies & Advanced Medicine, School of Veterinary Medicine, [2]Dept. of Bioengineering, [3]Dept. of Orthopaedic Surgery, [4]School of Arts and Sciences, University of Pennsylvania, Philadelphia, PA, [5]School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, [6]Translational Musculoskeletal Research Center, Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA
tif5353@seas.upenn.edu

Disclosures: TCW (N), DCS (N), WY (N), YL (N), SW (N), LH (8 – *Osteoarthritis Cartilage*), RLM (5 – 4WEB Medical), SWV (N)

INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease characterized by the breakdown of cartilage extracellular matrix (ECM).[1] Regenerative strategies for cartilage repair remain elusive due to a limited understanding of ECM assembly and chondrocyte-matrix interactions.[2,3] Recent evidence implicates type III collagen (COL3), an understudied fibril-forming collagen present in low concentrations (~1-5%) in healthy human cartilage, as a key regulator of ECM structural integrity and cartilage health.[4] Other work has shown a critical role for COL3 in regulating matrix architecture and mechanoresponses in other tissues.[5,6] In this study, we hypothesize that COL3 is a critical component of cartilage extra- and peri-cellular matrix maintenance and repair via its roles in neo-matrix assembly and integrin-mediated mechanotransduction.

METHODS: *Conditional* Col3a1 *knockout mice*: Systemic knockout of COL3 (*Col3a1* gene) was induced by intragastric injection of tamoxifen (TM) to neonatal (P0-2) conditional *Col3a1* knockout mice (*Col3a1*$^{F/F}$/RosaCre^{ER}, or F/F, n=22, 9♂, 13♀).[6] Control wild-type mice (*Col3a1*$^{B6/B6}$/RosaCre^{ER}, or B6/B6, n=22, 10♂, 12♀) were similarly injected with TM (IACUC approved). *RT-qPCR*: Femoral head articular cartilage was harvested either on post-natal day 21 (P21) or days 50-60 (P50-60) and snap frozen. Relative gene expression of *Col3a1*, *Col2a1, Col10a1, Acan*, *Itga10*, *Itga11*, *Mmp3, Mmp9*, and *Mmp13* were measured in F/F and B6/B6 mice (*Gapdh* was used as a housekeeper). *Histology*: Cartilage at P21 or P50-60 was fixed overnight in glutaraldehyde, embedded in paraffin, and 5 µm sections were stained with Safranin-O/Fast Green. Quantification of areal cell density (number of cells/unit area) and chondrocyte cell area was accomplished with wheat germ agglutin stained slides (to identify cell membranes) with DAPI, using ImageJ and CellProfiler in five regions of interest in the hypertrophic zone. *In situ zymography (ISZ)*: Cartilage sections were incubated with Invitrogen DQ™ collagen type IV (COL4) and collagen type I (COL1) to assess matrix metalloproteinase (MMP) activity against these substrates. *Statistical Analysis*: All statistics were conducted in GraphPad Prism with a significance level of α=0.05. Outliers were removed from each dataset using Grubbs' Test and significant differences were detected using unpaired Student's t-tests.

RESULTS: Efficient knockout of the floxed *Col3a1* allele in F/F mice was achieved, as demonstrated by a reduction of *Col3a1* mRNA levels to < 1% following neonatal TM treatment compared to TM-treated B6/B6 control mice at both P21 (p<0.0001, n≥12, 4♂, 8♀) and P50-60 (p=0.0031, n≥5, 2-3♂, 3-5♀). Histological analysis of cartilage revealed an expanded hypertrophic chondrocyte zone in P21 COL3-deficient mice compared to controls. (**Fig. 1A, 1B**). Both areal cell density and average cell area were increased in F/F relative to B6/B6 cartilage of P21 mice (**Fig. 1C, 1D)**, although these histomorphometric differences were resolved by P50-60 (**Fig. 1E, 1F**; images not shown). qPCR analysis revealed significant decreases in *Col2a1* (p=0.0164), *Col10a1* (p=0.0013), *Acan* (p=0.0315), and *Itga10* (p=0.0022) mRNA levels in F/F tissues compared to controls; no significant differences in *Itga11* (p=0.7022), *Mmp3* (p=0.1917), *Mmp9* (p=0.6250), or *Mmp13* (p=0.1766) expression levels were observed (n≥10, 4♂, 6♀). To further investigate induction of catabolic activity after COL3 loss, ISZ was performed, which revealed an increase in MMP activity against COL1 substrates (**Fig. 2A-C**), but not COL4 substrates (**Fig. 2D-F**) in COL3-deficient cartilage relative to controls. In contrast to the differential gene expression induced by COL3 loss at P21, at P50-60, qPCR data revealed no differences in *Col2a1* (p=0.4684), *Col10a1* (p=0.3334), *Acan* (p=0.9439), *Itga10* (p=0.7885), or *Mmp3* (p=0.3306) expression levels in COL3-deficient cartilage compared to controls (n≥5, 2-3♂, 3-5♀). However, there were suggested increases in *Itga11* (p=0.0908), *Mmp9* (p=0.0988), and *Mmp13* (p=0.0574, n ≥ 4, 2-3♂, 2-5♀). At this P50-60 timepoint, there was a significant increase in MMP activity in the ECM space assessed by ISZ against COL4 substrates in COL3-deficient cartilage compared to controls (**Fig. 3D-F**), but not against COL1 (**Fig. 3A-C**), in contrast to the observations at P21.

DISCUSSION: Our findings reveal a role for COL3 in temporally regulating chondrocyte maturation and ECM remodeling during postnatal development of murine femoral head cartilage. At P21, COL3 knockdown resulted in increased hypertrophic chondrocyte areal density and cell area and a reduction in *Col2a1*, *Acan*, and *Itga10* expression. This suggests an altered dynamic of how and when chondrocytes undergo and progress through hypertrophy in the ossification center. By late development (P50-60), both histology and altered cell phenotype markers normalized across genotypes, suggesting that COL3 is critical during early endochondral ossification but less so as the cartilage matures. COL3 also modulated ECM modification through temporally distinct MMP activity: COL3 loss induced an early increase in MMPs targeting COL1 substrates (relevant to cartilage: MMP-1, -13, and -14)[7] and shifted towards activation of COL4-degrading MMPs (MMP-2, -9, and -12)[8] later in development.

SIGNIFICANCE: These findings identify COL3 as a critical regulator of early chondrocyte phenotype and cartilage matrix remodeling during postnatal development and suggest a potential role for COL3 loss in accelerating cartilage degeneration and OA progression. Future studies will explore COL3's therapeutic relevance in cartilage repair and disease prevention.

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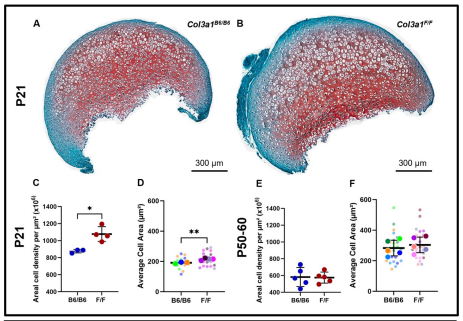

Fig. 1. Representative Safranin-O/Fast Green staining of P21 femoral head cartilage of P21 *Col3a1*$^{B6/B6}$ **(A)** and *Col3a1*$^{F/F}$ **(B)** mice, revealing expansion of hypertrophic chondrocytes. Quantification of areal cell density per µm² and average cell area (µm²) at P21 **(C, D)** and P50-60 **(E, F)**. Mean ± SD, n ≥ 3, 1-4♂, 2-3♀, unpaired Student's T-test (* p ≤ 0.05, ** p ≤ 0.01).

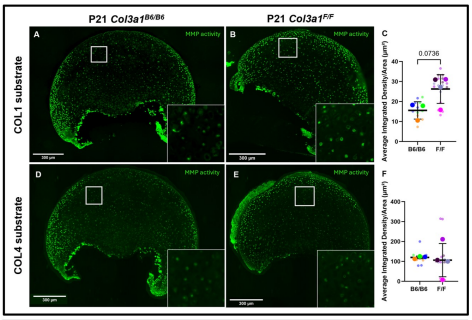

Fig. 2. Representative P21 in situ zymography staining of MMP activity against COL1 substrates (MMP-1, -13, -14) **(A, B)** and COL4 substrates (MMP-2, -9, -12) **(C, D)** in *Col3a1*$^{B6/B6}$ and *Col3a1*$^{F/F}$ murine femoral head cartilage. Quantification of staining intensity normalized to area (µm²) **(C, F)**. Mean ± SD, n ≥ 3, 1-2♂, 2-3♀, unpaired Student's T-test (* p ≤ 0.05).

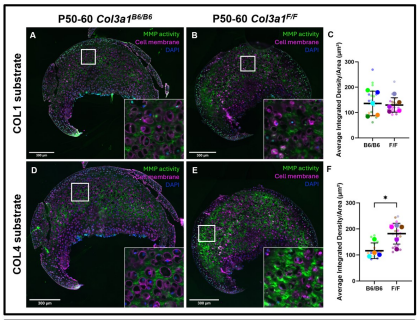

Fig. 3. Representative in situ zymography staining of MMP activity (green) against COL1 **(A, B)** and COL4 **(C, D)** substrates (green) in P50-60 murine femoral head cartilage. Quantification of staining intensity normalized to area (µm²) **(C, F)**. Mean ± SD, n ≥ 4, 3-4♂, 1-3♀, unpaired Student's T-test (* p ≤ 0.05).