# Cellular Dynamics and Zonal Specialization of the Murine Meniscus ECM during Postnatal Growth

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## Introduction

The molecular composition and organization of the extracellular matrix (ECM) is essential for the load bearing function of the adult knee meniscus. In large animals, the body of the meniscus is most often described as having a well-defined cartilaginous, collagen II-rich 'inner' region and fibrous, collagen I-rich 'outer' region. However, heterogeneity in the ECM is observed in both of these regions during aging[1] and with pathologic remodeling following injury.[2] This observation underscores that the understanding of how aberrant compositional changes emerge during meniscus degeneration requires an improved knowledge of how the mature meniscus ECM is established at the cellular and molecular level. To this end, we employ innovative transgenic murine meniscus models to study tissue maturation at the single cell length scale and with fine temporal resolution. Our previous work focused on defining changes in meniscus cell phenotypes throughout inner and outer zones and revealed regionalization of particular cell populations during maturation.[3] Here, we expand the analysis of postnatal maturation by quantifying both cellular and extracellular growth dynamics within the meniscus body.

## Methods

All animal work was approved by the UPenn IACUC.

### *Samples*

For all analyses, mouse hindlimbs (P0-P28, CD1 background) were harvested, fixed in 4% PFA, embedded in OCT, and cryosectioned (7μm) in the coronal plane as previously described.[4] Only sections containing the body region of the medial meniscus were analyzed.

### *EdU labeling*

3μg/g of EdU was IP injected into mice for two consecutive days prior to sacrifice at P4, P14, or P28. EdU incorporation was assessed using the Click Chemistry Tools kit and Calfluor Azide AF647, followed by DAPI counterstaining, and fluorescent imaging.

### *Quantification*

Tissue area, cell number, and EdU staining was quantified using Fiji plugins.[5] 3-5 sections were quantified and averaged per animal, with 3-5 animals/age group. One-way ANOVA with Tukey post-hoc ($p < 0.01$) was used to determine differences between age groups.

### *Second harmonic imaging (SHG)*

Tissue sections were imaged using a multiphoton microscope and 20X objective. Equal laser power was used between P7 and P28 samples for signal intensity comparison, but was increased to detect the lower signal in P0 samples.

## Results

The cross-sectional area of the meniscus body increased exponentially in the first two weeks post-birth (P0-P14) and plateaued thereafter —demonstrating rapid tissue growth (Fig. 1a, b). Cell proliferation did not contribute to the growth, as the cell count per tissue section was similar across timepoints, and minimal EdU incorporation was observed in meniscus cells after P4 (Fig. 1b, c). Increased SHG signal between P7 and P28 indicated accumulation of fibrillar collagen within the tissue (Fig. 2a, top panel), suggesting that matrix deposition is the likely cause of rapid size changes in the postnatal meniscus. Interestingly, proteoglycan (PG) and collagen staining showed a distinct PG-rich inner zone and collagen-rich outer zone already present at P7 (Fig. 2a, arrowheads). Indeed, appreciable inner/outer regional differences in ECM structure were present at birth (P0), with cells of the outer meniscus residing in a circumferentially aligned collagen matrix, while cells of the inner meniscus were not organized in this aligned fibrous matrix (Fig. 2b). By P28, however, PGs were more widespread, intercalated between the fibrous matrix, and enriched within the pericellular spaces (Fig. 2a).

## Discussion

As expected, postnatal meniscus growth is characterized by an accumulation of ECM proteins and the introduction of extrinsic mechanical forces (i.e. weightbearing). Given the fact that little cell division is detected within

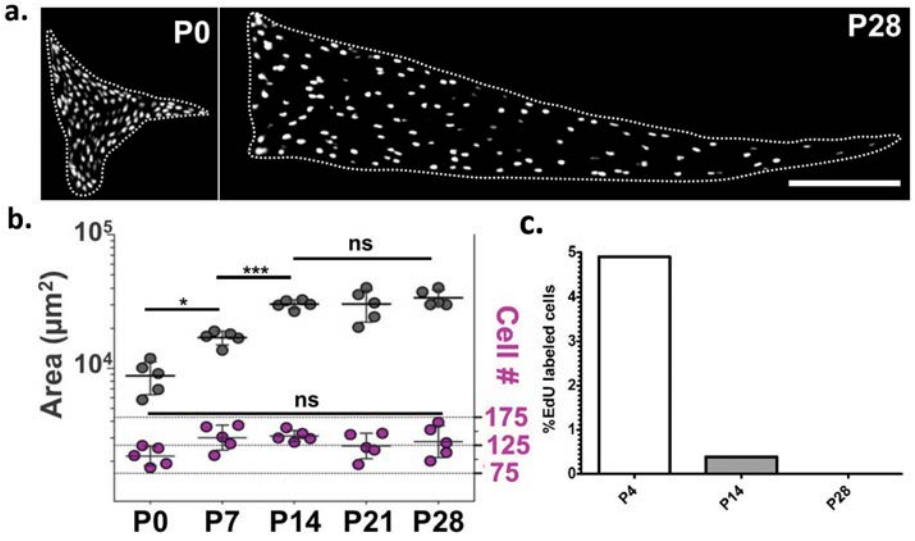


**Figure 1. (A)** Representative P0 and P28 meniscus body cross sections, with the tissue border outlined by a white dotted line, and cell nuclei stained (with DAPI) in grey. SB: 100mm. **(B)** Cross sectional area plotted on the left axis ($mm^2$, log10 scale, grey dots) and average cell number per measured cross section plotted on the right axis (linear scale, pink dots). n = 5 animals/age, mean ± s.d. shown. *$p < 0.01$, ***$p < 0.0001$, ns: not significant. **(C)** Average percentage of cells positive for EdU staining within meniscus body sections. n=4 animals/age, mean ± s.d. shown.

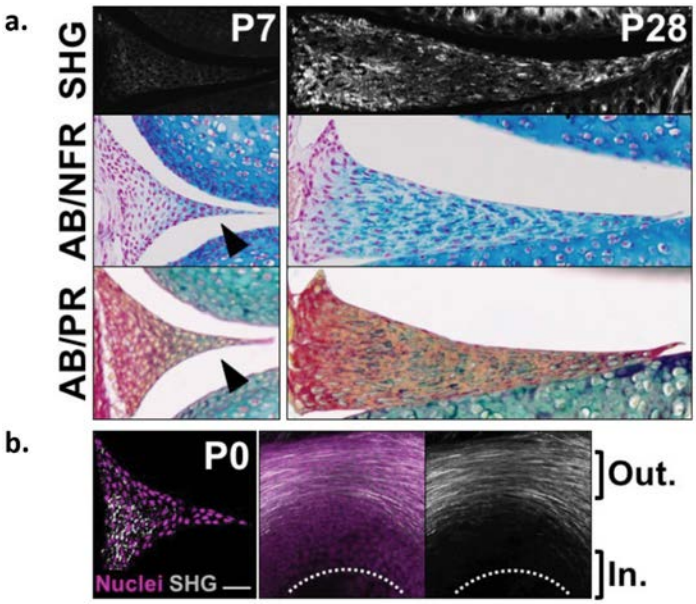


**Figure 2. (A)** Cross sections at P7 (left) and P28 (right), imaged for: fibrillar collagen using SHG with equivalent multiphoton laser settings (top panel), proteoglycans and nuclei using Alcian blue and nuclear fast red stains (AB/NFR, middle panel), and proteoglycan and collagen matrix using Alcian blue and Picrosirius red (AB/PR, bottom panel). **(B)** SHG imaging of P0 body region in the cross sectional plane (left) and circumferential transverse plane (right) with a DAPI counterstain. Dotted white line indicates inner edge of tissue. "Out": Outer region. "In." Inner region. SB: 20mm.

the tissue by P4, our data also indicate that the resident cells that orchestrate the establishment of the mature ECM are, in fact, present at the time the animal is born (Fig 1). Taken together, these observations show that the same population of meniscus cells is subject to tremendous changes in their microenvironment. As the principles of mechanobiology dictate, such shifts in biophysical cues can in turn alter the behavior of these cell populations. Thus, the observations of pericellular accumulation of PGs after P7 (Fig. 2a) and rapid tissue growth following full weightbearing (P0 to P14, Fig. 1b) may both be evidence of a cellular mechanobiologic response to elevated joint loading. Importantly, our data determine that ECM specialization in the inner and outer zones of the meniscus is present as soon as the animal is born (Fig. 2). In all, these findings suggest that while regional differences in loading patterns may drive changes in matrix deposition at *later* stages of maturation, other mechanisms—such as regional differences in cell origins[6]—may be at play in specifying inner and outer zones during early development.

## Significance

Ultimately, defining the cellular and mechanical regulators of hierarchical dense connective tissue formation is pivotal to guiding our repair strategies and providing success benchmarks to establish efficacy. This study is one of our first steps in establishing the cellular and matrix changes that occur during rapid tissue growth in the murine meniscus. Future studies will define the mechanisms that regulate cell fate and ECM organization.

## Acknowledgments

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## References

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