Single cell imaging of Col1/Col2 fluorescent reporters in the murine meniscus reveals marked spatial heterogeneity

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Introduction

The molecular composition and organization of the extracellular matrix (ECM) is essential for the function of load bearing tissues such as the knee meniscus. To better understand the mechanisms governing meniscus development, maturation, and remodeling, mouse models have become a prominent tools that provide a genetically tractable platform through which to both visualize and perturb molecular signaling and matrix formation [1][2]. Recently, a novel transgenic reporter mouse in which the expression of three distinct fluorescent reporter proteins is driven by promoters for the Col1a1, Col2a1, or Col10a1 genes [3] has been demonstrated as powerful system for studying spatiotemporal changes in expression of critical collagen types during tendon enthesis maturation [4]. Leveraging this mouse model, our goal is to investigate an important unanswered question of meniscus development and homeostasis. Namely, despite the fact that the meniscus is most often described as having a well-defined cartilaginous, Collagen II-rich 'inner' zone and fibrous, Collagen I-rich 'outer' zone, marked and emergent heterogeneity is observed in the ECM of both regions during aging [5] and pathologic remodeling following injury [6]. This is suggestive of multiple endogenous cell types with varying lineage capacities located within the tissue. To ultimately establish whether these cells are present and operative throughout development and maturation, the current study used the Col1/2/10 reporter mouse to assay expression profiles of individual meniscus fibrochondrocytes (MFCs) as a function of location within a juvenile meniscus.

Methods

Mouse model

This study utilized triple transgenic reporter mice with promoters for Col1a1, Col2a1, and Col10a1 driving CFP, YFP, and mCherry expression, respectively [2][3]. Animal use was approved by the University of Pennsylvania IACUC.

Sample preparation and staining

To characterize a time point prior to the ossification of the meniscal horns, knees from 2 week old mice (n = 4) were harvested, fixed, cryo embedded, sectioned with cryofilm [7], and their nuclei were counterstained with TO-PRO-3. Sections were taken from multiple cutting planes and levels throughout the menisci and imaged on an Axio Scan.Z1 microscope. To elucidate regional variation in expression profiles, both coronal and axial sections were imaged. Following fluorescent imaging, sections were stained with Alcian blue and Picrosirius red (for proteoglycans and collagen content, respectively) and imaged again to correlate matrix deposition with reporter expression.

Image quantification

Meniscus areas within coronal sections were segmented and defined as the regions of interest. Fluorescent intensity from the Col1 and Col2 channels was plotted from the inner margin to the outer boundary of the meniscus—with 0 marking the inner most and 1 the outermost boundary. In transverse sections, images were thresholded to segment individual nuclei. Reporter intensity for each cell was then measured and scatterplots were generated to correlate Col1 and Col2 expression in individual cells. Fluorescence intensities in both cases were scaled to the brightest cell detected within a slice. All image processing was performed using Fiji and custom written MATLAB scripts.

Results

High, uniform Col1 expression (Col1+) in cells located within the cruciate ligaments and high, uniform expression of Col2 (Col2+) in cells within the articular cartilage confirmed expected patterns of Col1/Col2 expression in the knee joint (data not shown). While Col10 positive cells (Col10+) were detected within the hypertrophic chondrocytes of the secondary ossification center, no Col10+ cells were detected within the meniscus at this time point. Cells within the meniscus did show a distinct zonal expression of Col1 and Col2 in the anterior horn, with Col1+ cells located in the outer region and a sharp transition to Col2+ cells in the center and inner zones (Figure 1a). This zonal distinction was much less evident, however, within the body and posterior horn of the meniscus. Rather than regions of uniform expression, Col1+ and/or Col2+ cells were

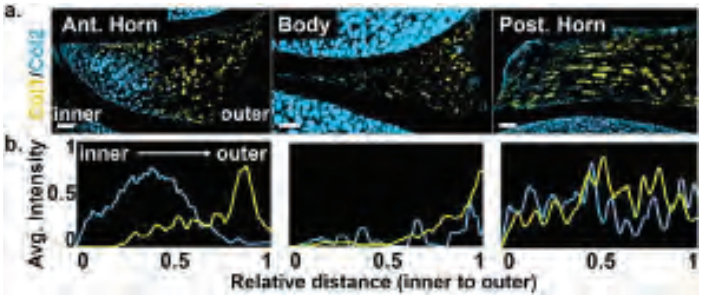

Figure 1. (A). Rep resentative coronal images of Coll (yellow) and Col2 (blue) expressing cells in the anterior hom, body and posterior hom of the meniscus. **(B)**. Fluorescence intensity plots for the corresponding images in **(C)**. Fluorescence intensity was scaled to the h ighest average valu e in the section. Distance scaled to total width of meniscus segment: O = inner-most, I = outer-most, Scale bars: 50µm.

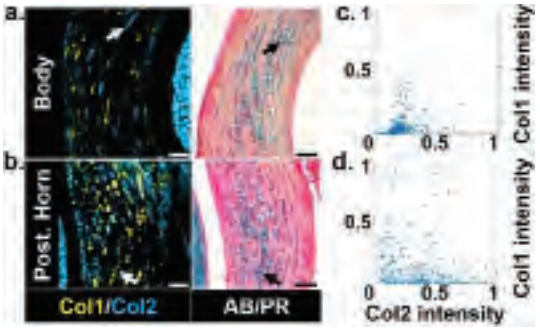

Figure 2) (A,B): Fluorescence and Alcian blue (AB)/ Picrosirius red (PR) images of the same section from the **(A)** body and **(B)** posterior hom region. Arrows point to the same cells in corresponding images. **(C,D):** Coll vs. Col2 fluorescence intensity in MFCs of the **(C)** body and **(D)** posterior horn images. Intensities scaled to the highest val ue meas ured in the section. Scale bar: 50µm.

interspersed throughout the inner and outer zones (Figure 1b,c). Indeed, transverse sections highlighted the presence of Col2+ MFCs within the body and posterior horn (Figure 2a,b), though much fewer cells within the body showed active Col1 or Col2 expression when compared to the posterior horn (Figure 2c,d). Interestingly, chromogenic staining of these same sections showed proteoglycan deposits in regions with either Col2 expressing or Col I expressing MFCs (Figure 2a,b arrows).

Discussion

Imaging the triple-reporter mouse meniscus through multiple planes of sectioning revealed that, while collagen expression within the anterior horn is consistent with the notion of distinct inner Col2-rich versus outer Col1-rich zones, the body and posterior horn portions display overlapping Col1/Col2 expression profiles. In fact, the clear zones observed in the anterior horn are likely due to the ongoing ossification process that will occur at a later age in these animals. In the body and posterior horn regions, numerous cells expressed Col1, Col2, or both simultaneously, and were interspersed with one another throughout the meniscus expanse (Figure 2c,d). This observation suggests that development emplaces cells of multiple and varying potentials throughout the meniscus as it forms, and this heterogeneity in cellular disposition may precipitate the accumulation of the proteoglycan-rich deposits seen in the outer regions during aging and degeneration.

Significance

This work evaluated the expression of key fibrocartilage associated collagens on an *individual* cell level within the developing meniscus. This provides a quantitative assessment of the distribution of Col1/2/10-expressing cells throughout the entire expanse of the tissue - establishing a baseline and validating a promising platform for future work involving cellular-level measurement of gene expression of ECM proteins in response to mechano- biologic perturbations in both *in vitro* and *in vivo* settings.

Acknowledgements

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References

1. Gamer+, 2017.
2. Hyde+, 2008.
3. Maye+, 2011.
4. Dyment+, 2015.
5. Han+ 2016.
6. Le Graverand+, 2001.
7. Dyment+, 2016.