Temporal Dynamics of Nascent ECM Production by Chondrocytes and MSCS via Multi-Color Protein Labeling

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Introduction

The extracellular matrix (ECM) of articular cartilage provides mechanical resilience over a lifetime of load-bearing use. Matrix synthesis and turnover occur continuously, and are requisite for cartilage homeostasis. In the context of engineered cartilage, the rates of ECM production, retention, and degradation define how rapidly an engineered construct can mature. However, most traditional methods to quantify the temporal dynamics of ECM formation rely on bulk biochemical measures that mask differences within the cell population.[1] Methods that resolve matrix at the single cell level, such as traditional staining or autoradiography coupled with histology, are unable to measure matrix synthesis rates in a single sample at multiple time points.[2] To overcome these limitations, we used a novel metabolic labeling approach in which artificial methionine analogs are included in the culture media. These analogs incorporate into proteins during synthesis and, following fixation, are fluorescently labeled via a 'click' reaction.[3] Sequential culture in the presence of different analogs allows for multi-color visualization of ECM formed at different times (analogous to how bone apposition rate is monitored by sequential exposure calcein and alizarin red.[4] This method allows one to quantify the temporal characteristics of ECM formation and visualize the structure and distribution of nascent ECM elements. In this study, we used this novel technique to quantify differences in the timing, heterogeneity, and localization of ECM produced by chondrocytes and mesenchymal stem cells (MSCs) in 3D culture.

Methods

Cell culture- Juvenile bovine MSCs and chondrocytes were isolated as in.[5] Passage 1 cells were encapsulated in 2% agarose micro-gels (μ-gels, ~400 μm in thickness) at 2×10^6 cells/mL. These μ-gels were cultured in a chemically defined, methionine-free media containing 10 ng/mL TGFβ-3 and 50 mM of the methionine analog homopropargylglycine (HPG) for days 1 to 7. On days 8 to 9, the HPG was replaced with 50 mM of a different methionine analog (azidohomoalanine (AHA)). Following fixation, Alexa488-azide and Alexa594-alkyne were reacted with HPG and AHA, respectively, to label proteins that had incorporated the methionine analogs during the two culture periods.[3] Nuclei were stained with Hoechst. *Imaging & analysis-* Individual cells were located via nuclear staining, and confocal sections of the cell midplane were captured at 100× (n = 20-30 cells/group). For each cell, 20 radial intensity profiles emanating from the cell center were mapped, truncated to include only the extracellular domain, and averaged over each cell. Total matrix intensity was calculated by integrating intensity over distance from the cell membrane. *Statistics-* Matrix radius and total intensity were compared via t-test.

Results

Methionine analogs metabolically labeled both intracellular proteins as well as proteins incorporated into the forming ECM (Figure 1A). At day 1, both cell types demonstrated intracellular labeling, but little extracellular labeling. During the first 7 days, ECM progressively extended from the cell border and into the extracellular space (Figure 1B-C). By day 7, both chondrocytes and MSCs accumulated a metabolically labeled extracellular matrix comprised of discrete fibers (Figure 1D). The chondrocyte matrix was both more extensive (radius = 19.8 vs 3.1 μm, $p < 0.01$) and more intensely labeled (total intensity = 165 vs 49 a.u., $p < 0.01$) than the MSC matrix. To compare the relative variability of the matrix, we computed the coefficient of variation (CV) of total matrix intensity. On day 7, MSCs demonstrated greater matrix variability than chondrocytes (MSC CV = 1.84, chondrocyte CV = 1.24), likely reflecting the heterogeneity that is characteristic of MSCs, as well as the slower kinetics of their differentiation and matrix formation process.

When the media was switched at day 8 from HPG- to AHA-containing media, two temporally distinct protein populations were labeled green and red respectively (Figure 2A). In chondrocytes, the nascent matrix accumulated over days 8-9 and labeled with AHA (red) was strikingly different from the matrix accumulated earlier in culture ('pre-existing' matrix incorporating HPG, green). In contrast to the discrete fibers formed at earlier time points, the nascent matrix formed by chondrocytes during this period lacked clear organization and

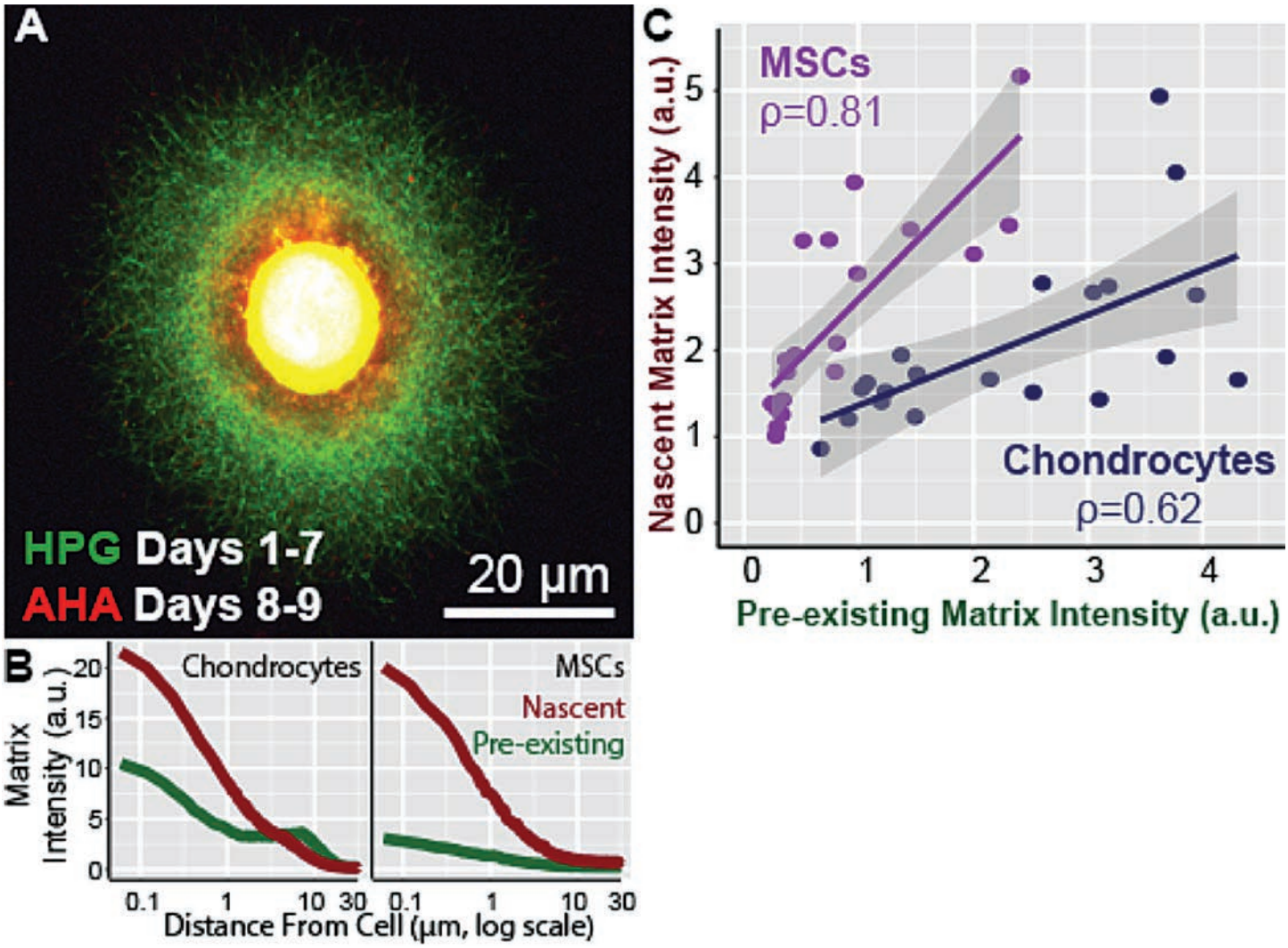

Figure 1. Metabolic labeling of ECM. (A) Day 7 chondrocyte labeled with HPG. (B) Radial profiles and (C) morphology of chondrocyte ECM with time. (D) Individual (grey) & average (blue/purple) profiles.

was primarily restricted to the pericellular space. Intensity profiles suggest chondrocyte nascent and pre-existing protein fractions segregated spatially (Figure 2B). Conversely, in MSCs, nascent matrix appeared to form discrete fibers that emanated from the cell and intermingled with pre-existing matrix. At day 9, the intensity of pre-existing matrix differed between cell types (chondrocyte = 116 a.u., MSC = 40 a.u., $p < 0.01$). However, the intensity of nascent matrix was similar between MSCs and chondrocytes. Because nascent and pre-existing matrix intensity were measured simultaneously, it was possible to examine the correlation between nascent and pre-existing matrix at the single cell level (Figure 2C). Nascent matrix was better correlated with pre-existing matrix in MSCs than in chondrocytes ($\rho_{MSC} = 0.81$, $\rho_{CH} = 0.62$, $p = 0.12$ via Fisher Z transformation).

Discussion

AHA and HPG incorporation into methionine-containing proteins (e.g. Col2, aggrecan) enabled the fluorescent labeling of temporally distinct protein fractions deposited in the extracellular space. One color labeling results were consistent with the expectation that ECM accumulates with culture time, and that chondrocytes produce more ECM than chondrogenically-induced MSCs.[1] Consistent with the notion that MSCs are comprised of multiple clonal sub-populations, ECM production by MSCs at the single cell level was more heterogeneous than chondrocytes. Sequential two color labeling showed that the matrix produced by chondrocytes at early time points differs in structure and organization from the nascent matrix produced later in culture. In contrast, the nascent and pre-existing matrix fractions were similar in MSCs, reflecting differences in the trajectory and rate of matrix accumulation between these cell types. Nascent matrix positively correlated with pre-existing matrix, suggesting that cells that initially produced extensive matrix continued to do so later in culture. The strong nascent-to-pre-existing correlation in MSCs may indicate that matrix accumulation from days 7-9 is a temporally steady process in MSCs. In contrast, the weaker correlation in chondrocytes

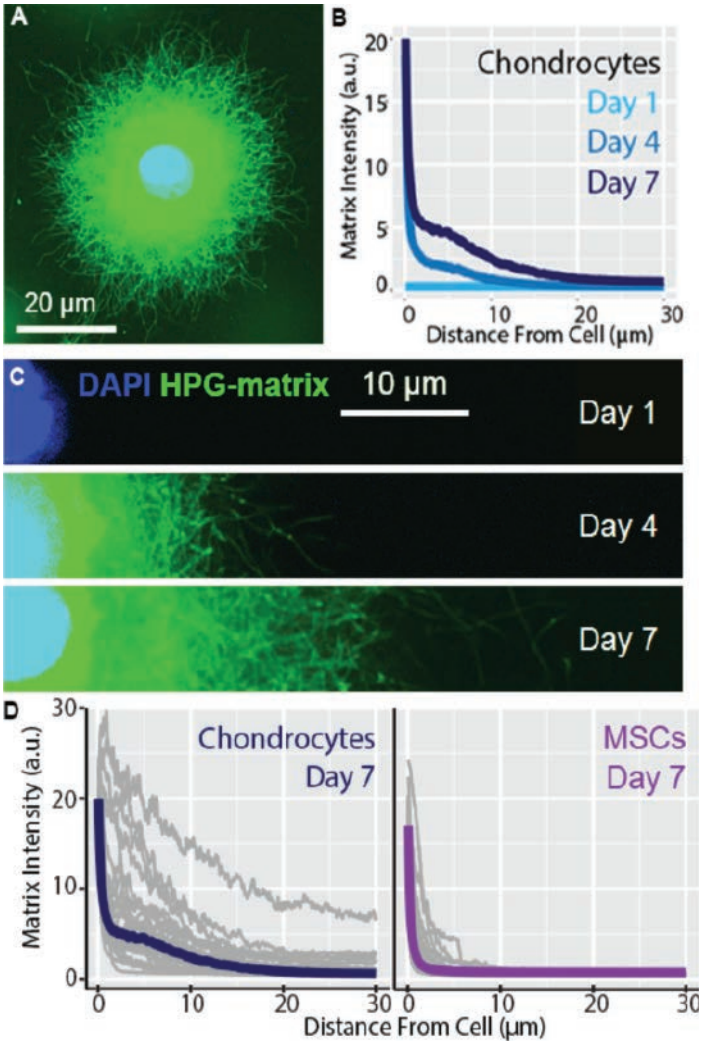

Figure 2. Sequential labeling. (A) Day 9 chondrocyte. (B) Matrix intensity profiles. (C) Correlation between nascent & pre-existing matrix.

may indicate a temporally variable (potentially switch-like) process. This difference could be inherent to cell type (MSC vs. chondrocyte), or could reflect the influence of the existing matrix on nascent matrix formation: as a cell accumulates a biologically 'sufficient' matrix, ECM production may undergo a shift in dynamics. To distinguish between these possibilities, future work will utilize two-color labeling over a time course to better ascertain matrix dynamics and heterogeneity at the single cell level.

Significance

Matrix dynamics are crucial for cartilage development and maintenance *in vivo* and *in vitro*. This technique enables interrogation of these dynamics at the single cell level.

Acknowledgements

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