Proliferating Chondrocytes are Present in Situ in Immature Bovine Articular Cartilage Explants: Localization near the Articular Surface with EdU

Nasim Eshragh Nia[1], Erica G Gacasan[1], Alborz Jelvani[1], Xueyi Wan[1], Robert L Sah[1]
[1]University of California-San Diego, La Jolla, CA
naeshrag@eng.ucsd.edu

Disclosures: NE (N), EGG (N), AJ (N), XW (N), RLS (5-Restoration Biologics; 6-JRF Ortho; 8-Cartilage, Osteoarthritis & Cartilage)

INTRODUCTION: The surface zone of articular cartilage contains a progenitor cell population that proliferates and give rise to cells of adult cartilage.[1,2] Such cells have been studied primarily *in vivo*[2,3] and by isolation and culture *in vitro*.[1,3] Immature articular cartilage explants increase in cell number *in vitro*, during serum-stimulated culture,[4] and thus likely contain such cells. To detect proliferating cells, 5-ethynyl-2′-deoxyuridine (EdU) can be used, based on its incorporation into newly synthesized DNA.[5] *In vitro* and *in vivo* studies with EdU have been limited to 24 hours of labeling due to possible cytotoxic effects in monolayer cultures.[6] The objective of this study was to localize proliferating cells in immature bovine articular cartilage with EdU. Specifically, the aims were to characterize EdU-incorporating cells (1) nuclear morphology *en face* and (2) spatial distribution in vertical sections, and the dose and labeling duration effects of EdU on chondrocyte (3) viability and (4) function.

METHODS: Studies were performed on a total of 72 halved cartilage disks, 2 mm diameter by 1 mm thick, from 7 immature (1-3 wk old) bovine stifle joints with 3 samples per condition. Cartilage samples were incubated in medium with 20% FBS for up to 8 days. Chondrocyte viability was assessed with Live/Dead™ and fluorescence microscopy. Proliferating cells were localized by reacting EdU with AlexaFluor™ 594 (red fluorescence). All cells were stained with Hoechst 33342 (blue fluorescence). Total and EdU/Total cells were quantified in *en face* and vertical orientations. Exp 1. The effect of label duration (0.25, 1, 2, 4 and 8 day) with 5μM EdU was determined. Exp 2. The effects of EdU dose (0, 0.5, 5 and 10μM) and label duration (3 vs. 6 days) on [35S]sulfate incorporation and chondrocyte viability were assessed as indices of cartilage function and fate. Exp 3. The fate of EdU labeled cells was assessed by EdU pulse and 3 or 6 days chase (5μM EdU). ***Statistics.*** 1- or 2-way ANOVA with Tukey *post-hoc*. Data are shown as Mean±SE, with significance set as $p<0.05$.

RESULTS: Exp 1. In *en face* images **(Fig 1)**, EdU uptake indicated classical stages of mitosis, prophase **(Fig 1A)**, pro-metaphase **(Fig 1B)**, metaphase **(Fig 1C)**, anaphase **(Fig 1D)**, telophase (**Fig 1E**). There was a marked increase with label duration (between ¼ and 8 days) of EdU+ cells at the surface seen both *en face* **(Fig 2A-E)** and at the surface of vertical sections (**Fig 2F-J**). Quantitatively, *en face* EdU+ cells increased by 8 days label to 2,300/mm^2 ($P<0.01$) corresponding to 56±7% of total cell nuclei ($P<0.001$, **Fig 3A**), as fluorescence signal intensity increased. EdU+ cell density varied (**Fig 3B**) with distance from the articular surface ($P<0.0001$), in an interactive way with culture duration ($P<0.0001$). Although EdU+ incorporation was highest nearest the articular surface (**Fig 3A,B,C**), some EdU+ cells were also evident deeper in the tissue (**Fig 3D,E**). Exp 2. EdU did not affect cell viability, which was maintained at 95%. In addition, EdU+ chondrocytes co-localized with live cells at day 6 of labeling with the highest 10 μM EdU dose. In addition, 35S incorporation was not affected by EdU concentration. Exp 3. EdU-labeled cells appeared to progress through mitosis, as EdU cell doublets increased with chase for 3 and 6 days.

DISCUSSION: These results demonstrate that immature bovine cartilage explants contain proliferating chondrocytes, consistent with a progenitor cell population. EdU and DNA staining was consistent with classical descriptions of chromatin dynamics in a dividing cell. The localized band of proliferating cells is consistent with those described rabbit knee articular cartilage.[2] The lack of adverse effect of EdU at 5 and 10 μM suggests it may be useful not only for localizing proliferating cells, but also for tracking cells. The depth-associated variation in EdU+ cells may reflect both tissue growth and cell movement.

SIGNIFICANCE: (1) Immature bovine cartilage explants may serve as a model to study progenitor cells during tissue maturation. (2) EdU may be useful not only as a marker of proliferating cells, but also to track their fate.

REFERENCES: [1] Dowthwaite GP+, *J Cell Sci*, 2004. [2] Hunziker EB+, *Osteoarthritis Cartilage*, 2007. [3] Chan CKF+, *Cell*, 2015. [4] Williamson AK+, *Tissue Eng*, 2003. [5] Salic A+, *PNAS* 2008. [6] Angelozzi M+, *Methods Mol Biol* 2021.

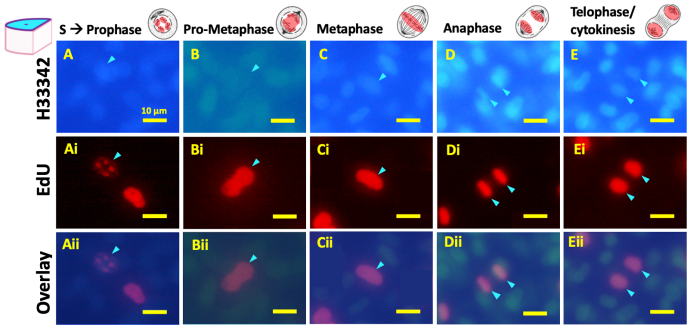

Figure 1. Mitotic patterns of EdU in chondrocytes in calf cartilage. (A-E) *En face* fluorescence images of **(A-E)** Hoechst 33342 and **(i)** EdU and **(ii)** their overlay. Samples were labeled with 5μM of EdU for ¼ - 2 days.

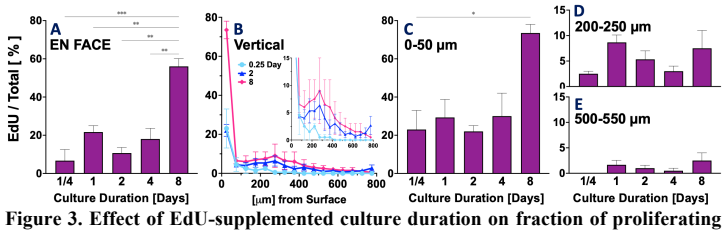

Figure 3. Effect of EdU-supplemented culture duration on fraction of proliferating chondrocytes in (A) *En face* and **(B-E)** vertically at various depths from the articular surface.

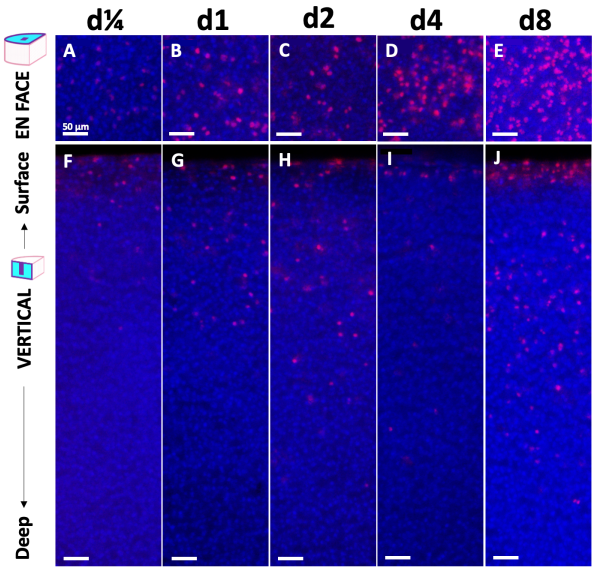

Figure 2. Localization of proliferating chondrocytes in calf cartilage *in situ*. (A-E) *En face* and **(F-J)** vertical digital zooms of EdU (red) and Hoechst (blue) nucleus fluorescence in representative samples labeled with 5μM EdU with 20% serum.