Degree of Cell Cycle Synchrony Directly Influences Chondrogenic Potential of Equine Adipose Stem Cells

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INTRODUCTION: Mesenchymal stem cells (MSCs) are an attractive cell source due to their increased availability and intrinsic ability to differentiate down desired lineages. Their success in tissue engineering, however, has been limited by variation in differentiation potential and proliferation capacity and there is poor understanding how to select the most potent cells for clinical application. Toward this end, we have previously shown that controlling the cell cycle phase of both terminally differentiated cells and MSCs prior to 3D encapsulation or pelleting yields more chondrogenic tissue. In particular, synchronized cells entering the S phase (synthesis) are more responsive to exogeneous cues (growth factor stimulation) than cells in G1, G2 (growth), or M (mitosis) [1,2]. To determine if this is due to an increased percentage of proliferating cells (total cells in S, G2, M phases) or a direct consequence of cells residing in the S phase, here we controlled the percentage of cells in each cell cycle phase, varying the % of S phase cells while maintaining a similar overall % of proliferating cells, hypothesizing that increasing the relative proportion of S phase cells in a cell pellet would induce greater biosynthetic output in the presence of chondrogenic factors. We couple this technique with the use of transcriptional reporter systems to better understand the influence of S phase cells on chondrogenic potential.

METHODS: Cell isolation and transfection Following euthanization for other studies (IACUC-University of Florida), adipose tissue was harvested from healthy, skeletally mature horses (2-5 years). MSCs were isolated and plated for two passages before being transduced with dual gene lentiviral report constructs encoding fluorescent reporters, as previously described [3]. Briefly, the pcDH-GFP lentiviral expression plasmid, encoding td Tomato under constitutive control of a CMV promoter was modified to include GFP under control of a human COL2A1 promoter. Transfection efficiencies were >90% based on flow cytometry. Cell Synchronization Following transduction, cells were returned to monolayer culture for 2 additional passages. At 70% confluence, cells were trypsinized and one subset of cells was used to create 3D cell pellets (asynchronous). Another subset of cells was suspended in 0.5% methylcellulose solution (1M cells/mL) for 48 hours to arrest cells in the G1 phase. Cells were extracted from suspension culture and confirmed to be synchronized to the G1 phase through flow cytometry analysis. Cells were plated to allow for cell reattachment and re-entry into the cell cycle. After 22 hours (post-methylcellulose (PMC) 22) and 28 hours (PMC 28), cells were trypsinized from monolayer culture to produce populations composed of varying % S phase cells. Cells from each group were then used to create cell pellets. Flow Cytometry A subset of cells were fixed, stained, and analyzed on a flow activated cell sorting (FACS) machine to characterize the population of cells in each phase for each synchronizadon technique [2]. Pellet formation and culture 1.0 mL of a 0.5×10⁶ cell suspension from each group was aliquoted into 1.5 mL sterile screw-top tube, formed into pellets by centrifugation, and cultured for 28 days in chondrogenic medium supplemented with 10 ng/mL TGF-β3. At days 3, 14, and 28, pellet samples were harvested and saved for COL2A1 reporter expression imaging or biochemical and histological analysis. Florescence microscopy GFP and tdTomato expression was characterized by confocal microscopy (Leica LSM 700). Biochemistry: GAG, collagen, and DNA content were quantified [4]. Histology Acid formalin-fixed samples were embedded, sectioned (5µm), and stained with Safranin O, Picrosirius Red, and Hematoxylin & Eosin to assess GAG, collagen and cellular distribution, respectively. Statistics Two-way ANOVA (α<0.05) with Tukey’s HSD post-hoc tests was used to compare groups. Linear regression was performed to assess the relationship between % S phase cells and % proliferating cells vs. biosynthetic output.

RESULTS: Cell synchronization via methylcellulose suspension successfullly arrested cells in the G1 phase and modulating the length of replating time significantly altered the percentage of S phase cells (asynchronous: ~6%, PMC 22: ~69%, PMC 28: ~28%, Figure 1a, b). As a consequence, the variation in population composition of the various cell pellets directly influenced the biochemical synthetic output of the cells. By day 28, PMC22 cells were more biosynthetically active, producing 1.7x more GAG/DNA and 2.6x more COL/DNA than asynchronous cells and 1.2x more GAG/DNA and 2.8x more COL/DNA than PMC28 cells (p<0.05, Figure 1c). Furthermore, the positive linear relationship between biosynthetic output and % S phase cells was found to be strong for both GAG/DNA and COL/DNA (r²=0.9336, r²=0.8587, Figure 1d top). In contrast, the relationship between biosynthetic output and total % proliferating cells was found to vary depending on the biochemical marker. While there was a strong positive relationship for GAG/DNA, there was a weak relationship for COL/DNA (Figure 1d bottom). The modulation of cell cycle phase produced varying changes in histological staining and COL2A1 reporter expression in the various groups. Notably, GFP expression was found to be homogeneously distributed for both asynchronous and PMC 28 pellets, corresponding to streaky collagen staining throughout the cell pellet (Figure 2). In contrast, GFP expression for PMC 22 pellets was much more uniform and intense across the sample, paralelling the increased intense collagen staining. DISCUSSION: While the total percentage of proliferating cells was approximately constant for synchronized cells, increased chondrogenic potential was observed when there was an increase of S phase cells (~2.5x greater in PMC 22 vs. PMC 28), confirming our hypothesis that priming cells to reside in their S phase prior to pelleting leads to enhanced cartilage-like tissue. Future studies will examine the differential response of S phase cell pellets to additional physiologic stimuli (e.g. osmotic loading, hydrostatic pressure, growth factors and cytokines) to further elucidate the translational potential of synchronized cell populations in repair tissue.

SIGNIFICANCE: Modulating the composition of cell pellets by controlling the % of cells residing in each cell cycle phase has a direct effect on the differentiation of equine adipose MSCs to produce cartilage-like tissues. Increasing the % of S phase cells in the cell pellet has the potential to yield enhanced chondrogenic tissues and may provide a technique for selecting the most potent cells for clinical application.


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