**INTRODUCTION:** Current tissue engineering strategies for intervertebral disc (IVD) repair require large quantities of primary cells. Since IVD tissues have low cell densities, large numbers are difficult to obtain. Traditionally, serial monolayer passaging has been utilized to increase cell numbers. Although cells from other cartilaginous tissues have been shown to exhibit changes in cell morphology and collagen expression with passage, the response of IVD cells to serial passaging has not been fully described [1,2,3]. Therefore, the objective of this study was to characterize the effect of serial monolayer passaging on the expression of types I and II collagen by outer and inner annulus fibrosus (AF) cells and to compare expression profiles of cells isolated from different levels of the spine. We hypothesized that serial monolayer passaging would result in increased type I and decreased type II collagen expression in both outer and inner annulus fibrosus cells.

**METHODS:** Primary Cell Isolation: Lumbar IVDs were harvested from the spines of 2 adult sheep (S6, S7) at level L1-L2 (upper, U) and level L6-S1 (lower, L). The outer (OA) and inner (IA) annulus were separated and the cells isolated by enzymatic digestion with 0.2% pronase and 0.1% collagenase in DMEM w/ 20% FBS, 2.5 μg/mL Fungizone reagent and antibiotics. Released cells were designated passage 0 (P0). **Serial Passaging:** Passage 0 (P0) and passage 1 (P1) cells were plated at a concentration of 1x10^4 cells/75cm^2 for 6 days, which corresponded to 2-3 doublings per passage. Subsequent passages up to P6 were plated at a concentration of 3x10^4 cells/75cm^2 for 4 days, which corresponded to 1-2 doublings per passage. At each passage, cultures were analyzed for gene expression and evaluated histologically. All cultures were maintained in DMEM with 10% FBS, and antibiotics.

**RT-PCR:** Total RNA was extracted using the TRIZOL isolation system (Invitrogen, Carlsbad, CA). Reverse transcription (RT) was performed using First-Strand Synthesis System for RT-PCR (Invitrogen). Primers for type II collagen (COL II) were as published [4] while those for type I collagen (COL I) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed based on sequences in GenBank. **Histology:** Monoclonal antibodies to types I (Sigma, St. Louis, MO) and II (II-IB2, DSHB) collagen were used with a peroxidase-based detection system (Vectorstain Elite ABC, Vector Labs, Burlingame, CA) and 3,3’diaminobenzidine as the substrate chromagen to detect protein localization. Non-immune controls were performed without primary antibody. **Statistical Analysis:** A three-way ANOVA with a Tukey post-hoc test was performed to determine effect of passage, cell type and location. Data represent mean ± s. d. (n=3-4).

**RESULTS:** At P1, monolayer cultures of inner AF cells exhibited a more rounded, chondrocyte-like morphology (Fig 1C) as compared to the spindle-shaped, fibroblastic morphology of outer AF cells (Fig 1A). Gene expression profiles supported this observation through highly significant differences in type I and II collagen expression, with inner AF cells producing more type II collagen and outer AF cells producing more type I collagen (p<0.0001) (Fig 2). However, with serial monolayer passaging, at P6 both cell types displayed similar fibroblast-like morphologies (Fig 1B and 1D) and levels of type I collagen expression (Fig 2). Differences in outer and inner AF cells were consistent in both upper and lower discs. The dramatic morphological changes in inner AF cells corresponded to a significant decrease in type II collagen after P1 (p<0.05) (Fig 2). Similarly, outer AF cells exhibited a significant decrease in types I and II collagen after P2 (p<0.05) (Fig 2). These trends were also observed in complementary samples from a separate animal (S7, data not shown).

Immunohistochemical staining of outer and inner AF cells for type II collagen supported the morphological observations and collagen gene expression results. Inner AF cells displayed punctate intracellular type II collagen staining which decreased with passage (Fig 3C and 3D), whereas outer AF type II collagen staining was dramatically less at P1 and almost undetectable at P6 (Fig 3A and 3B). Conversely, type I collagen staining was more intense in outer AF compared to inner AF cultures (data not shown).

When comparing expression profiles of IVD cells isolated from different vertebral levels and at low passage, both outer (P0-P2) and inner AF cells (P0-P1, S7, data not shown) exhibited a significant difference in type I collagen expression (p<0.05) but no significant difference in type II collagen expression.

**DISCUSSION:** This study demonstrates that IVD cell morphology and collagen expression are altered with serial monolayer passaging. Specifically, we confirmed the hypothesis that both outer and inner AF cells exhibit decreased type II collagen expression with passage. However, neither cell type showed dramatic increases in type I collagen expression as originally hypothesized. Previous studies on serial monolayer passaging of chondrocytes reported similar changes in cell morphology and type II collagen expression, with a concomitant increase in type I collagen [1,2,3]. In addition, we found that outer and inner AF cells were markedly different from each other in terms of morphology and collagen expression profile, which supports previous investigations analyzing cells from different regions of the disc [5,6].

Cells isolated from two levels of the spine showed only differences in type I collagen expression at early passages. While loads likely differ between vertebral levels, whether this difference in mechanical environment directly influences collagen expression cannot be confirmed from this study [7]. Future analyses may include cells from all levels to better correlate collagen expression with vertebral level.

To the best of our knowledge, this is the first study to specifically investigate the effects of serial passaging on collagen expression of IVD cells. Our findings suggest that outer and inner AF cells are distinct cell populations within the same tissue and that monolayer passages up to P1-P2 should be used for cell-based experimental treatments.


**ACKNOWLEDGEMENTS:** Louis J. Soslowsky for providing animal tissue and NIH for support.