Bovine Annulus Fibrosus Cell Phenotypes are Driven by Both Substrate Stiffness and Ligand Presentation

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INTRODUCTION: Back pain is a leading global disability, and this multifactorial disease affected 577M individuals in 2017 [1]. Intervertebral disc (IVD) degeneration contributes to ~40% of back pain cases, and annulus fibrosus (AF) defects are a degenerative phenotype exhibiting cellular exhaustion, disorganized matrix, poor healing, and painful conditions [2,3]. Cell delivery for IVD repair is a research priority, yet tunable biomaterials that recapitulate native extracellular matrix microenvironments to drive distinct cell differentiation patterns of stem cells *in vitro* are lacking for the AF. Modular cell culture platforms that characterize AF cell phenotypes on different substrate conditions will improve our understanding of AF cell biology to inform a cell-instructive scaffold that ideally maintains an AF progenitor phenotype for clinical delivery. Previous studies have controlled substrate stiffness and ligand concentration to promote a juvenile NP cell phenotype [4], and our work follows a similar methodology for investigating AF cell phenotypes. The objectives of this study were to: (1) determine effects of stiffness and ligand type on AF cell growth and morphology using engineered synthetic substrates, and (2) evaluate cell phenotypic response using bulk transcriptomic profiling on these substrates to determine the potential for promoting an AF progenitor phenotype. Substrate stiffnesses approximated neonatal (soft) and mature (stiff) mouse AF matrix properties, while ligands target integrins for fibronectin (RGD) and collagen (GFOGER). We postulate that Soft-GFOGER (GFO) substrates may promote AF cell phenotypes more representative of neonatal AF cell progenitors.

METHODS: 2D poly-(ethylene glycol)-norbornene (PEG-NB) hydrogels were engineered by varying the number of PEG arms (4 or 8) and concentrations (4% or 10% w/v) to alter the material's stiffness. Unconfined compression testing (0.2% strain/s) was performed to calculate Young's moduli (Fig. 1C). Soft 8kPa (4-arm, 4% w/v) and stiff 94kPa (8-arm, 10% w/v) hydrogels were fabricated with 2mM ligand (RGD or GFO), 3kDa PEG-dithiol, lithium phenyl-2,4,6-trimethylbenzoylphosphate photo initiator, and ultraviolet light (λ=365 nm, 10 mW/cm^2). Hydrogels were seeded with AF cells isolated from caudal bovine IVDs (n=3 donors) at 1,000 cells/cm^2 and cultured for 7 days at 37°C in supplemented DMEM. Brightfield and immunofluorescent (IF) imaging enabled cell density and morphology data collection and visualization at day 7. Cell density, cell area, and aspect ratio were quantified in ImageJ, and statistical differences ($p<0.05$) between groups were detected using two-way ANOVA with Tukey's multiple comparisons. On day 7, RNA was also extracted using TRIzol and purified via RNA capture spin columns. Quality control, library preparation, sequencing (Illumina NovaSeq6000), and alignment were done by Azenta Life Sciences. Principal component analysis (PCA) and differential gene expression were conducted in R with DESeq2.

RESULTS: Cell density decreased as stiffness increased, and RGD-based hydrogels promoted greater cell growth compared to their GFO-based counterparts (Fig. 1A, D). Cell area and elongation decreased as stiffness increased for RGD-based conditions (Fig. 1B, E, F). PCA clustering was group-dependent with soft hydrogels exhibiting more distinctive gene signatures with greater separation on PC1 and PC2 than the stiff substrate conditions that mostly separated along PC2 (Fig. 2A). *IGDCC3, TNXB, ADGRB1, SCX, GATA2, and EN2* expression were up-regulated in RGD- vs. GFO-based substrates, suggesting regulatory functions in developmental processes and cell adhesion, that was most apparent on soft substrates (Fig. 2B, C).

DISCUSSION: AF cells were sensitive to substrate stiffness and ligand presentation, exhibiting large differences in cell morphology and gene expression signatures on different substrate conditions. Soft substrates and RGD ligand promoted greater cell density with larger, more elongated cells. Soft-RGD substrate cells also differentially express *SCX*, an AF cell and tendon phenotypic marker, and *SCX*-positive AF cells were involved in functional regeneration of neonatal mouse AF tissue following injury [5]. Soft-RGD up-regulated *GATA2* and *EN2*, which are implicated in transcriptional regulation during development, suggesting a role in AF phenotypic differentiation. The high cell growth rates also imply a role in promoting proliferation. In contrast, AF cells on Soft-GFO exhibited cell clustering, a stellate morphology, and limited growth. The AF cells on Soft-GFO did not differentially express AF-specific markers, yet they expressed various collagens and formed cell networks with matrix deposition similar to previously described AF cell cord-like structures [6]. AF cells on Soft-GFO were rounded and expressed survival genes *B4GALNT3*, *ALOX12,* and *FOSB* known to influence cell cycle and stemness, which may suggest a larger phenotypic switch and some progenitor-like characteristics needing more study. Next steps include using pathway analyses to more clearly elucidate biological functions, and transcriptional entropy scoring to quantify progenitor characteristics [7]. Bovine IVD single-cell transcriptomics identified multiple AF cell clusters including progenitor populations [8], that will also be compared with these results to identify similarities of AF cells on these substrates to native AF populations. We conclude that AF cells can be driven to distinct phenotypes based on both substrate stiffness and ligand type, which provide novel insights into AF cell-ECM interactions for the identification of progenitor-like AF cells and eventual development of cell-instructive biomaterials for AF repair.

SIGNIFICANCE: This study improved understanding of AF cell-ECM interactions to inform development of a cell-instructive biomaterial to drive AF cells toward a progenitor phenotype and deliver those cells to promote AF repair and slow IVD degeneration.

REFERENCES: [1] Wu+ *Ann Transl Med* 2020; [2] Ohtori+ *Spine* 2015; [3] Guterl+ *Eur Cell Mater* 2013; [4] Barcellona+ *Biomater* 2020; [5] Torre+ *NPJ Regen Med* 2019; [6] Nakai+ *JOR* 2016; [7] Teschendorff+ *Nat Comm* 2017; [8] Rodriguez+ *ORS* 2023.

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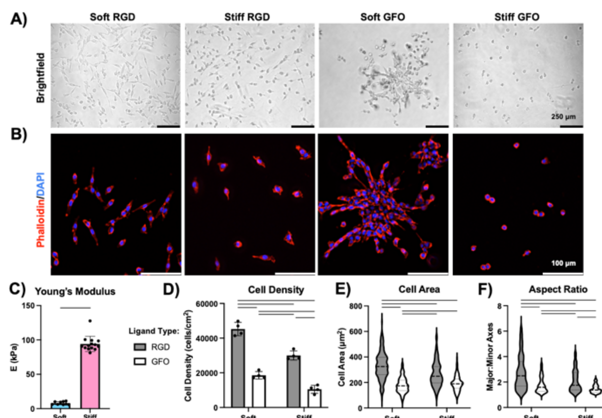

Fig. 1: ***Substrate stiffness and ligand influence AF cell growth and morphology.*** (A) Brightfield images and (B) cytoskeletal and nuclear IF images of bovine AF cells on PEG-NB formulations of different (C) compressive moduli caused cells to exhibit distinct (D) cell density, (E) cell area, and (F) aspect ratio. Bar = $p<0.05$.

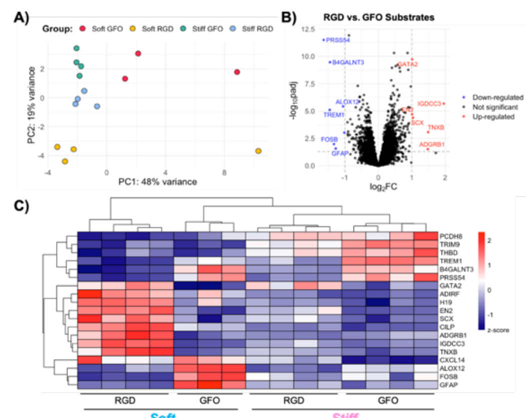

Fig. 2: ***RNA-seq analysis of bovine AF cells show substrate stiffness and ligand have distinct expression patterns.*** (A) PCA plot of distinct clusters. (B) Volcano plot of up- and down-regulated DEGs for RGD vs. GFO substrates. (C) Heatmap of top DEGs for each PEG-NB condition.