Fibronectin Synergy Site Sensitizes Activation of YAP and NF-κB in Nucleus Pulposus Cells

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INTRODUCTION: Degenerative disc disease involves breakdown of the intervertebral discs of the spine due to genetics, ageing, or faulty mechanical loading [1]. The innermost nucleus pulposus (NP) loses its phenotypic characteristics, driving inflammation and extracellular matrix (ECM) reorganization that stiffens the tissue as hydration and proteoglycan are lost. Fibronectin and its fragments have long been associated with osteoarthritis and have also been implicated in degenerative disc disease [2, 3]. The mechanosensitive transcription factor YAP is influenced by these changes, translocating to the nucleus in response to high stiffness and cell spreading. The inflammatory transcription factor NF-κB is also activated with degeneration, resulting in nuclear translocation of the of a complex that includes the p65 subunit. Previous work has shown that manipulation of integrin interactions can regulate NP cell phenotype [5,6] and that fibronectin can influence both fibrotic and inflammatory signaling [2,7]. Our group has recently shown that fibronectin sensitizes the activation of contractility, YAP, and p65 in NP cells [7]. On 2D ECM coated surfaces, fibronectin resulted in significantly higher traction forces and nuclear translocation of YAP and p65 than laminin or collagen coated surfaces [7]. Here we implement a peptide modified hydrogel system to identify specific domains of fibronectin that drive this fibrotic and inflammatory signaling. We identify a role for the fibronectin synergy domain in regulation of nucleus pulposus fibrotic and inflammatory signaling. This was assessed through quantification of activation of the mechanosensitive transcription factor YAP, the inflammatory transcription factor NF-κB, sulfated glycosaminoglycan (sGAG) production, and gene expression (rt-PCR).

METHODS: NP cells were isolated from fresh bovine caudal discs the same day of slaughter (Westville Meats, FL) and cultured in high glucose DMEM with 10% FBS and 1% pen-strep for 2 passages. Dextran vinyl sulfone (DexVS) was synthesized as previously described [8,9] and used to generate hydrogels with 8% w/v by crosslinking with 20mM DTT. Gels were modified with RGD peptide (CGRGDS, 2mM, GenScript), synergy site peptide (Syn: CGGRPREDRVPPSRNSITLTN, 2mM, GenScript), or both (RGD-Syn, 2mM of each) by overnight incubation in triethanolamine buffer (pH 8.0, sigma). Peptide modified gels (Fig 1C) were rinsed with PBS and NP cells were seeded and allowed to spread for one day. Cells were fixed with 4% PFA and stained with DAPI, 488-Phalloidin, YAP/Alexa-568-α-mouse and p65/Alexa-647-α-rabbit antibodies then imaged at 20x 0.8NA. Cell area (ImageJ) and nuclear to cytoplasmic ratio for YAP and p65 was quantified in MATLAB by taking the ratio of average nuclear signal to average cytoplasmic signal based on edge detected nuclear masks. Sulfated glycosaminoglycans (sGAG) production was measured by the DMMB assay and aggrecan gene expression normalized to GAPDH was measured using qPCR for samples isolated from cells grown on 2D gels for 1 day (Aurum total RNA kit, iScript and iTaq, Bio-Rad).

RESULTS: To assess the specific domains within fibronectin that induce the previously observed inflammatory and fibrotic signaling, we assessed responses on DexVS gels modified with peptides from two integrin binding regions of fibronectin [10]. YAP and p65 nuclear localization were highest for the RGD-Syn combination group (Fig 1A-C), indicating the combination of these two sites can activate the fibrotic and inflammatory signals that were previously observed to be activated on full length fibronectin [5]. Cell spread area showed less spreading for the synergy only group as expected, likely because this site requires RGD for integrin binding and stabilization of adhesive interactions. However, we did not observe significant changes in sGAG production by DMMB assay or aggrecan gene expression on RGD gels compared to RGD-Syn gels (Fig 1D-E). Longer time point experiments may be required to observe significant changes in sGAG production and gene expression.

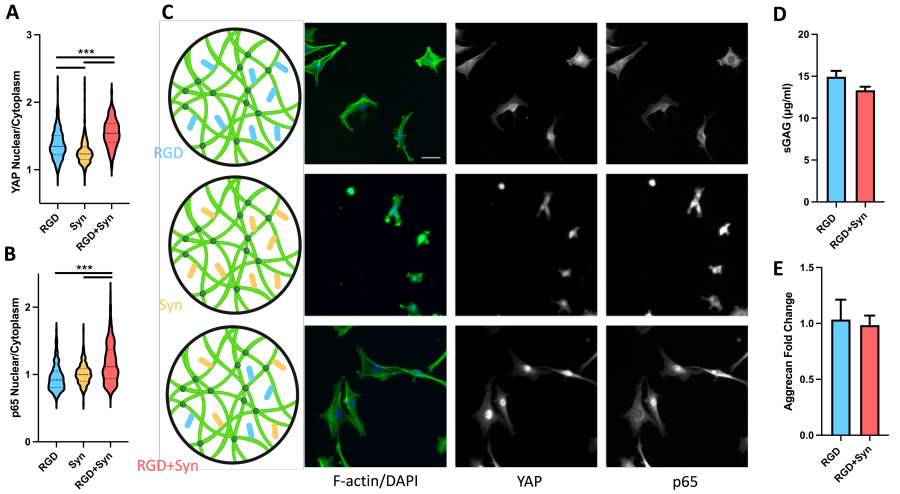

Figure 1: Quantification of YAP (A) and p65 (B) nuclear to cytoplasmic ratio in NP cells seeded on 8% DexVS gels modified with RGD, synergy peptide, or both for 24hr. Violin plot indicates relative distribution, dotted lines indicate mean and quartiles. One-way ANOVA with Tuckey's post hoc. n=233-432 cells/group. *p<0.05, ****p<0.0001. Representative images (C) of YAP and p65. Scale= 20μm. sGAG production (D) and qPCR for aggrecan fold change (E). Mean +/- SEM, n=6 samples.

DISCUSSION: Fibronectin expression has been shown to increase in degenerate discs [4]. However, the role that this plays in the progression of the disease is unclear. Here, we show that the synergy domain of fibronectin activates fibronectin specific integrin signaling, increasing spreading and activation of YAP. Additionally, this activates inflammatory NF-κB signaling, an important contributor to disc degeneration. This indicates that the fibronectin synergy domain may be important in the fibronectin induced activation of contractility, YAP, and NF-κB [7]. This synergy domain may provide a target for regenerative approaches. Since these cell-ECM interactions are integrin dependent, this may indicate that fibronectin accelerates degenerative changes through binding of α5β1, which is known to strongly interact with the FNIII9-10 domains that the RGD and synergy peptides are derived from [10].

SIGNIFICANCE/CLINICAL RELEVANCE: The fibronectin synergy site may be a promising therapeutic target for slowing or reversing fibrotic or inflammatory changes in the intervertebral disc. Blocking synergy site interactions may improve healthy NP cell phenotype and sGAG production.

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