Priscilla Y. Hwang
Department of Biomedical Engineering,
Duke University,
136 Hudson Hall, Box 90281,
Durham, NC 27708
e-mail: ph17@duke.edu

Jun Chen
Department of Orthopaedic Surgery,
Duke University,
375 Medical Science Research Building, Box 3093,
Durham, NC 27710
e-mail: junchen@duke.edu

Liufang Jing
Department of Biomedical Engineering,
Duke University,
136 Hudson Hall, Box 90281,
Durham, NC 27708
e-mail: lfjing@duke.edu

Brenton D. Hoffman
Department of Biomedical Engineering,
Duke University,
136 Hudson Hall, Box 90281,
Durham, NC 27708
e-mail: brenton.hoffman@duke.edu

Lori A. Setton[1]
Department of Biomedical Engineering and
Orthopaedic Surgery,
Duke University,
136 Hudson Hall, Box 90281,
Durham, NC 27708
e-mail: setton@duke.edu

The Role Of Extracellular Matrix Elasticity and Composition In Regulating the Nucleus Pulposus Cell Phenotype in the Intervertebral Disc: A Narrative Review

Intervertebral disc (IVD) disorders are a major contributor to disability and societal health care costs. Nucleus pulposus (NP) cells of the IVD exhibit changes in both phenotype and morphology with aging-related IVD degeneration that may impact the onset and progression of IVD pathology. Studies have demonstrated that immature NP cell interactions with their extracellular matrix (ECM) may be key regulators of cellular phenotype, metabolism and morphology. The objective of this article is to review our recent experience with studies of NP cell-ECM interactions that reveal how ECM cues can be manipulated to promote an immature NP cell phenotype and morphology. Findings demonstrate the importance of a soft (<700 Pa), laminin-containing ECM in regulating healthy, immature NP cells. Knowledge of NP cell-ECM interactions can be used for development of tissue engineering or cell delivery strategies to treat IVD-related disorders.
[DOI: 10.1115/1.4026360]

Introduction

The intervertebral disc (IVD) is a heterogeneous, fibrocartilaginous tissue that provides load support, energy dissipation, and flexibility in the spine. The IVD, which is composed of the nucleus pulposus (NP), anulus fibrosus (AF), and cartilage endplate (Fig. 1), is situated between adjacent vertebral bodies and acts as the main joint of the spinal column, occupying approximately 1/3 of its total height [1,2]. The cells within each of the regions of the IVD are subjected to a variety of signals from both physical and biochemical stimuli from their surrounding extracellular matrix (ECM) microenvironment [3–8]. These cues are believed to play critical roles in regulating development, maintenance, and repair of the IVD, but in ways that are poorly understood.

During disc degeneration or aging, significant changes are observed in IVD cell phenotype and density in parallel with changes in ECM composition and structure. A dramatic decrease in cell density and multicell clusters in both the NP and AF regions is observed [9–11] with increased prevalence of cells with cytoplasmic projections [12–14]. While the exact factors resulting in decreased NP cell clustering with age in vivo are not fully understood, it is likely that cell death associated with decreased nutrient oxygen and glucose transport to the IVD can contribute to these decreased cell numbers and cell clusters [2]. With decreases in cell density, the large, vacuolated cells in the NP, which are normally arranged in cell clusters, transition to a sparse population of smaller, isolated chondrocyte-like cells [15]. The loss of proteoglycan matrix causes changes in proteoglycan structure [16–18], which results in decreased negative fixed-charge density, decreased water content, and a loss of swelling pressure [19,20], impairing the tissue's ability to resist and redistribute compressive loads. Corresponding with these compositional changes are structural alterations including loss of disc height and increased anulus lamellar disorganization. Changes in ECM composition and structure may also result in substantially altered mechanics and kinematics for the entire IVD motion segment, with decreased internal pressurization and disc height resulting in higher compressive loads transferred to the AF, compromising its structure and function (e.g., overload leading to clefts, buckling, or rupture). Nerve compression, spinal canal impingement, and altered spinal loading configurations can also occur, which can contribute to symptomatic back pain [21]. These dramatic shifts in ECM mechanical environment can be expected to impact NP cell health, metabolism and survival, although the direct links between environmental factors and NP cell behaviors are still under study.

The purpose of this article is to review our experience with studies of NP cell interactions with their surrounding ECM, as this knowledge can be useful in the development of treatments for disc-related ailments. The first section of this article covers what we have learned of how NP cells interact with select proteins of the native ECM. We then describe how changes in the surrounding ECM can alter NP cell phenotype and morphology, and summarize recent work performed to reveal how NP cells sense,

[1]Corresponding author.

Contributed by the Bioengineering Division of ASME for publication in the Journal of Biomechanical Engineering. Manuscript received October 3, 2013; final manuscript received December 13, 2013; accepted manuscript posted December 26, 2013; published online February 5, 2014. Editor: Beth Winkelstein.

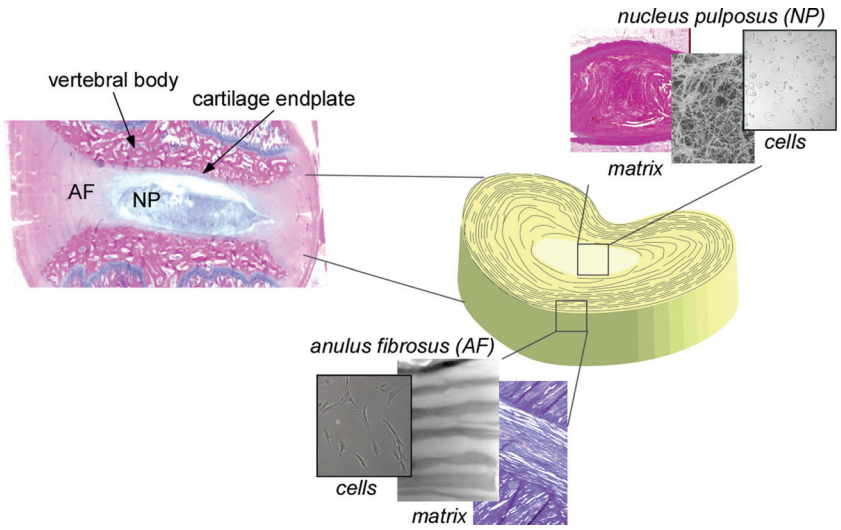

Fig. 1 The intervertebral disc is situated between vertebral bodies in the spinal column, and acts to support loads, provide flexibility, and dissipate energy in the spine. The disc is comprised of distinct anatomic zones: the anulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplates. The AF consists of concentric lamella of highly-aligned collagen fibers, with cells typically aligned along the fiber direction. The NP is a gelatinous, highly-hydrated tissue, with cells typically exhibiting rounded, unaligned morphologies. Staining is safranin O and fast green. Images of specific cell morphology in each region were obtained via light microscopy.

interpret, and respond to different mechanical and biochemical cues in their ECM.

NP Cells and Their Native ECM Microenvironment

Immature NP Cells. Cells within the developing and immature NP are derived from embryonic notochord [15,22–24], and exhibit morphologic features that reflect this unique embryonic origin: notochordal NP cells are large in diameter [25–27] containing large intracellular vacuoles, are organized in interconnected cell clusters, and exhibit strong cell-cell interactions characterized by gap junctions [26,27], cadherins [28–30], and desmosomal cell-cell adhesions [31,32]. Recent characterizations of immature NP cells (bovine, rat, juvenile human) via cDNA microarrays, flow cytometry, real time PCR, and immunohistochemistry, have identified new phenotype markers that are specific to immature NP cells (Table 1, specific references displayed in table) [28,33–36]. Of note, the transcription factor, *T*-brachyury, and adherens junction protein, *N*-cadherin, are two markers that have high expression in immature NP cells compared to mature or degenerate NP cells, or even neighboring AF cells [34–36].

Immature NP cells exhibit a well-developed cytoskeleton; NP cells have been shown to express F-actin (distributed in a punctuate or cortical arrangement [37,38]), microtubules [37], and high levels of both vimentin and cytokeratin intermediate filament proteins (e.g., cytokeratins 8/2018/19, [13,32,37–41]). Quantitative analysis of regional variations in cytoskeletal protein expression in bovine IVDs indicates that NP cells express significantly higher levels of vimentin as compared to AF cells, with these filaments traversing from the NP cell's plasma membrane to the nucleus [37]. The intermediate filament cytoskeletal network is known to support cell shape and resist mechanical loads [42,43], and has been shown to be associated with tissue regions which experience high levels of compressive loading and increased polymerized vimentin [44–46]. The observed intermediate filament expression patterns in the IVD appear to correspond with this notion.

Mature NP Cells. Cells with notochordal-like morphologic features are retained into adulthood or throughout life in some animal species [41,47], but in the human many of these morphologic cell features are lost by early adulthood, with only a sparse population of nonclustering, rounded, chondrocyte-like cells remaining in the mature human NP. Mature human NP cells do, however, exhibit phenotypic characteristics distinct from chondrocytes [48,49], and recent evidence indicates that mature NP cells retain at least some phenotypic features of notochordal cells [28,39,50], suggesting that the chondrocyte-like cells of the mature human NP may have notochordal origins. NP cell morphology in the mature human IVD has been described as rounded or ellipsoidal, with cell sizes and shapes similar to chondrocytes [23,51,52]. Similarly, quantitative studies of in situ NP cell morphology in rat IVDs found NP cell shape to be nearly spheroidal, with cells exhibiting no preferred orientation within the tissue [53]. This is distinct from the elongated and spindle-like morphologies noted for AF cells, that align with the principal collagen fiber direction [53]. NP cells may also extend cytoplasm-filled processes of varying length and number away from cell bodies [54].

NP Cell-Matrix Interactions. The extracellular matrix of the NP is a highly hydrated, gelatinous tissue [55–57] that acts mechanically to resist and to redistribute spinal compressive loads. In the young, healthy human, the NP ECM elasticity ranges from 0.3–5 kPa [56,57] and is comprised primarily of water (70–90% of wet weight), proteoglycans (65% of dry weight), and randomly oriented type II collagen (15-20% dry weight) [58–60]. Other compositionally minor (though potentially critical functionally) ECM components of the NP include elastin, small proteoglycans, and minor collagens (types III, VI, IX) [61–63], and laminins [25,64,65]. Many of these ECM constituents can directly interact with NP cells through cell-surface receptors, if resident within the cell pericellular matrix or multicell cluster (e.g., type VI collagen, N-cadherin [53]); alternately, these ECM constituents can exert a mechanical influence upon NP cells through regulating nutrient transport, hydration (e.g., fixed charge density) and swelling pressure [66]. Each of these studies demonstrates the NP ECM environment interacts very closely with NP cells to regulate cell response.

Prior studies in other cell types have demonstrated an influential role of matrix composition or tissue elasticity in regulating cell functions and behaviors, including cell differentiation, metabolism

Table 1 Listing of molecular markers identified as present in immature nucleus pulposus tissue or cells. A majority of these markers are elevated in immature nucleus pulposus tissue as compared to adjacent anulus fibrosus, and may persist in elevated expression patterns into maturity

Marker	Species[a]	Presence in NP	Reference
Cell Surface Receptors			
Integrin α6 (CD49e)	R, P, H	protein	[64, 82]
Integrin αα3 (CD49c)	R, P, H	protein	[64]
Integrin β4 (CD104)	R, P, H	protein	[64]
Lu (BCAM, CD239)	R, P, H	mRNA and protein	[64]
CD24	R, H, B	mRNA and protein	[34,35,48,101,102]
N-cadherin (CDH2, CD325)	R, H, B	mRNA and protein	[31,33,35,36]
Transcription Factors			
T-brachyury	R, H, B, M	mRNA and protein	[34,102]
Cytoskeleton			
KRT8	P, H, B	mRNA and protein	[29,35,39,40,103]
KRT19	R, H, B	mRNA and protein	[35,36,48]
Vimentin	R, P, H, B	mRNA and protein	[29,31,37,40,48,82]
Matrix-related Proteins			
Laminin α5	R, P, H	protein	[64]
Laminin γ2	P	protein	[58]
Type II collagen	P	mRNA and protein	[58,82,104]
Aggrecan	P, B	mRNA	[35,82]

[a]R = rat, P = porcine, H = human, M = mouse, B = bovine.

and cell death [30,67–69]. Relatively little is known of how matrix composition or tissue elasticity can regulate NP cell differentiation, metabolism and more, nor the cell signaling and associated mechanisms that regulate these interactions. The following sections describe our current knowledge of how the NP ECM may regulate cell behavior via matrix elasticity and matrix composition.

NP Cell and Matrix Elasticity. Studies in different cell types have indicated that cells seek to obtain information and signals to determine cell fate via mechanical input from their environment, either from cell-substrate or cell-cell interactions [70]. Generally, cells adhere and maintain their desired phenotype in ECM that is closest to their tissue's physiological elasticity [30]. Prior studies have identified NP tissue elasticity to range from 0.3–5 kPa [71,72]. As NP cells are known to form cell clusters in vivo, studies have also been performed to determine similarity of NP cell elasticity to its neighboring matrix. NP cell elasticity has been determined via micropipette aspiration and also by atomic force microscopy (AFM) testing. Both of these studies have shown that porcine NP cell stiffness is also in a similar range to its native tissue: 0.345–0.8 kPa [38,70].

NP Cell and Matrix Ligand Interactions. In addition to sensing matrix elasticity, cells also sense the presence of various ECM ligand proteins via both integrin and nonintegrin cell surface receptors. Integrins are membrane-spanning heterodimeric proteins consisting of α and β subunits (18 α and 8 β subunits which form 24 known heterodimers) [73] with specific α-β pairings determining ligand-binding specificity. The extracellular domains of integrin receptors bind to various ECM ligand proteins (i.e., collagens, laminins, fibronectin). The cytoplasmic tail of the integrin receptor interacts with a wide range of intracellular proteins, including scaffolds, kinases and phosphatases [74]. A subset of these proteins interact with the actin-myosin cytoskeleton to transduce mechanical signals into intracellular biochemical signals to direct a variety of downstream signaling cascades that control cytoskeletal organization, gene regulation, and other cellular processes and functions [30,68,75]. The integrin and non-integrin (i.e., syndecans) receptors NP cells utilize to interact with their

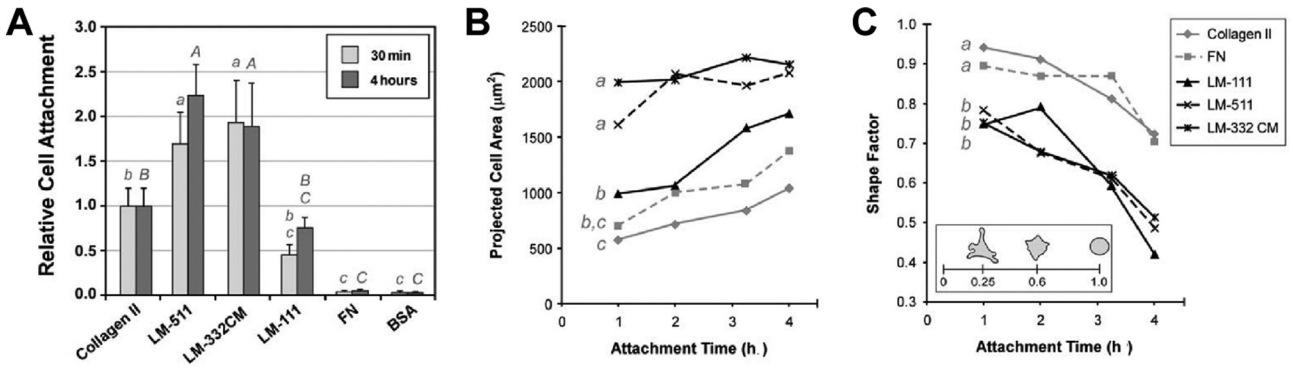

Fig. 2 Porcine NP cells preferentially attach and spread upon laminin-containing substrates. (*a*) Fraction of adherent cells remaining attached to ECM substrates following application of centrifugal detachment force. Higher numbers of NP cells resist detachment when adherent to laminin ligands (isoforms LM-332, LM-511, LM-111), as compared to collagen and fibronectin ECM ligands ((*b*) and (*c*)) NP cell spreading and NP cell shape dynamics on ECM substrates. NP cells on laminin isoforms LM-332 and LM-511 spread rapidly and to a greater extent as compared to other matrix substrates NP cells on laminin isoforms. Additionally, NP cells lost their original shape factor as the cells spread on laminin isoforms (error bars omitted for clarity, significant effects of substrate and time were detected via two-way ANOVA, $p < 0.05$; substrates not labeled with same letter were statistically different. (LM = laminin, FN = fibronectin, BSA = bovine serum albumin, CM = cultured media) Specific methods described in detail and image adapted from Gilchrist et al. 2011 [83].

surrounding ECM may play critical roles in modulating these same cell processes.

Cell surface receptors that modulate cell-matrix interactions have been identified at various stages of IVD development, maturation, and in degeneration. These receptors consist primarily of the integrin class of cell-matrix receptors, which are known to play critical roles in cell adhesion, signaling, and mechanosensing in a variety of tissues [76,77]. In immature and mature IVD tissues (porcine and human tissue), integrins that bind various ECM ligands are expressed. Studies have identified many integrins interacted with collagens ($\alpha1$, $\alpha2$, $\beta1$ integrin subunits) and fibronectin ($\alpha5$, αv, $\beta3$, $\beta5$ integrin subunits) in both AF and NP regions of immature and mature IVD tissues [65,78]. Cell assays on fibronectin substrates indicate the $\alpha5\beta1$ integrin mediates IVD cell attachment for both NP and AF cells (bovine, human, rabbit) that result in different signaling effects in each of the cell types [25,79,80]. For NP cells, studies encapsulating NP cells (bovine [81], rabbit [80]) in alginate beads with fibronectin-*f* fragment have resulted in decreased cell proliferation and proteoglycan synthesis, indicating fibronectin may be a main contributor to disc degeneration.

NP Cell-Laminin Interactions. As prior studies have demonstrated, several isoforms of laminin are present in the native immature NP cell ECM for many different species, including human, that are unique to the NP region of the disc [64]. However, the presence of these laminin ligands and receptors are altered and some disappear with aging and disc degeneration [64,70]. Immature NP cells appear to also uniquely express a number of laminin (LM) binding integrins ($\alpha6$, $\beta4$) [25,65,82] and non-integrin (CD239, CD151) [64] cell surface receptors. Flow cytometric analyses of isolated IVD cells have confirmed this NP-specific expression of LM receptors in immature NP cells [25,64], with differential expression between NP and AF cells maintained in vitro, suggesting distinctly different roles for AF and NP cells in interacting with LM proteins. How immature NP cells interact LM through these LM-binding integrins, and how aging-related changes in receptor expression and ECM protein composition, may be important for understanding events that regulate NP cell pathobiology. Experiments have shown immature porcine and adult human NP cells to attach to surfaces coated with specific LM ligands, and have been performed with selective integrin blocking antibodies to determine the role of LM-integrin subunits in attachment to specific matrix proteins [25,70]. NP cells were found to adhere to two LM isoforms (LM-511, LM-332) at two-fold or greater numbers than other ECM ligands (collagen, fibronectin, LM-111), and to show significantly higher resistance to detachment forces on laminins as compared to other substrates (Fig. 2(*a*)). NP cells have also been found to attach to these laminin ligands principally through integrin subunits $\alpha6$ and $\beta1$ in the immature porcine, but through $\alpha3$, $\alpha5$ and $\beta1$ in the mature human NP cell [25,78,83]. Additionally, NP cells exhibited significantly higher levels of spreading on these laminin ligands compared to other ECM proteins (Figs. 2(*b*) and 2(*c*)), as recorded by measures of cell spread area and shape factor, which is defined as $4A\pi/p^2$ (A = projected cell area, p = cell perimeter) [83]. These NP cell adhesion behavior studies highlight the NP cells' ability to interact with laminins as an important constituent of the immature NP tissue, and the importance of LM integrin subunits in regulating NP cell attachment.

Substrate Elasticity and Matrix Protein Ligand Effects on NP Cell Phenotype

Given the important role that laminin proteins appear to play in mediating immature NP cell adhesion to surfaces, recent work in our group has focused on studying if and how physical cues associated with laminin ligand presentation serve to regulate behaviors of the immature NP cell phenotype. Studies have been performed to determine if a physical stiffness similar to that of the native NP tissue (Young's modulus of 200–300 Pa) can help maintain an immature NP phenotype [83]. Immature porcine NP cells have been cultured upon a mechanically-tunable polyacrylamide (PAAm) gel system (E = 100–15200 Pa) [84] functionalized with a laminin-111 rich extract (basement membrane extract or BME, purified from Engelbreth-Holm-Swarm tumor [85]) or type II collagen (as a control) [70]. Findings in this study showed maintenance of the immature NP cell behavior, specifically cell clustering, on soft (<700 Pa), BME-functionalized PAAm gels (Fig. 3).

By contrast, studies have revealed anulus fibrosus (AF) cells require different substrate stiffness and ECM ligand presentation to maintain cell survival [86]. Rat AF cells were cultured upon the same PAAm gel system described earlier but gels were functionalized with type I collagen. Findings in this study demonstrate AF

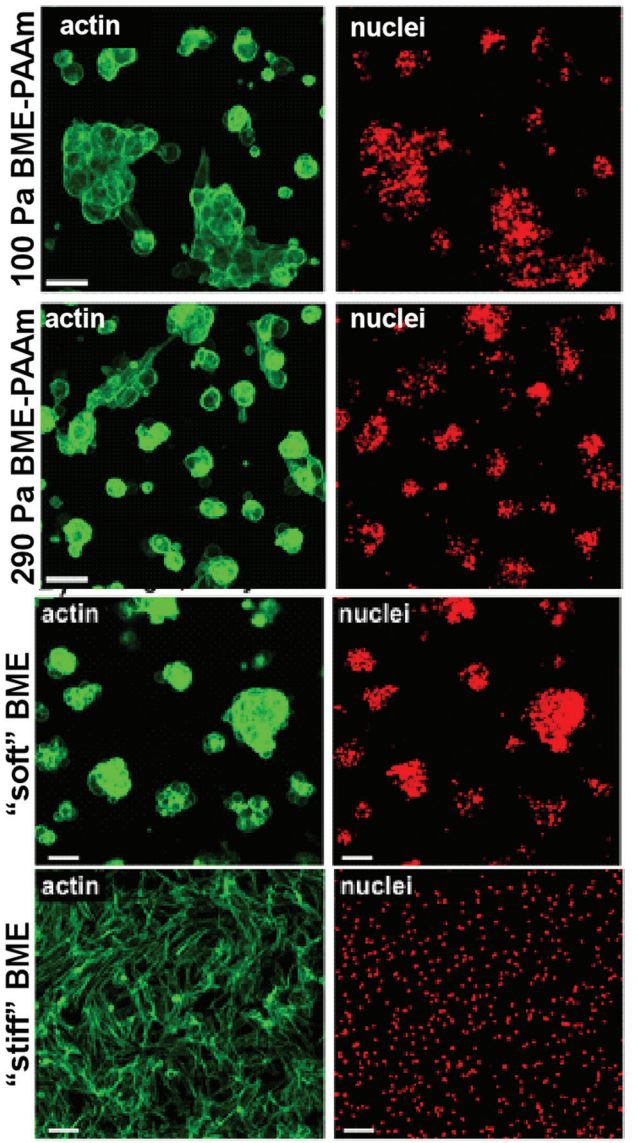

Fig. 3 Soft laminin-containing substrates promote immature NP cells to form multicell clusters, while retaining cell dimensions and rounded morphology. Actin immunostaining of immature porcine NP cell behavior on BME-functionalized polyacrylamide gel (BME-PAAm) (100 and 290 Pa), "soft" BME (300 Pa), and "stiff" BME (2900 Pa) substrates after 7 days of culture (green = actin (phalloidin), red = cell nuclei (propidium iodide), bar = 100 μm). Specific methods and image adapted from Gilchrist et al. 2011 [70].

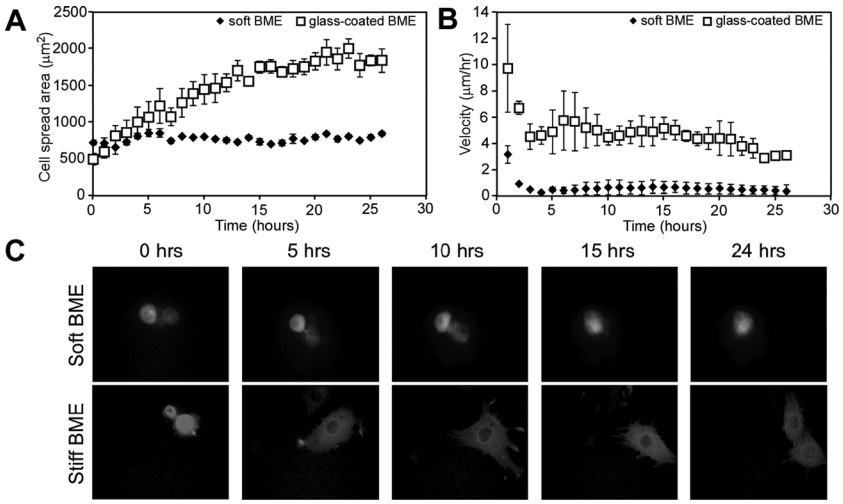

Fig. 4 Changes in immature porcine NP cell morphology on substrates. (*a*) Immature porcine NP cells spread out on stiff BME but maintain rounded morphology on soft BME. (*b*) Immature porcine NP cells have significantly decreased cell velocity on soft BME upon formation of cell cluster. On stiff BME, NP cells continue to send out lamellipodia and filopodia as if sensing the underlying substrate. (*c*) Immature porcine NP cells transfected with GFP-actin display distinct actin fibers as the cell spreads and attaches to the underlying stiff BME substrate. On soft BME, NP cells remain rounded and do not have any actin stress fiber formation. Methods for substrate development were adapted from Gilchrist et al. 2011 [70]. Imaging and analysis performed using the Olympus VivaView Fluorescent Incubator Microscope and Metamorph Software, in collaboration with the Duke Light Microscopy Core Facility.

cells cultured upon a 0.1 kPa type I collagen-functionalized PAAm gel adopted rounded, filopodia-poor morphologies with increased cell apoptosis, while AF cells cultured upon a corresponding stiffer PAAm gel (63 kPa) promoted cell spreading with a significantly higher percentage of cell survival. These findings, along with findings in other cell types (i.e., mesenchymal stem cells [87], endothelial cells [88], fibroblasts [88,89]), confirm the idea that matrix elasticity and composition can be used to manipulate cell behavior.

Based on prior work indicating laminin-containing substrates can promote some features of an immature NP cell, "soft" (300 Pa, 13.8 mg/ml self-polymerizing) and "stiff" (>2900 Pa, 200 μg/ml, glass-coated) surfaces coated with BME were created to further understand the influence of these physical cues on the immature NP phenotype. As observed on the PAAm gel system, on soft BME substrates, porcine NP cells also showed minimal spreading and assembled into multicell clusters over 7 days, with over 98% of cells in large clusters [83] (Fig. 3).

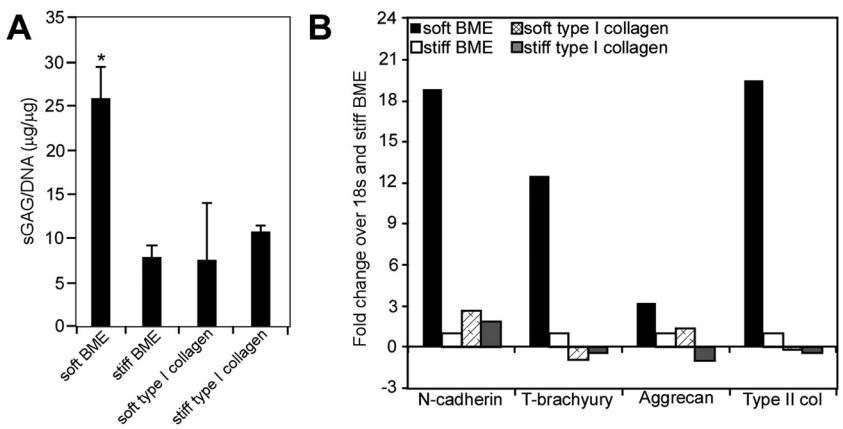

Fig. 5 Matrix production and changes in gene expression in immature porcine NP cells cultured upon various substrates (*a*) Matrix production in immature porcine NP cells on soft BME substrates is significantly higher (*$p < 0.05$, One-way ANOVA, with Tukey's post hoc analysis) than matrix production in NP cells on all other substrates. (*b*) Gene expression was calculated relative to values for 18 s mRNA and normalized by values for stiff BME. mRNA values for NP-specific and NP-matrix-related markers were higher in immature porcine NP cells on soft BME substrates compared to all other substrates Methods for biochemical assays are adapted from Gilchrist et al. 2011 [70], and methods for gene expression are adapted from Tang et al. 2012 [34]

These findings were confirmed via live-cell imaging of immature porcine NP cells cultured upon the same soft-BME substrates (Fig. 4). In addition to the same "soft" and "stiff" BME substrates as described in the previous paragraph, corresponding "soft" (300 Pa, self-polymerizing 4 mg/ml) and "stiff" (>2900 Pa, 50 μg/ml, glass-coated) type I collagen substrates were created as control. Immature porcine NP cells (45,000 cells/well) were cultured upon these four substrates for 24 h with live-cell imaging (20× objective, Olympus VivaView Fluorescent Incubator Microscope, Duke Light Microscopy Core Facility). Quantitative analysis of NP cell spread area (Fig. 4(*a*)) and velocity (Fig. 4(*b*)) on the four substrates demonstrated significantly higher cell spread area and constant protrusions of cell lamellipodia when cells were exposed to "stiff" BME substrates (Fig. 4). On "soft" BME substrates, NP cells moved with higher velocities until forming a cell-cell contact; after which, cell velocity decreased significantly. In contrast, cells on any other "stiff" substrate were motile with a steady velocity over the 24-hour duration of cell imaging. This behavior of cell clustering was not observed when NP cells were exposed to type I collagen surfaces (data not shown). Transfection of NP cells with actin-GFP (Bacmam 2.0, Molecular Probes, Life Technologies) showed formation of stress fibers in NP cells during culture upon "stiff" BME substrates (Fig. 4(*c*)). Actin stress fibers did not form when NP cells were cultured upon "soft" BME substrates, as cells formed multicell clusters.

In addition to studying changes in cell morphology during culture on "soft" BME substrates, NP cells have been analyzed for their ability to maintain an immature NP cell phenotype. Higher matrix production was observed for NP cells on "soft" BME substrates compared to "stiff" BME or type I collagen substrates (Fig. 5(*a*)). Gene expression analysis of NP-matrix proteins confirmed high levels of type II collagen and aggrecan in NP cells cultured on "soft" BME (Fig. 5(*b*)). Also, higher gene expression of NP-specific markers, N-cadherin and T-brachyury, were observed on "soft" BME as compared to other substrates (Fig. 5(*b*)).

Together, these findings demonstrate the importance of physical cues, specifically substrate stiffness and laminin ligand presentation, in promoting morphologies and metabolism characteristic of the immature NP cell. Future work would need to be done to determine if adult NP cells that exhibit more fibroblast-like

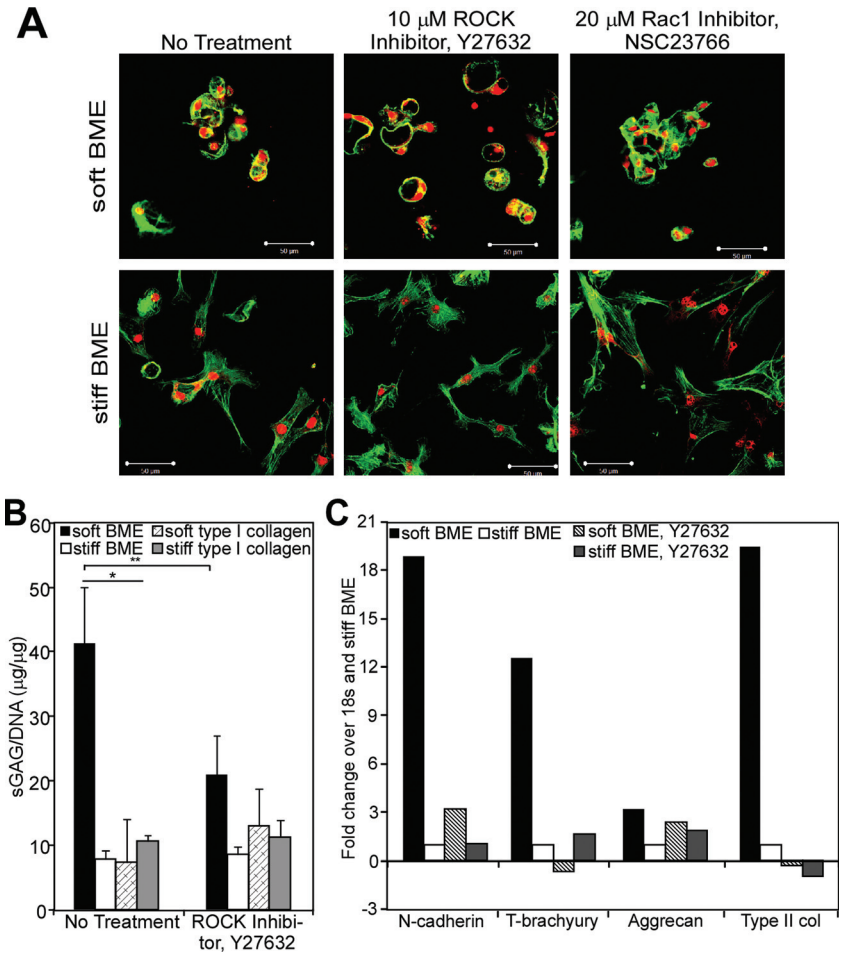

Fig. 6 Treatment of immature porcine NP cells with Rho GTPase inhibitors, ROCK (Y27632) and Rac1 (NSC23766). (*a*) Immature porcine NP cells are unable to form cell clusters on soft BME substrates after treatment with ROCK inhibitor but not Rac1 inhibitor (green = phalloidin, red = propidium iodide, bar = 50 μm). (*b*) Decreased matrix production in NP cells after 4-day treatment with ROCK inhibitor on soft BME substrates (*$p<0.01$, **$p<0.05$, Two-way ANOVA with Tukey's post hoc analysis); matrix production is unaffected by ROCK inhibitor in NP cells on all other substrates. (*c*) Gene expression was calculated relative to values for 18 s mRNA and normalized by values for stiff BME. mRNA values for NP-specific and NP-matrix-related markers are decreased in immature porcine NP cells after 4-day treatment with ROCK inhibitor on soft BME substrates. Methods for biochemical assays are adapted from Gilchrist et al. 2011 [70], and methods for gene expression are adapted from Tang et al. 2012 [34].

characteristics, including the synthesis of type I collagen and few cell-cell contacts, could be promoted to express a juvenile NP cell phenotype upon substrates of controlled stiffness and laminin presentation.

Importance Of NP Cell Clustering In Regulating An Immature NP Phenotype. The ability to form multicell clusters in vivo and on soft, laminin-containing substrates is unique to the immature NP cell [70]. This cell clustering behavior appears important for promoting the immature NP phenotype, as preservation of the immature NP phenotype has only been observed when NP cells are able to form clusters. Still, the mechanisms that promote the formation of a stable cell cluster for immature NP cells have not yet been studied.

Rho family GTPases are known to orchestrate actin reorganization during both cell-ECM adhesion formation and cell-cell adherens junction assembly [90,91]. Two of the major Rho GTPases are Rac1 (often acting with CDC42) and RhoA, which play very cell type-specific roles in regulating the cell cytoskeleton and cell-cell contacts [92,93]. Rac1 and RhoA are primary regulators of the cytoskeleton as well as focal adhesions and adherens junctions. In particular, RhoA has been identified to regulate myosin contractility, and actin polymerization, which leads to changes in intracellular contractility, stress fiber assembly, focal adhesion maturation, and adherens junction formation and disruption [94–98]. Rac is a primary regulator of lamellipodia formation, as well as the initiation of focal complex formation and of adherens junction dynamics [98]. Additionally, Rho family GTPases are also involved with cell cycle control and regulation of transcription factor activity [99,100].

Therefore, to study the effects of cell clustering in regulating NP cell phenotype, we treated immature porcine NP cells with ROCK/Rho kinase (Y27632, 10 μM) and Rac1 (NSC23766, 20 μM) inhibitors upon "soft" BME surfaces, in order to study the processes that regulate stable NP cell cluster formation. Results demonstrate that NP cells lose their ability to form cell clusters when treated with ROCK/Rho kinase, but not Rac1 inhibitor (Fig. 6(*a*)). These findings indicate ROCK-dependent RhoA GTPase signaling as a main regulator of cell clustering behavior in immature porcine NP cells. Additionally, treatment with ROCK/Rho kinase inhibitor resulted in decreased matrix production in porcine NP cells on "soft" BME substrates, with associated decreases in gene expression of NP-matrix-related markers, aggrecan and type II collagen (Figs. 6(*b*) and 6(*c*)). NP-specific markers, *N*-cadherin and *T*-brachyury, also showed decrease in gene expression in porcine NP cells on "soft" BME after treatment with ROCK/Rho kinase inhibitor. These findings suggest that immature NP cells unable to form clusters on soft BME substrates are also unable to maintain their immature NP phenotype as observed by decrease in both matrix production and presence of NP-specific markers.

Conclusion

In summary, intervertebral disc degeneration is believed to arise in part by aging-associated changes in primary cells of the NP region, including a loss of cell morphology, decreases in cell number and change in cell phenotype towards a more fibroblast-like cell type. During disc degeneration, NP cells that have lost their ability to form functional and stable cell-cell contacts may also lose their ability to produce NP-specific matrix and NP-matrix-related proteins that contribute to an inability for self-repair of the aging NP matrix. The NP cell interacts with the matrix throughout growth and aging, and receives physical cues from its ECM that help regulate and maintain the NP cell phenotype.

Work by our group and others have shown NP cells form functional interactions with ECM proteins, including collagens, fibronectin and laminin. Functional interactions studied include integrin-mediated cell adhesion to these proteins as measured by cell attachment number and strength, with ECM protein effects observed upon cell shape, velocity, and spread area. Findings from these functional interactions demonstrate NP cells have higher cell attachment numbers and strength to laminin compared to fibronectin or collagen. In addition to the importance of ECM protein, ECM elasticity also regulates NP cell phenotype. Studies have established that a soft ($<$0.5 kPa), laminin-111 rich ECM is capable of promoting NP cell clustering behaviors that are associated with higher matrix production and gene expression of NP-specific markers. This knowledge is just beginning to reveal how cell-matrix interactions can be engineered to successfully support cell-based therapies or tissue engineering strategies for generating a healthy intervertebral disc.

Acknowledgment

The authors thank the Duke Light Microscopy Core Facility for use of the live-cell imaging microscope and Yasheng Gao for helping with all the live-cell imaging work. This work was funded by the National Institutes of Health (NIH) (AR047442, EB002263, AR057410), the Searle Scholars Program, and a National Science Foundation (NSF) Graduate Research Fellowship.

References

[1] Bogduk, N., 2005, *Clinical Anatomy of the Lumbar Spine and Sacrum*, Elsevier, New York.
[2] Urban, J. P., and Roberts, S., 2003, "Degeneration of the Intervertebral Disc," Arthritis Res. Ther., **5**(3), pp. 120–30.
[3] Baer A. E., Laursen, T. A., Guilak, F., and Setton, L. A., 2003, "The Micromechanical Environment of Intervertebral Disc Cells Determined by a Finite Deformation, Anisotropic, and Biphasic Finite Element Model," ASME J. Biomech. Eng., **125**(1), pp. 1–11.
[4] Hsieh, A. H., Wagner, D. R., Cheng, L. Y., and Lotz, J. C., 2005, "Dependence of Mechanical Behavior of the Murine Tail Disc on Regional Material Properties: A Parametric Finite Element Study," ASME J. Biomech. Eng., **127**(7), pp. 1158–1167.
[5] Cao, L., Guilak, F., and Setton, L. A., 2009, "Pericellular Matrix Mechanics in the Anulus Fibrosus Predicted by a Three-Dimensional Finite Element Model and In Situ Morphology," Cell. Mol. Bioeng., **2**(3), pp. 306–319.
[6] Jackson, A. R., Huang, C. Y., Brown, M. D., and Gu, W. Y., 2011, "3D Finite Element Analysis of Nutrient Distributions and Cell Viability in the Intervertebral Disc: Effects of Deformation and Degeneration," ASME J. Biomech. Eng., **133**(9), p. 091006.
[7] Cao, L., Guilak, F., and Setton, L. A., 2011, "Three-Dimensional Finite Element Modeling of Pericellular Matrix and Cell Mechanics in the Nucleus Pulposus of the Intervertebral Disk Based on In Situ Morphology," Biomech. Model Mechanobiol., **10**(1), pp. 1–10.
[8] Korecki, C. L., MacLean, J. J., and Iatridis, J. C., 2008, "Dynamic Compression Effects on Intervertebral Disc Mechanics and Biology," Spine, **33**(13), pp. 1403–1409.
[9] Boos, N., Weissbach, S., Rohrbach, H., Weiler, C., Spratt, K.F., and Nerlich, A.G., 2002, "Classification of Age-Related Changes in Lumbar Intervertebral Discs: 2002 Volvo Award in Basic Science," Spine, **27**(23), pp. 2631–2644.
[10] Liebscher, T., Haefeli, M., Wuertz, K., Nerlich, A.G., and Boos, N., 2011, "Age-Related Variation in Cell Density of Human Lumbar Intervertebral Disc," Spine, **36**(2), pp. 153–159.
[11] Roberts, S., Evans, H., Trivedi, J., and Menage, J., 2006, "Histology and Pathology of the Human Intervertebral Disc," J. Bone Joint Surg. Am., **88**(2), pp. 10–14.
[12] Johnson, W. E., Eisenstein, S. M., and Roberts, S., 2001, "Cell Cluster Formation in Degenerate Lumbar Intervertebral Discs is Associated With Increased Disc Cell Proliferation," Connect Tissue Res., **42**(3), pp. 197–207.
[13] Johnson, W. E., and Roberts, S., 2003, "Human Intervertebral Disc Cell Morphology and Cytoskeletal Composition: A Preliminary Study of Regional Variations in Health and Disease," J. Anat., **203**(6), pp. 605–612.
[14] Hastreiter, D., Ozuna, R. M., and Spector, M., 2001, "Regional Variations in Certain Cellular Characteristics in Human Lumbar Intervertebral Discs, Including the Presence of Alpha-Smooth Muscle Actin," J. Orthop. Res., **19**, pp. 597–604.
[15] Choi, K. S., Cohn, M. J., and Harfe, B. D., 2008, "Identification of Nucleus Pulposus Precursor Cells and Notochordal Remnants in the Mouse: Implications for Disk Degeneration and Chordoma Formation," Dev. Dyn., 2008; **237**(12), pp. 3953–3958.
[16] Roughley, P. J., 2004, "Biology of Intervertebral Disc Aging and Degeneration: Involvement of the Extracellular Matrix," Spine, **29**(23), pp. 2691–2699.
[17] Urban, J. P., 2002, "The Role of the Physicochemical Environment in Determining Disc Cell Behaviour," Biochem. Soc. Trans., **30**(6), pp. 858–864.
[18] Johnstone, B., and Bayliss, M. T., 1995, "The Large Proteoglycans of the Human Intervertebral Disc. Changes in Their Biosynthesis and Structure With Age, Topography, and Pathology," Spine, **20**(6), pp. 674–684.

[19] Nachemson, A., 1992, "Lumbar Mechanics as Revealed by Lumbar Intradiscal Pressure Measurements, *The Lumbar Spine and Back Pain*, 4th ed., M. I. V. Jayson, Churchill Livingstone, New York, pp. 157–171.
[20] McNally, D. S., and Adams, M. A., 1992, "Internal Intervertebral Disc Mechanics as Revealed by Stress Profilometry," Spine, **17**, pp. 66–73.
[21] Hurri, H., and Karppinen, J., "Discogenic Pain," Pain, **112**(3), pp. 225–228.
[22] Peacock, A., 1951, Observations on the Prenatal Development of the Intervertebral Disc in Man," J. Anat., **85**(3), pp. 260–274.
[23] Walmsley, R., 1953, *The Development and Growth of the Intervertebral Disc*, Edinburgh Med. J., **60**(8), pp. 341–364.
[24] Ellis, K., Bagwell, J., and Bagnat, M., 2013, "Notochord Vacuoles are Lysosome-Related Organelles That Function in Axis and Spine Morphogenesis," J. Cell. Biol., **200**(5), pp. 667–679.
[25] Gilchrist, C. L., Chen, J., Richardson, W. J., Loeser, R. F., and Setton, L. A., 2007, "Functional Integrin Subunits Regulating Cell-Matrix Interactions in the Intervertebral Disc," J. Orthop. Res., **25**(6), pp. 829–840.
[26] Hunter, C. J., Matyas, J. R., and Duncan, N. A., 2003, "The Three-Dimensional Architecture of the Notochordal Nucleus Pulposus: Novel Observations on Cell Structures in the Canine Intervertebral Disc," J. Anat., **202**(3), pp. 279–291.
[27] Trout, J. J., Buckwalter, J. A., Moore, K. C., and Landas, S. K., 1982, "Ultrastructure of the Human Intervertebral Disc I Changes in Notochordal Cells With Age," Tissue Cell, **14**(2), pp. 359–369.
[28] Minogue, B. M., Richardson, S.M., Zeef, L. A., Freemont, A. J., and Hoyland, J. A., 2010, "Characterization of the Human Nucleus Pulposus Cell Phenotype and Evaluation of Novel Marker Gene Expression to Define Adult Stem Cell Differentiation," Arthritis Rheum., **62**(12), pp. 3695–3705.
[29] Gotz, W., Kasper, M., Fischer, G., and Herken, R., 1995, "Intermediate Filament Typing of the Human Embryonic and Fetal Notochord," Cell Tissue Res., **280**(2), pp. 455–462.
[30] Buxboim, A., Ivanovska, I. L., and Discher, D. E., 2010, "Matrix Elasticity, Cytoskeletal Forces and Physics of the Nucleus: How Deeply do Cells 'Feel' Outside and In?" J. Cell. Sci., **123**(3), pp. 297–308.
[31] Hayes, A. J., Benjamin, M., and Ralphs, J. R., 1999, "Role of Actin Stress Fibres in the Development of the Intervertebral Disc: Cytoskeletal Control of Extracellular Matrix Assembly," Dev. Dyn., **215**(3), pp. 179–189.
[32] Lehtonen, E., Stefanovic, V., and Saraga-Babic, M., 1995, "Changes in the Expression of Intermediate Filaments and Desmoplakins During Development of Human Notochord," Differentiation, **59**(1), pp. 43–49.
[33] Rodrigues-Pinto, R., Richardson, S. M., and Hoyland, J. A., 2013, "Identification of Novel Nucleus Pulposus Markers: Interspecies Variations and Implications for Cell-Based Therapies for Intervertebral Disc Degeneration," Bone Joint Res., **2**(8), pp. 169–178.
[34] Tang, X., Jing, L., and Chen, J., 2012, "Changes in the Molecular Phenotype of Nucleus Pulposus Cells With Intervertebral Disc Aging," PLoS One, **7**(12), pp. e52020–e52020-7.
[35] Minogue, B. M., Richardson, S. M., Zeef, L. A., Freemont, A. J., and Hoyland, J. A., 2010, "Transcriptional Profiling of Bovine Intervertebral Disc Cells: Implications for Identification of Normal and Degenerate Human Intervertebral Disc Cell Phenotypes," Arthritis Res. Ther., **12**(1), pp. R22-1–R22-20.
[36] Lv, F., Leung, V. Y., Huwang, S., Huang, Y., Sun, Y., and Cheung, K. M., 2013, "In Search of Nucleus Pulposus-Specific Molecular Markers," Rheumatology: Advance Access published online: September 18, 2013.
[37] Li, S., Duance, V. C., and Blain, E. J., 2008, "Zonal Variations in Cytoskeletal Element Organization, mRNA and Protein Expression in the Intervertebral Disc," J. Anat., **213**(6), pp. 725–732.
[38] Guilak, F., Ting-Beall, H. P., Baer, A. E., Trickey, W. R., Erickson, G. F., and Setton, L. A., 1999, "Viscoelastic Properties of Intervertebral Disc Cells Identification of Two Biomechanically Distinct Cell Populations," Spine, **24**(23), pp. 2475–2483.
[39] Gilson, A., Dreger, M., and Urban, J. P., 2010, "Differential Expression Level of Cytokeratin 8 in Cells of the Bovine Nucleus Pulposus Complicates the Search for Specific Intervertebral Disc Cell Markers," Arthritis Res. Ther., **12**(1), p. R24.
[40] Stosiek, P., Kasper, M., and Karsten, U., 1988, "Expression of Cytokeratin and Vimentin in Nucleus Pulposus Cells," Differentiation, **39**(1), pp. 78–81.
[41] Hunter, C. J., Matyas, J. R., and Duncan, N. A., 2004, "Cytomorphology of Notochordal and Chondrocytic Cells From the Nucleus Pulposus: A Species Comparison," J. Anat. **205**(5), pp. 357–362.
[42] Brown, M. J., Hallam, J. A., Colucci-Guyon, E., and Shaw, S., 2001, "Rigidity of Circulating Lymphocytes is Primarily Conferred by Vimentin Intermediate Filaments," J. Immunol., **166**(11), pp. 6640–6646.
[43] Herrmann, H., Bar, H., Kreplak, L., Strelkov, S.V., and Aebi, U., 2007, "Intermediate Filaments: From Cell Architecture to Nanomechanics," Nat. Rev. Mol. Cell. Biol., **8**(7), pp. 562–573.
[44] Benjamin, M., Archer, C. W., and Ralphs, J. R., 1994, "Cytoskeleton of Cartilage Cells," Microsc. Res. Tech., **28**(5), pp. 372–377.
[45] Eggli, P. S., Hunziker, E. B., and Schenk, R. K., 1988, "Quantitation of Structural Features Characterizing Weight- and Less-Weight-Bearing Regions in Articular Cartilage: A Stereological Analysis of Medial Femoral Condyles in Young Adult Rabbits," Anat. Rec., **222**(3), pp. 217–227.
[46] Chen, J., Yan, W., and Setton, L. A., 2004, "Static Compression Induces Zonal-Specific Changes in Gene Expression for Extracellular Matrix and Cytoskeletal Proteins in Intervertebral Disc Cells In Vitro," Matrix Biol., **22**(7), pp. 573–583.
[47] Butler, W. F., 1988, "Comparative Anatomy and Development of the Mammalian Disc," *The Biology of the Intervertebral Disc*, P. Gosh, ed., CRC, Boca Raton, pp. 39–82.
[48] Lee, C. R, Sakai, D., Nakai, T., Toyama, K., Mochia, J., Alini, M., and Grad, S., 2007, "A Phenotypic Comparison of Intervertebral Disc and Articular Cartilage Cells in the Rat," Eur. Spine J., **16**(12), pp. 2174–2185.
[49] Sakai, D., Nakai, T., Mochia, J., Alini, M., and Grad, S., 2009, "Differential Phenotype of Intervertebral Disc Cells: Microarray and Immunohistochemical Analysis of Canine Nucleus Pulposus and Anulus Fibrosus," Spine, **34**(14), pp. 1448–1456.
[50] Risbud, M. V., Schaer, T. P., and Shapiro, I. M., 2010, "Toward an Understanding of the Role of Notochordal Cells in the Adult Intervertebral Disc: From Discord to Accord," Dev. Dyn., **239**(8), pp. 2141—2148.
[51] Roberts, S., Ayad, S., and Menage, P. J., 1991, "Immunolocalisation of Type VI Collagen in the Intervertebral Disc," Ann. Rheum. Dis., **50**(11), pp. 787–791.
[52] Trout, J. J., Buckwalter, J. A., and Moore, K. C., 1982, "Ultrastructure of the Human Intervertebral Disc: II. Cells of the Nucleus Pulposus," Anat. Rec., **204**(4), pp. 307–314.
[53] Cao, L., Guilak, F., and Setton, L. A., 2007, Three-Dimensional Morphology of the Pericellular Matrix of Intervertebral Disc Cells in the Rat," J. Anat., **211**(4), pp. 444–452.
[54] Errington, R. J., Puustjarvi, K., White, I. R., Roberts, S., Urban, J. P., 1998, "Characterisation of Cytoplasm-Filled Processes in Cells of the Intervertebral Disc," J. Anat., **192**(3), pp. 369–378.
[55] Iatridis, J. C., Setton, L. A., Weidenbaum, M., and Mow, V. C., 1997, "The Viscoelastic Behavior of the Non-Degenerate Human Lumbar Nucleus Pulposus in Shear," J. Biomech., **30**(10), pp. 1005–1013.
[56] Iatridis, J. C, Weidenbaum, M., Setton, L. A., and Mow, V. C., 1996, "Is the Nucleus Pulposus a Solid or a Fluid? Mechanical Behaviors of the Nucleus Pulposus of the Human Intervertebral Disc," Spine, **21**(10), pp. 1174–1184.
[57] Cloyd, J. M., Malhotra, N. R., Weng, L., Chen, W., Mauck, R. L., and Elliott, D. M., 2007, Material Properties in Unconfined Compression of Human Nucleus Pulposus, Injectable Hyaluronic Acid-Based Hydrogels and Tissue Engineering Scaffolds," Eur Spine J., 16(11), pp. 1892–1898,
[58] Eyre, D. R., and Muir, H., 1977, "Quantitative Analysis of Types I and II Collagens in Human Intervertebral Discs at Various Ages," Biochim. Biophys. Acta. **492**(1), pp. 29–42.
[59] Gower, W. E., and Pedrini, V., 1969, "Age-Related Variations in Proteinpolysaccharides From Human Nucleus Pulposus, Annulus Fibrosus, and Costal Cartilage," J Bone Joint Surg. Am., **51**(6), pp. 1154–1162.
[60] Taylor, J. R., and Twomey, L. T., 1988, "The Development of the Human Intervertebral Disc," *The Biology of the Intervertebral Disc*, P. Gosh, ed., CRC, Boca Raton, FL., pp. 39–82.
[61] Roberts, S., Menage, J., Duance, V., Wotton, S., and Ayad, S., 1991, "Volvo Award in Basic Sciences Collagen Types Around the Cells of the Intervertebral Disc and Cartilage End Plate: An Immunolocalization Study," Spine, **16**(9), pp. 1030–1038.
[62] Melrose, J., Ghosh, P., and Taylor, T. K., 2001, "A Comparative Analysis of the Differential Spatial and Temporal Distributions of the Large (Aggrecan, Versican) and Small (Decorin, Biglycan, Fibromodulin) Proteoglycans of the Intervertebral Disc," J. Anat., **198**(1), pp. 3–15.
[63] Yu, J., 2002, "Elastic Tissues of the Intervertebral Disc," Biochem. Soc. Trans, **30**(6), pp. 848–852.
[64] Chen, J., Jing, L., Gilchrist, C. L., Richardson, W. J., Fitch, R. D., and Setton, L. A., 2009, "Expression of Laminin Isoforms, Receptors and Binding Proteins Unique to Nucleus Pulposus Cells of Immature Intervertebral Disc," Connect Tissue Res., **50**, pp. 294–306.
[65] Nettles, D. L., Richardson, W. J., and Setton, L. A., 2004, "Integrin Expression in Cells of the Intervertebral Disc," J. Anat. **204**(6), pp. 515–520.
[66] Natarajan, R. N., Williams, J. R., and Andersson, G. B., 2004, "Recent Advances in Analytical Modeling of Lumbar Disc Degeneration," Spine, **29**(23), pp. 2733–2741.
[67] Discher, D. E., Janmey, P., and Wang, Y. L., 2005, "Tissue Cells Feel and Respond to the Stiffness of Their Substrate," Science, **310**(5751), pp. 1139–1143.
[68] Pelham, R. J., Jr., and Wang, Y., 1997, "Cell Locomotion and Focal Adhesions are Regulated by Substrate Flexibility," Proc. Natl. Acad. Sci. U.S.A, **94**(25), pp. 13661–5.
[69] Guo, W. H., Frey, M. T., Burnham, N. A., and Wang, Y. L., 2006, "Substrate Rigidity Regulates the Formation and Maintenance of Tissues," Biophys. J., **90**(6), pp. 2213–2220.
[70] Gilchrist., C. L., Darling, E. M., Chen, J., and Setton, L. A., 2011, "Extracellular Matrix Ligand and Stiffness Modulate Immature Nucleus Pulposus Cell-Cell Interactions," PLoS One, **6**(11), pp. e27170-1–e27170-9.
[71] Johannessen, W., and Elliott, D. M., 2005, "Effects of Degeneration on the Biphasic Material Properties of Human Nucleus Pulposus in Confined Compression," Spine, **30**(24), pp. E724–E729.
[72] Iatridis, J. C., Setton, L. A., Weidenbaum, M., and Mow, V. S., 1997, "Alterations in the Mechanical Behavior of the Human Lumbar Nucleus Pulposus With Degeneration and Aging," J. Orthop. Res., **15**(2), pp. 318–322.
[73] Miner, J. H., and Yurchenco, P. D., 2004, "Laminin Functions in Tissue Morphogenesis," Annu. Rev. Cell. Dev. Biol., **20**, pp. 255–284.
[74] Zaidel-Bar, R,. Shalev, I., Ma'ayan A., Iyengar, R., Geiger, B., 2007, "Functional Atlas of the Integrin Adhesome," Nat. Cell. Biol., **9**(8), pp. 858–867.
[75] Hoffman, B. D., Grashoff, C., and Schwartz, M. A., 2011, "Dynamic Molecular Processes Mediate Cellular Mechanotransduction," Nature, **475**(7356), pp. 316–323.
[76] Hynes, R. O., 1992, "Integrins: Versatility, Modulation, and Signaling in Cell Adhesion," Cell, **69**(1), pp. 11–25.
[77] Schwartz, M. A., 2010, "Integrins and Extracellular Matrix in Mechanotransduction," Cold Spring Harb. Perspect. Biol., **2**(12), pp. a005066-1–a005066-13.

[78] Bridgen, D. T., Gilchrist, C. L., Richardson, W. J., Isaacs, R. E., Brown, C. R., Yang, K. L., Chen, J., and Setton, L. A., 2013, "Integrin-Mediated Interactions With Extracellular Matrix Proteins for Nucleus Pulposus Cells of the Human Intervertebral Disc,". J. Orthop. Res., **31**(10), pp. 1661–1667.
[79] Le Maitre, C. L., Frain, J., Millward-Sadler, J., Fotheringham, A. P., Freemont, A. J., and Hoyland, J. A., 2009, "Altered Integrin Mechanotransduction in Human Nucleus Pulposus Cells Derived From Degenerated Discs," Arthritis Rheum., **60**(2), pp. 460–469.
[80] Anderson, D. G., Li, X., and Balian, G., 2005, "A Fibronectin Fragment Alters the Metabolism by Rabbit Intervertebral Disc Cells in vitro," Spine, **30**(11), pp. 1242–1246.
[81] Aota, Y., An, H. S., Homandberg, G., Thonar, E. J., Andersson, G. B., Pichika, R., and Masuda, K., 2005, "Differential Effects of Fibronectin Fragment on Proteoglycan Metabolism by Intervertebral Disc Cells: A Comparison With Articular Chondrocytes," Spine, **30**(7), pp. 722–728.
[82] Chen, J., Yan, W., and Setton, L. A., 2006, "Molecular Phenotypes of Notochordal Cells Purified From Immature Nucleus Pulposus," Eur. Spine J., **15**(3), pp. S303–S311.
[83] Gilchrist, C. L., Francisco, A. T., Plopper, G. E., Chen, J., and Setton, L. A., 2011, "Nucleus Pulposus Cell-Matrix Interactions With Laminins," Eur. Cell. Mater, **21**, pp. 523–532.
[84] Wang, Y. L., and Pelham, R. J., Jr., 1998, "Preparation of a Flexible, Porous Polyacrylamide Substrate for Mechanical Studies of Cultured Cells," Methods Enzymol., **298**, pp. 489–496.
[85] Hassell, J. R., Robey, P. G., Barrach, H. J., Wilczek, J., Rennard, S. I., and Martin, G. F., 1980, "Isolation of a Heparan Sulfate-Containing Proteoglycan From Basement Membrane," Proc. Natl. Acad. Sci. U.S.A., **77**(8), pp. 4494–4498.
[86] Zhang, Y.H., Zhao, C.Q., Jiang, L.S., and Dai, L.Y., 2011, "Substrate Stiffness Regulates Apoptosis and the mRNA Expression of Extracellular Matrix Regulatory Genes in the Rat Annular Cells," Matrix Biol., **30**(2), pp. 135–144.
[87] Engler, A. J., Sen, S., Sweeney, H. L., and Discher, D. E., 2006, "Matrix Elasticity Directs Stem Cell Lineage Specification," Cell, **126**(4) pp. 677–689.
[88] Yeung, T., Georges, P. C., Flanagan, L. A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P. A., 2005, "Effects of Substrate Stiffness on Cell Morphology, Cytoskeletal Structure, and Adhesion," Cell. Motil. Cytoskeleton, **60**(1), pp. 24–34.
[89] Wang, H. B., Dembo, M., and Wang, Y. L., 2000, "Substrate Flexibility Regulates Growth and Apoptosis of Normal but not Transformed Cells," Am. J. Physiol. Cell. Physiol., **279**(5), pp. C1345–C1350.
[90] Weber, G. F., Bjerke, M. A., and DeSimone, D. W., 2011, "Integrins and Cadherins Join Forces to Form Adhesive Networks," J. Cell. Sci., **124**(8), pp. 1183–1193.
[91] Schwartz, M. A., and DeSimone, D. W., 2008, "Cell Adhesion Receptors in Mechanotransduction," Curr. Opin. Cell. Biol., **20**(5), pp. 551–556.
[92] Braga, V. M., 2002, "Cell-Cell Adhesion and Signalling," Curr. Opin. Cell. Biol., **14**(5), pp. 546–556.
[93] Arthur, W. T., Noren, N. K., and Burridge, K., 2002, "Regulation of Rho Family GTPases by Cell-Cell and Cell-Matrix Adhesion," Biol. Res., **35**(2), pp. 239–246.
[94] Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K., 1996, "Phosphorylation and Activation of Myosin by Rho-Associated Kinase (Rho-Kinase)," J. Biol. Chem., **271**(34), pp. 20246–20249.
[95] Amano, M., Chihara, K., Kazushi, K., Fukata, Y., Nakamura, N., Matsuura, Y., and Kaibuchi, K., 1997, "Formation of Actin Stress Fibers and Focal Adhesions Enhanced by Rho-Kinase," Science, **275**(5304), pp. 1308–1311.
[96] Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K., 1996, Regulation of Myosin Phosphatase by Rho and Rho-Associated Kinase (Rho-Kinase), Science, **273**(5272), pp. 245–248.
[97] Hall, A., 2005, "Rho GTPases and the Control of Cell Behaviour," Biochem. Soc. Trans., **33**(5), pp. 891–895.
[98] Burridge, K., and Wennerberg, K., 2004, "Rho and Rac Take Center Stage," Cell, **116**(2), pp. 167–179.
[99] Yamada, S., and Nelson, W. J., 2007, "Localized Zones of Rho and Rac Activities Drive Initiation and Expansion of Epithelial Cell-Cell Adhesion," J. Cell. Biol., **178**(3), pp. 517–527.
[100] Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K., 2001, "Cadherin Engagement Regulates Rho Family GTPases," J. Biol. Chem., **276**(36), pp. 33305–33308.
[101] Fujita, N., Miyamoto, T., Imai, J., Hosogane, N., Suzuki, T., Yagi, M., Morita, K., Ninomiya, K., Miyamoto, K., Takaishi, H., Matsumoto, M., Morioka, H., Yabe, H., Chiba, K., Watanabe, S., Toyama, Y., and Suda, T., 2005, "CD24 is Expressed Specifically in the Nucleus Pulposus of Intervertebral Discs," Biochem Biophys Res Commun, **338**(4), pp. 1890–1896.
[102] Tang, X. J. L., Setton, L. A., Richardson, W. J., Isaacs, R. E., Fitch, R. D., Brown, C. R., and Chen, J., 2013, "Identifying the Molecular Phenotype of Cells in the Human Intervertebral Disc Reveals the Existence of a Unique Notochordal-Like Cell Population," Trans. Orthop. Res. Soc., **38**(859), p. 0859.
[103] Weiler, C., Nerlich, A.G., Schaaf, R., Bachmeier, B.E., Wuertz, K., and Boos, N., 2010, "Immunohistochemical Identification of Notochordal Markers in Cells in the Aging Human Lumbar Intervertebral Disc," Eur. Spine J., **19**(10), pp. 1761–170.
[104] Nerlich, A. G., Schleicher, E. D., and Boos, N., 1997, "Volvo Award Winner in Basic Science Studies. Immunohistologic Markers for Age-Related Changes of Human Lumbar Intervertebral Discs," Spine, **22**(24), pp. 2781–2795.