Mechanosensitive transcriptional coactivators MRTF-A and YAP/TAZ regulate nucleus pulposus cell phenotype through cell shape

Bailey V. Fearing,* Liufang Jing,* Marcos N. Barcellona,* Savannah Est Witte,* Jacob M. Buchowski,† Lukas P. Zebala,‡ Michael P. Kelly,§ Scott Luhmann,∥ Munish C. Gupta,† Amit Pathak,‡ and Lori A. Setton*†,1
*Department of Biomedical Engineering, †Department of Orthopaedic Surgery, and ‡Department of Mechanical Engineering and Materials Science, Washington University in St. Louis, St. Louis, Missouri, USA

ABSTRACT: Cells of the adult nucleus pulposus (NP) are critically important in maintaining overall disc health and function. NP cells reside in a soft, gelatinous matrix that dehydrates and becomes increasingly fibrotic with age. Such changes result in physical cues of matrix stiffness that may be potent regulators of NP cell phenotype and may contribute to a transition toward a senescent and fibroblastic NP cell with a limited capacity for repair. Here, we investigate the mechanosignaling cues generated from changes in matrix stiffness in directing NP cell phenotype and identify mechanisms that can potentially preserve a biosynthetically active, juvenile NP cell phenotype. Using a laminin-functionalized polyethylene glycol hydrogel, we show that when NP cells form rounded, multicell clusters, they are able to maintain cytosolic localization of myocardin-related transcription factor (MRTF)-A, a coactivator of serum-response factor (SRF), known to promote fibroblast-like behaviors in many cells. Upon preservation of a rounded shape, human NP cells similarly showed cytosolic retention of transcriptional coactivator Yes-associated protein (YAP) and its paralogue PDZ-binding motif (TAZ) with associated decline in activation of its transcription factor TEA domain family member–binding domain (TEAD). When changes in cell shape occur, leading to a more spread, fibrotic morphology associated with stronger F-actin alignment, SRF and TEAD are up-regulated. However, targeted deletion of either cofactor was not sufficient to overcome shape-mediated changes observed in transcriptional activation of SRF or TEAD. Findings show that substrate stiffness-induced promotion of F-actin alignment occurs concomitantly with a flattened, spread morphology, decreased NP marker expression, and reduced biosynthetic activity. This work indicates cell shape is a stronger indicator of SRF and TEAD mechanosignaling pathways than coactivators MRTF-A and YAP/TAZ, respectively, and may play a role in the degeneration-associated loss of NP cellularity and phenotype.—Fearing, B. V., Jing, L., Barcellona, M. N., Witte, S. E., Buchowski, J. M., Zebala, L. P., Kelly, M. P., Luhmann, S., Gupta, M. C., Pathak, A., Setton, L. A. Mechanosensitive transcriptional coactivators MRTF-A and YAP/TAZ regulate nucleus pulposus cell phenotype through cell shape. FASEB J. 33, 14022–14035 (2019). www.fasebj.org

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Pathology of the intervertebral disc (IVD) has emerged as a major healthcare concern and contributor to disability, with more than 80% of the population experiencing back pain within their lives (1, 2). Within the IVD, considerable changes to the extracellular matrix (ECM) occur with age and degeneration that are believed to induce cellular changes, including phenotype and cellularity. Many IVD disorders likely originate in the nucleus pulposus (NP) (Fig. 1A). NP cells generate a highly hydrated and gelatinous ECM during development and maturation (3–5) and

ABBREVIATIONS: AGC, aggrecan; AF, anulus fibrosus; CDH2, N-cadherin; COL2, collagen type II; ECM, extracellular matrix; FBS, fetal bovine serum; GLUT1, glucose transporter 1; IVD, intervertebral disc; Lat B, latrunculin B; LM, laminin; MRTF, myocardin-related transcription factor; NP, nucleus pulposus; PEG, polyethylene glycol; PEG-LM, LM-conjugated PEG; qPCR, quantitative PCR; ROCK, Rho-associated protein kinase; sGAG, sulfated glycosaminoglycan; siRNA, small interfering RNA; SRE, serum-response element; SRF, serum-response factor; TAZ, PDZ-binding motif; TEAD, TEA domain family member–binding domain; V27, Y27632; YAP, Yes-associated protein

1 Correspondence: Department of Biomedical Engineering, Washington University in St Louis, One Brookings Dr., Campus Box 1097, St. Louis, MO 63130, USA. E-mail: setton@wustl.edu

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are distinct from cells of the adjacent anulus fibrosus (AF) in morphology, phenotype, and biosynthetic profile (6–10). With age, NP cells transition to a sparser distribution with a very poor ability to maintain or repair the NP-specific ECM (9–11). These age-related cell changes may be linked to changes in the ECM of the IVD, including loss of hydration and swelling pressure, and an increase in stiffness [from 0.5 to 1 kPa to \( \geq 5 \) kPa (12, 13); Fig. 1B], such that the IVD becomes more fibrocartilaginous and cannot maintain proper IVD function. These changes may contribute to the generation of tissue fissures and decreased IVD height that is characteristic of the pathologic IVD (3, 14).

The ECM of the NP largely consists of type II and other collagen species, with a highly diverse population of proteoglycans (9). One of the distinguishing features of young NP tissue is the presence of multiple laminin (LM) isoforms (15–17) that are thought to be remnants of the developing notochord (17, 18). LM111, LM511, and LM332 are present in tissues of the juvenile NP, along with LM-specific integrin-attachment subunits \( \alpha_3, \alpha_5, \alpha_6, \beta_1 \), and CD239 (19–21). In degenerate human NP, all LM expression is decreased or absent. Previous studies have shown that porcine and human NP cells prefer attachment to LMs compared with other ECM proteins in vitro (15, 22, 23). Furthermore, when the stiffness of LM- or LM-rich substrates is close to that of native NP tissue [0.3 kPa (12, 24)], NP cells form distinct clustered morphologies, increase expression of brachyury, collagen type II (COL2), and aggrecan (AGC), and maintain intracellular vacuoles, all of which are morphologic and molecular markers of a healthy, biosynthetically active NP-specific phenotype (25, 26).

In many cell types, initial cell-ECM attachment has been shown to mature into discrete sites of focal contacts and clustered integrin attachments that connect to a polymerized F-actin cytoskeleton (27–34). Assembly of these focal contacts depends, in part, on substrate conditions such as stiffness and ligand presentation that can impact the extent of activation of multiple intracellular signaling pathways. Soft substrates can promote reduced focal adhesions, F-actin formation, and cell contractility in many cell types, as compared with stiff conditions (35, 36). Changes in the actin cytoskeleton formation can directly interfere with signaling events through binding of F-actin to transcription and transcriptional coactivators. In particular, myocardin-related transcription factor (MRTF)-A translocates to the nucleus during F-actin turnover or other events that disrupt actin polymerization (37). MRTF-A is a potent coactivator of serum-response factor (SRF) transcription factor, which binds to serum-response elements (SREs) present in \( > 200 \) genes to induce transcriptional changes that promote fibroblastic differentiation in myofibroblasts, keratinocytes, and other cells (38, 39). SRF regulates the activity of immediate early genes, and a disproportionate number of those target genes encode elements of actin cytoskeleton (38, 40).
Additionally, the transcriptional coactivator Yes-associated protein (YAP) and its parologue PDZ-binding motif (TAZ) are known to similarly affect cells upon substrates of varying rigidity or confined cell shape (41). YAP/TAZ regulates cell signaling through binding to TEA domain family member–binding domain (TEAD) transcription factor, present in hundreds of gene pathways. The regulatory mechanisms of this pathway involve phosphorylation that confines YAP/TAZ to the cytosol, resulting in the inability of YAP/TAZ to translocate to the nucleus. Once in the nucleus, YAP/TAZ functions as a coactivator of TEAD and further induces expression of cell proliferation– and apoptosis–associated genes. Under increased F actin and contractile forces, YAP/TAZ functions as a coactivator and translocates to the nucleus where it binds TEAD, allowing transcriptional activation of downstream gene targets that drive increased proliferation and differentiation (27, 41–44). Both SRF and TEAD represent dual controlled pathways. The regulatory mechanisms of this pathway involve SRF and TEAD translocation to the nucleus; this rounded morphology may also be altered through integrin signaling, with evidence that mechanical stress can activate increased TGF-β via αvβ3 and lead to decreased vacuoles and other characteristics of degeneration (50). Cell shape is also a factor that has been shown to regulate NP cell phenotype, with evidence that a rounded cell morphology, preserved when cells are cultured upon a soft, LM-conjugated polystyrene glycol (PEG-LM) substrate, led to functional N-cadherin (CDH2) interactions with associated attenuation of β-catenin translocation to the nucleus; this rounded morphology and associated cell-cell contacts preserve biosynthetic activity and expression of NP-specific markers (51). In contrast, culture upon stiff substrates has been shown to induce a characteristic flattened cell shape, with associated formation of polymerized F-actin cytoskeleton. Given known interactions of the F-actin cytoskeleton with transcriptional coactivators that promote a fibroblast-like phenotype, we hypothesize that MRTF-A and YAP/TAZ association with F-actin stress fibers in NP cells may be an additional regulator of the differentiation of the adult, human NP cell toward a pathologic and fibroblastic phenotype in the degenerate and stiffened NP ECM. In this study, we sought to determine if substrate stiffness regulation of SRF and TEAD play a role in primary human NP cell morphology, phenotype, and biosynthetic activity.

MATERIALS AND METHODS

Primary human NP cell culture

Surgical waste samples from to-be-discarded tissue (Washington University Institutional Review Board exempt) were obtained from patients undergoing surgical treatment for degeneration or scoliosis (ages 25–75), and NP regions were identified and used to isolate primary cells using a pronase-collagenase digestion method (15). Each patient sample was collected separately from lumbar and thoracic regions and, in accordance with the nonhuman subjects research designation, only age, race, and gender information was collected, whereas grade of pathology and vertebral level remained unknown. NP tissue was separated from the surrounding AF and cartilaginous tissue and digested with agitation for 2–4 h in 25 ml digestion medium per gram of NP tissue (0.3% collagenase type II, 0.2% pronase). Cells were cultured up to passage 3 in culture medium composed of Ham’s F-12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin under 5% CO2, and atmospheric oxygen at 37°C.

PEG-LM hydrogel synthesis

PEG-LM hydrogels were synthesized as previously described in refs. 52 and 53. Briefly, full-length LM111 (Trevigen, Gaithersburg, MD, USA) was conjugated with acrylate-PEG-hydroxy succinimide (10 kDa; Creative PEGWorks, Chapel Hill, NC, USA) to produce a PEGylated-LM solution. PEG-LM was dialyzed against PBS to remove any unreacted acrylate-PEG-hydroxy succinimide, and the final LM concentration in the resulting PEG-LM conjugate precursor solution was determined via absorbance reading at 280 nm. Hydrogels were synthesized by combining 500 μg/ml PEG-LM precursor solution with varying amounts of 8-arm PEG acrylate (20 kDa; Creative PEGWorks) in chamber slides (Millicell EZ Slide; MilliporeSigma, Burlington, MA, USA) and polymerized in the presence of a photoinitiator (0.1% w/v; Irgacure 2959; BASF, Ludwigshafen, Germany) upon UV exposure (5–4 mW × cm–2). Hydrogels were produced at either 4% (w/v; soft 0.3 kPa) or 20% (stiff, 20 kPa; Fig. 1C, D).

Mechanical testing

PEG-LM hydrogels were synthesized as previously described, then placed in PBS (1 time; pH 7.4) and allowed to swell to equilibrium volume. Then, gels were cut into discs 8 mm in diameter and roughly 2 mm thick. All samples were tested in oscillatory shear using an AR-G2 Rheometer (TA Instruments, New Castle, DE, USA). Samples were first placed on a preheated plate, allowed to reach 37°C, and subjected to a preloading step of 0.015 N. Then, a 10% compressive strain was applied, and the samples were allowed a 2-min conditioning step for relaxation. Samples were then subjected to oscillatory torsional strains [1–10 rad/s with a constant shear strain (γ) of 0.01], and complex shear modulus (G*–1) was reported for all samples at an angular frequency of 10 rad/s.

Primary human NP cell culture on PEG-LM

Primary human NP cells maintained in monolayer culture were collected using 0.05% trypsin-EDTA (Thermo Fisher Scientific) and resuspended in F-12 growth medium. NP cells

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were seeded at a density of $2.5 \times 10^5$ cells/ml and cultured upon 4 and 20% PEG-LM substrates for up to 4 d (37°C, 5% CO$_2$). LM-coated glass substrate controls were coated at upon 4 and 20% PEG-LM substrates for up to 4 d (37°C, 5% serum, 4°C overnight. Wells were then rinsed once with PBS, and lentiviral cells were seeded in 6-well plates and allowed to attach overnight. 105 cells were centrifuged, washed, and fixed with 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA, USA). Percentage of positive cells was determined by flow cytometry was removed after 18 h of incubation and replaced with normal room temperature for 30 min. Following a wash step, cells were diluted 1:200 in PBS containing 0.1% saponin and incubated at 12 h post-transfection, the medium was changed and, subsequently, the viral supernatant was collected at 24 and 48 h. The resulting virus was amplified and purified from both plasmids using a Maxi Prep Kit (Qiagen, Germantown, MD, USA). Lentiviral production was conducted in the Hope Center Viral Vectors Core following standard viral production methods. Briefly, HEK293T cells were transfected with the transfer vector, pMD2.G, and psPAX2 (Addgene) packaging gene vectors using Lipofectamine LTX (Thermo Fisher Scientific). At 12 h post-transfection, the medium was changed and, subsequently, the viral supernatant was collected at 24 and 48 h. The resulting supernatant was filtered (0.45 μm), pooled, and concentrated by ultracentrifugation.

**Transcription factor activity**

**SRF**

An HIV lentivirus vector was used that expressed destabilized green fluorescent protein 2 from the copepod *Pontellina plumata* (copGFP, and firefly luciferase under the control of 4 ETS-like 1 transcription factor (ELK1)-SRF response elements and minimal cytomegalovirus promoter (pGreenFire1-ELK1-SRF-EF1-Neo; Systems Biosciences, Palo Alto, CA, USA). Sequencing was performed to confirm location and order of the SRE, minimal cytomegalovirus reporter, and luciferase reporter gene.

**TEAD**

A plasmid containing 4 TEAD-binding sites upstream of a firefly luciferase reporter gene and under control of a minimal chicken troponin T promoter was purchased from Addgene (8xGTHIC; Watertown, MA, USA). The plasmid was cloned into a third-generation HIV–1-based lentiviral vector by cutting out the sequence upstream of 8xGTHIC and downstream of luciferase.

**Lentivirus production**

DNA was amplified and purified from both plasmids using a Maxi Prep Kit (Qiagen, Germantown, MD, USA). Lentiviral production was conducted in the Hope Center Viral Vectors Core following standard viral production methods. Briefly, HEK293T cells were transfected with the transfer vector, pMD2.G, and psPAX2 (Addgene) packaging gene vectors using Lipofectamine LTX (Thermo Fisher Scientific). At 12 h post-transfection, the medium was changed and, subsequently, the viral supernatant was collected at 24 and 48 h. The resulting supernatant was filtered (0.45 μm), pooled, and concentrated by ultracentrifugation.

**Lentiviral transduction of primary human NP cells**

Following 1 passage of monolayer culture, $5 \times 10^5$ human NP cells were seeded in 6-well plates and allowed to attach overnight. Wells were then rinsed once with PBS, and lentiviral transduction medium was added containing the following: F-12 with 10% serum, 4 μg/ml polybrene (MilliporeSigma), and 2 μl concentrated lentivirus (titer 5.5 × 10^5). Transduction medium was removed after 18 h of incubation and replaced with normal F-12 growth medium and cells cultured for 4 additional days. Percentage of positive cells was determined by flow cytometry (Guava EasyCyte; MilliporeSigma) using an antifirefly luciferase antibody (ab21176; Abcam, Cambridge, MA, USA). Briefly, $5 \times 10^5$ cells were centrifuged, washed, and fixed with 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA, USA). Cells were washed twice again in PBS containing 0.1% saponin (MilliporeSigma). The primary luciferase antibody was diluted 1:200 in PBS containing 0.1% saponin and incubated at room temperature for 30 min. Following a wash step, cells were treated with the secondary antibody (Alexa Fluor 488; Thermo Fisher Scientific) diluted in PBS containing 0.1% saponin (1:250) for 30 min at room temperature. Percentage of luciferase-positive cells was determined by setting a threshold using an isotype control to gate the population of positive cells (>90% positive; unpublished results).

**Luciferase assay**

Reporter cells were serum starved (0.5%) overnight prior to hydrogel culture. Fifty thousand cells were seeded into each well of an 8-well chamber slide containing soft and stiff PEG-LM and cultured for 4 d in low-serum (2%) F-12 growth medium. Following the culture period, Bright-Glo Luciferase Assay (Promega, Madison, WI, USA) was performed according to the manufacturer’s instructions. Briefly, medium was replaced with regular F-12 medium followed by Bright-Glo reagent (1:1). Cultures were incubated for 5 min at room temperature and contents were transferred to a 96-well white-bottom plate and luminescence read on an Enspire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Statistical differences were determined using a 2-way ANOVA (stiffness and time) with Tukey’s post hoc test.

**Immunocytochemistry**

**Cofactor localization**

Following the culture period, samples were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS+/− for 10 min, rinsed with PBS, and blocked for 1 h with 3.75% bovine serum albumin (MilliporeSigma) and 5% nonimmune goat serum (Thermo Fisher Scientific). Samples were then immunolabeled with antihuman mouse MRTF-A antibody (1:100; sc32909; Santa Cruz Biotechnology, Dallas, TX, USA), antihuman rat YAP-TAZ (1:100; sc101199; Santa Cruz Biotechnology), or an appropriate isotype-matched control overnight at 4°C. After an additional wash step, cells were treated with goat antimouse or goat antirat Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific) for 1 h at room temperature, protected from light. Cell nuclei were counterstained with DAPI (2 μg/ml; MilliporeSigma) for 10 min at room temperature. Gels were immediately imaged using a long-working-distance ×40 objective via confocal microscopy (SPE DM6; Leica Microsystems, Buffalo Grove, IL, USA) and analyzed to obtain the ratio of nuclear intensity to the corrected cytosolic intensity (ImageJ; National Institutes of Health, Bethesda, MD, USA). Differences in the nuclear-cytosolic ratio for NP cells cultured upon soft and stiff PEG-LM were tested using Student’s t test for significance ($P < 0.05$).

**Actin alignment**

Another set of cells was stained for F-actin fibers using a conjugated phalloidin antibody (1:200 for 2 hours; room temperature; phalloidin–Alexa Fluor 488; Thermo Fisher Scientific). Cells were similarly counterstained for nuclei visualization using DAPI, and F-actin orientation was analyzed from microscopic images (ImageJ plugin, Orientation). Differences in F-actin orientation for NP cells cultured upon different substrates were determined using a 1-way ANOVA and Tukey’s post hoc test.

**siRNA interference**

siRNA pools of gene-specific 19–25 nt sequences targeting YAP and MRTF-A designed to knock down mRNA were
purchased from Santa Cruz Biotechnology (sc-38637 and sc-43944, respectively) along with a nontargeting 20-25-nt siRNA designed as a negative scramble control (sc-44236). Primary human NP cells, cultured no longer than passage 1, were trypsinized, counted, and centrifuged at 0.5 × 10^6 cells per siRNA construct. Nucleofection was used to deliver the siRNA using the Amaxa Nucleofector II (Lonza, Basel, Switzerland) using a modified protocol from the manufacturer for primary human chondrocytes. Cells were resuspended in 100 ml of room-temperature Human Chondrocyte Nucleofector Solution (Lonza). Five micrograms of DNA was added and immediately transferred to a cuvette for nucleofection (program T-030). Following nucleofection, 500 ml of prewarmed 20% FBS containing F-12 medium was added and gently transferred to a prewarmed 6-well plate. Following 24 h incubation, medium was replaced with 10% FBS F-12 medium. All siRNA experiments were conducted 48 h postnucleofection.

mRNA extraction and real-time quantitative PCR

Following the culture period, mRNA was extracted from all samples. Cells cultured atop hydrogels were detached using 0.05% trypsin and lysed upon addition of Trizol (Thermo Fisher Scientific). Cells cultured on glass substrates with coatings were lysed by addition of RLT buffer with β-mercaptoethanol. mRNA extraction was performed on all samples using an RNeasy Mini Kit with DNase I Digestion (Qiagen). Quality and concentration of mRNA was determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific) to verify absorbance at 260 and 280 nm. mRNA was reverse transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Samples were diluted to a final concentration of 10 ng/µl in RNase- and DNase-Free water. Real-time quantitative PCR (qPCR) was performed using TaqMan primer probes (Thermo Fisher Scientific) for human NP phenotype and matrix-related primers (Table 1). qPCR was performed using a StepOnePlus Thermal Cycler (Thermo Fisher Scientific) in duplicate under standard conditions (12.5 µl 2× Universal Master Mix, 1.25 µl TaqMan primer probes, 9.25 µl ddH2O, and 2 µl 10 ng/µl cDNA). 18s and glyceraldehyde 3-phosphate dehydrogenase (Thermo Fisher Scientific) were used as an internal control housekeeping gene. Using the 2^-ΔΔCt method, fold-changes were calculated with the initial Δ accounting for fold-change over the respective housekeeping gene and the second Δ accounting for change over patient-matched NP cells on LM-coated glass or LM-coated glass with vehicle latrunculin B (Lat B) or scramble [small interfering RNA (siRNA)] controls. Differences in expression level for each gene were tested with a 1-way ANOVA with Tukey’s post hoc test.

TABLE 1. List of qPCR primers used with corresponding common name

| Primer probe | Common name | Product no. |
|--------------|-------------|-------------|
| SLC2A1a     | GLUT1       | Hs00892681-m1 |
| ACANa       | AGC         | Hs00153936_m1 |
| COL2A1a     | COL2        | Hs00156568_m1 |
| GAPDH       | Housekeeping gene | 402869 |
| 18s         | Housekeeping gene | Hs99999901_s1 |

aNP-specific markers. AThermo Fisher Scientific. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Biochemical analysis

Sulfated glycosaminoglycan (sGAG) production was analyzed from primary human NP cell cultures using the dimethyl-methylene blue spectrophotometric method as previously described in Gilchrist et al. (35). Medium overlay was collected from cultures, and corresponding gels were uniformly minced and placed in microcentrifuge tubes. Hydrogels without cells and cells alone were used as controls. All control and experimental groups were digested in papain solution (125 µg/ml in PBS with 5 mM EDTA and 5 mM cysteine) for 2 h at 65°C. sGAG was quantified from absorbance readings (535 nm) using a chondroitin-4-sulfate (MilliporeSigma) standard curve and corrected using the cell-free control. Total sGAG was determined by medium overlay plus cell digests and then normalized to total DNA content (Quant-iT PicoGreen dsDNA Kit; Thermo Fisher Scientific). Differences in sGAG and DNA (µg/µg) were detected with a 1-way ANOVA and Tukey’s post hoc test.

Inhibition of F actin and cytoskeletal tension

To test for YAP/TAZ and MRTF-A signaling independent of culture substrate, Lat B was used to drive changes in F actin and thus cell shape. Lat B is an inhibitor of F-actin polymerization and disrupts existing F-actin cytoskeletal networks (54). NP cells carrying the TEAD-luciferase construct, SRE-luciferase construct, or nontransduced NP cells were cultured upon LM-coated glass-chamber slides. After an overnight attachment period, cultures were treated with Lat B (1 µM; MilliporeSigma) for 30 min followed by exchange of medium with normal growth medium. Cultures were analyzed for transcriptional activation, gene expression, and actin coherency. To assess the role of cytoskeletal tension in modulation of YAP/TAZ and MRTF-A, the Rho-associated protein kinase (ROCK)–Rho GTPase inhibitor Y27632 (Y27; 10 µM; MilliporeSigma) was used to treat NP cells upon glass substrates. Primary human NP cells carrying the TEAD luciferase or SRE luciferase were cultured atop LM-coated glass and treated with Y27 for 24 and 48 h. Nontransduced cells were similarly cultured for immunofluorescent staining of nuclear-cytoplasmic quantification of coactivator localization and actin coherency.

RESULTS

Soft PEG-LM hydrogels promote phenotypic expression and radial actin

Soft and stiff PEG-LM hydrogels that were synthesized in accordance with previously published data (Fig. 1C and 2A) (51, 52) demonstrate similar effects on primary human NP cells. Cells cultured on top of soft PEG-LM substrates showed a distinct morphology with rounded, multicell aggregates that were more highly aligned in cells grown on LM-coated glass compared with those on soft PEG-LM and stiff PEG-LM, which display more radial actin (Fig. 2E).

Soft PEG-LM hydrogels promote phenotypic expression and radial actin
Soft PEG-LM hydrogels attenuate SRF and TEAD in primary human NP cells

Primary human NP cells containing an SRF-luciferase reporter construct demonstrated increased SRF transcriptional activity when cultured upon LM-coated glass after 24 and 48 h (Fig. 2F). Glass substrates also promote increased nuclear localization of its cofactor MRTF-A (Fig. 2G, H), suggesting soft PEG-LM is able to prevent cofactor translocation to keep SRF transcriptionally quiet.

In primary human NP cells transduced with the 8xGTIIC TEAD response element, cultures grown atop LM-coated glass and stiff PEG-LM show significantly increased TEAD transcriptional activity compared with those on soft PEG-LM (Fig. 2I). When stained for...
subcellular YAP/TAZ localization, NP cells on stiff PEG-LM and LM-coated glass show increased nuclear presence of YAP/TAZ staining intensity (Fig. 2J). This was further observed as distinct gaps in cofactor staining that matched the nuclear area in cells upon soft PEG-LM (Fig. 2K), indicating YAP/TAZ remains inactive within the cytosol.

YAP and MRTF-A knockdown have no effect on transactivation or phenotype

In highly efficient siRNA-mediated knockdown of MRTF-A and YAP in human NP cells (Fig. 3A), with no observed changes in cell shape, there were no differences in gene expression of the NP markers AGC, COL2, and GLUT1 (fold-change normalized to scramble control). SRF transcriptional activation does not change when MRTF or YAP are knocked down; a significant decrease in transactivation was observed with a double knockdown of both MRTF and YAP on LM-coated glass compared with the scramble control. TEAD transactivation is not affected by the absence of YAP or MRTF and although it did not reach significance, there was a slight downward trend on LM-coated glass in double-knockdown cells. Data shown from replicates for 3 human cell isolations and presented as means ± sd. *P < 0.05 [significant difference between siRNA knockdown cells and scramble control (Dunnet’s test)].

Figure 3. A) Fold-change normalized to the scramble control showing efficiency of siRNA knockdown of MRTF and YAP. B) YAP and MRTF knockdown do not promote changes in NP phenotype expression of AGC, COL2, and GLUT1 (fold-change normalized to scramble control). C) SRF transcriptional activation does not change when MRTF or YAP are knocked down; a significant decrease in transactivation was observed with a double knockdown of both MRTF and YAP on LM-coated glass compared with the scramble control. D) TEAD transactivation is not affected by the absence of YAP or MRTF and although it did not reach significance, there was a slight downward trend on LM-coated glass in double-knockdown cells. Data shown from replicates for 3 human cell isolations and presented as means ± sd.*P < 0.05 [significant difference between siRNA knockdown cells and scramble control (Dunnet’s test)].

ROCK-Rho inhibition does not attenuate YAP/TAZ–TEAD or MRTF-A–SRF signaling in primary human NP cells

Upon treatment of cells on LM-coated glass with the ROCK inhibitor Y27, there were no observed differences in SRF transactivation at either 24 or 48 h (Fig. 4A). This was further confirmed with MRTF-A nuclear colocalization (Fig. 4B, C), indicating it remains primarily nuclear throughout the treatment period and in the absence of ROCK-Rho signaling. This effect was also seen in TEAD transcriptional activity, which showed no significant difference compared with the vehicle (PBS) control (Fig. 4D). There was an increase in transactivation at 48 h compared with 24 h, but this difference was not significant (P > 0.05). YAP/TAZ coactivator localization was also predominantly nuclear (Fig. 4E, F), suggesting these pathways remain transcriptionally active in primary human NP cells even with inhibition of ROCK-Rho signaling. Of note, NP cell morphology did not change under these conditions and appears closely to what is observed on very stiff glass substrates with no statistical difference observed in F-actin alignment between the Y27 treatment group and vehicle control treatment group (Fig. 4G).
F-actin depolymerization promotes improved NP phenotype and inhibited transactivation

SRF reporter cells that were treated with Lat B cultured upon LM-coated glass (i.e., substrates shown to produce maximal transcriptional activation and fibroblast-like spreading in morphology) show a significant loss of SRF transcriptional activity (Fig. 5A). TEAD (8xGTIIC) reporter cells indicate a similar trend, with Lat B treatment inhibiting TEAD transcriptional activation (Fig. 5B). Changes in cell shape were also observed in these Lat B–treated cells with a more rounded, clustered morphology appearing on glass (Fig. 5C) and a significant decrease in F-actin alignment (Fig. 5D). These changes in cell shape...
and decreased transactivation occurred simultaneously with NP marker expression, where Lat B allowed for enhanced expression of AGC, COL2, and GLUT1 NP phenotypic markers (Fig. 5E).

**DISCUSSION**

The findings of this study suggest the importance of cell shape in regulating SRF and TEAD-mediated mechanotransduction that promotes characteristics of the healthy NP cell phenotype and bioactivity. Degenerate primary human NP cells transduced with transcriptional reporter sequences for SRF were shown to decrease activation of the transcription factor when cultured upon soft PEG-LM hydrogel substrates as compared with cells cultured on stiff PEG-LM and LM-coated glass, which show a relatively high basal level of SRF transactivation across time points. Degenerate primary human NP cells transduced with a TEAD transcriptional reporter displayed a similar trend with peak TEAD activation on LM-coated glass substrates and inhibition of TEAD activation upon soft PEG-LM. The dampened signaling observed on soft PEG-LM appeared concurrently with changes in cell shape, with more cortical actin appearing in rounded, multicell clusters, as opposed to the spread, fibrotic shape observed on LM-coated glass. Upon changes in morphology from circular to more flattened, the subcellular localization of transcriptional coactivators YAP/TAZ and MRTF-A also follows this trend by shuttling into the...
nucleus, where they bind to transcription factors. This suggests that upon soft PEG-LM when a rounded, multicell cluster morphology is present, human NP cells are able to sequester YAP/TAZ and MRTF-A within the cytosol, leaving TEAD and SRF, respectively, quiescent and inactive. Based on these findings, maximum activation of TEAD and SRF signaling in NP cells may be observed on very stiff substrates, which in this case is LM-coated glass.

siRNA constructs targeting YAP and MRTF-A, where no observable differences in NP cell morphology occurred, were unable to overcome this shape- and stiffness-induced activation of TEAD and SRF. These data suggest that YAP and MRTF-A do not regulate TEAD and SRF in the absence of changes in NP cell shape. This corresponded with a lack of NP marker expression. These novel findings demonstrating MRTF-A and YAP/TAZ translocation to the nucleus upon soft substrates suggest that NP cells in multicell clusters can inhibit MRTF-A and YAP/TAZ coactivation of SRF and TEAD, respectively, because of decreased actin turnover.

A surprising finding of this study was the inability of a targeted YAP and MRTF siRNA knockdown to elicit any effect on TEAD or SRF transactivation in the absence of changes in cell shape. Prior literature has linked YAP/TAZ to TEAD and MRTF-A to SRF (38, 41, 42, 57); however, no such studies have been conducted in primary NP cells. Much of this previous work, in particular for YAP/TAZ, has been focused in cancer cell lines. The NP, being of notochordal origin, thus represents a unique tissue and cell type to apply this mechanotransduction work and may suggest that these observations are cell type–dependent. Some recent work indicates that TEAD in fact contains some of the weaker transcription-factor binding motifs targeted by YAP, which may preferentially bind to other sites associated with different transcription factors, such as runt-related transcription factors 1 and 2 (RUNX1, RUNX2) and repressor element-1 silencing transcription factor (REST) (58). Conversely, multiple cofactors are known to control TEAD, including the vestigial-like (VGLL) family proteins (59–62), p160 (63), and activator protein 1 (AP-1) (64, 65).

It is also known that ternary complex factors (TCFs) compete with MRTFs for binding to SRF transcriptional sites (66), and SRF activation may require a combination of a cofactor complex, such as GATA proteins, C-reactive protein (CRP), and Nkx (67–69). This body of work clearly indicates that regulation of SRF and TEAD transcription factors is far more complex than arising from a single cofactor, and findings here further suggest that multiple factors control this response to changes in cell shape in a tissue- and cell-dependent manner. Further work must be completed to better understand the mechanisms governing cell shape–induced changes in transcripational activity and downstream gene expression and protein function outcomes.

Interestingly, a double knockdown of MRTF and YAP did show a small (and only significant for SRF) trend in decreased transactivation. This could indicate the ability of YAP to interact with MRTF to potentiate its effects (70–72) and suggests there may be some degree of crosstalk occurring between the pathways in human NP cells. In fact, several recent studies have begun to elucidate the complex and multilevel regulation of these pathways in other cell types (55, 56, 73). Previously, it was shown that not only do SRF and TEAD share similar target gene signatures, such as ankyrin repeat domain 1 (ANKRD1) and Cysteine-rich angiogenic inducer 61 (Cyr61), but the genes themselves also contain binding sites for both MRTF-SRF and YAP/TAZ–TEAD (74–76). The exact transcriptional mechanisms of MRTF-SRF and YAP/TAZ–TEAD crosstalk remain unclear, but early studies suggest MRTF-YAP binding may be a potent activator to achieve full YAP/TAZ–TEAD target gene activation and that the 2 pathways may act in coordination to respond to various extracellular stimuli to exert a context-dependent switch during mechanical or chemical signaling. Our work here indicates a similar trend was found with SRF and TEAD inactivation occurring in rounded, compact cells where 2 different methods of actin cytoskeletal disruption and remodeling (i.e., substrate stiffness and Lat B), but not with a single gene knockdown, were shown to promote changes in transcriptional activation that lead to similar downstream gene expression.

ROCK-Rho GTPases are known to sustain transcriptional activation of both TEAD and SRF through their ability to promote actin polymerization and thus potentiate cytoskeletal tension and nuclear shuttling of YAP/TAZ and MRTF-A (37, 41, 74). Earlier studies have demonstrated the use of ROCK inhibitor Y27 to reduce MRTF–SRF signaling and decrease transactivation in mesenchymal stem cells and YAP/TAZ nuclear localization (41, 77). However, results here show ROCK inhibition has no effect on YAP/TAZ or MRTF-A translocation and activation of their respective transcription factors. Importantly, NP cell shape and morphology was not altered with ROCK inhibition. This may be because of the state of these aged, pathologic cells cultured on very stiff glass that have formed a spread, fibroblast-like shape where morphology and the actin cytoskeleton cannot be diminished by ROCK inhibition. Indeed, a prior study in juvenile porcine NP cells treated with Y27 indicated cells were unable to form clusters on a soft substrate, a hallmark of healthy NP cells, and maintained a fibrotic morphology with strong F-actin staining on a stiff substrate (78). This study also indicated that ROCK inhibition prevented a juvenile phenotype, as observed through decreased matrix (sGAG) production and presence of NP markers (e.g., AGC, COL2, and brachyury). This may further suggest that the inability of ROCK inhibition to overcome cell shape and morphology sustains YAP/TAZ and MRTF-A activation in NP cells, which ultimately leads to a loss of phenotype and bioactivity.

In this study, it was determined that blocking MRTF-A coactivation of SRF and YAP/TAZ coactivation of TEAD by subjecting human NP cells to soft PEG-LM substrates and thereby forcing less aligned F-actin and a more rounded, clustered morphology, can promote NP-specific marker expression and elevated biosynthesis for adult human NP cells. Given the lack of regulation of TEAD and SRF by YAP and MRTF-A alone and in the absence of ROCK signaling, we chose to expand upon the observed lack of change in cell shape and used Lat B, which targets and disrupts polymerized F-actin to manipulate these pathways in a cell shape–dependent manner. Indeed,
addition of Lat B to NP cells cultured atop LM-coated glass, which produces maximal activation of SRF and TEAD, resulting in a spread, fibroblastic-like cell shape, produced a robust morphologic change with a significant decrease in F-actin alignment and a return to the more rounded, multicell clusters closer to that observed on soft PEG-LM. This change in cell shape promoted a decrease in SRF and TEAD transcriptional activity. Furthermore, NP phenotype following Lat B–induced attenuation of SRF and TEAD suggests that human NP cells, even in those with maximum activation of TEAD and SRF on glass substrates, can recover expression of NP-specific markers and bioactivity when SRF and TEAD are inactivated because of an actin cytoskeleton-induced change in cell shape.

Although substrate stiffness effects on TEAD and SRF signaling have been previously shown in other cell types (38, 40, 41, 58, 76, 79–82), a novel finding of this study was their role in primary human NP cells. Published studies of NP cells cultured atop soft and stiff PEG-LM– (51, 52) and LM-rich substrates (15, 35) have demonstrated consistent changes in morphologic and cytoskeletal observations where NP cells become more flattened and fibroblast-like on stiff substrates or in the absence of LM (23, 83). Soft and stiff hydrogels, which mimic healthy and pathologic tissue, respectively, were used in this study because there does not appear to be an intermediate stiffness effect. Previously published studies suggest NP cells behave similarly on 100 and 210 Pa as soft and correspondingly alike as stiff on 720 and 15,200 Pa, which may suggest an NP-specific effect, because we have previously shown that intermediate stiffness of ~8000 Pa LM-functionalized PEG hydrogel showed no difference in metabolite concentration compared with a soft (~300 Pa) hydrogel, and appeared more dependent on ligand presentation (35, 52). Indicative of dedifferentiation of NP cells, the transition to a more fibroblastic state in NP cells upon stiff substrates is also closely associated with a loss of NP cellularity and biosynthetic activity. This is believed to replicate the observed loss of phenotypic markers with age and degeneration as the NP tissue itself becomes stiffened, less hydrated, and loses its ability to withstand mechanical demands (84). Data from this study suggest these changes in SRF and TEAD signaling are driven by these cellular changes and the shift to a more flattened, fibroblast-associated cell shape. This concept may indeed be further supported by the finding here of highly aligned actin fiber organization in NP cells on LM-coated glass, whereas those on soft PEG-LM or in the presence of the F-actin inhibitor Lat B display more radial actin, which appears to drive changes in morphology from spread and flattened to one that is more round and compact, respectively. This is consistent with previously published data showing that NP cell roundedness is associated with functional CDH2 interactions, and that suppressing such interactions causes loss of biosynthetic ability and phenotypic marker expression (51). Just as that study demonstrated the presence of CDH2+ rounded cell clusters only appear when matrix synthesis and gene expression are at levels equivalent to healthy, juvenile NP cells, the present work indicates that soft substrate–induced changes in NP cell morphology are required for TEAD and SRF inactivation and also appear in parallel with increased phenotype expression and sGAG production. It has also been shown that inhibition of ROCK–RhoA signaling (which is known to occur with both YAP/TAZ and MRTF-A translocation) with the ROCK inhibitor Y27 is sufficient to disrupt multicell rounded, cluster morphology in immature porcine NP cells and was observed along with loss of phenotype and biosynthesis (78). This further suggests NP cell shape and roundedness are necessary to attenuate specific mechanosignaling events in NP cells that regulate phenotype and bioactivity.

Although integrins and other molecules that modulate matrix interactions are not the subject of the current study, they represent a possible regulating mechanism. Prior studies have suggested that integrins mediate NP cell interactions through a combination of known integrin interactions (e.g., α3, β1, and α5) (21, 22, 85–87). These prior integrin-blocking studies did not investigate the role of SRF or TEAD, although results from this study may at least be partly caused by interactions with a combination of such LM peptides being presented via PEG-LM hydrogels and coatings. These interactions may be necessary to drive changes in NP cell shape and thus changes in SRF and TEAD activation, although more work is needed to examine this potential role.

Overall, the findings from this study demonstrate that rounded, clustered morphology leads to an attenuation of TEAD and SRF in primary human NP cells with radial actin organization, increased NP-specific marker expression, and enhanced biosynthetic activity. Separate methods to disrupt actin cytoskeletal organization and signaling were utilized to consider the multilevel control exerted on TEAD- and SRF-signaling pathways. Transcriptional luciferase reporters, qPCR, and immunofluorescence suggest Lat B–induced inactivation of TEAD and SRF yields similar results as soft PEG-LM substrates. During degeneration and aging, the NP tissue becomes stiffened and loses hydration and disc height. Thus, soft and stiff PEG-LM hydrogels are designed to mimick this transition that NP cells undergo in vivo. But when a targeted knockdown of these transcriptional cofactors independent of PEG-LM hydrogels was employed, we observed an inability to regulate TEAD and SRF and to recover healthy NP gene expression without cells returning to a rounded, clustered morphology. Accordingly, nonstiffness-regulated attenuation of SRF and TEAD via F-actin inhibition with Lat B is able to overcome the substrate-mediated differences observed in NP cell shape and thus phenotype. However, in NP cells where ROCK (i.e., tension) was inhibited, there was no change in SRF and TEAD, and importantly, no difference in cell morphology where cells behaved as they do on very stiff substrates as spread, fibroblast-like cells. Because of the changes observed in actin orientation and morphology with TEAD and SRF inactivation, the cytoskeletal organization in clustered cells versus flattened cells may be a key mechanism regulating healthy biosynthetic and phenotypic expression patterns and may play a role in promoting the regeneration of pathologic NP cells. Given the known changes in NP tissue stiffening that occur with...
aging and degeneration; this may indicate an initiating event in which changes in the tissue lead to changes in NP cell shape, promoting a self-sustaining loop pattern during progressive worsening of IVD during degeneration and aging. The results of this study indicate that inhibition of F-actin polymerization, retention of a rounded, clustered morphology, and subsequent transcriptional inactivation of SRF and TEAD are involved in maintaining the healthy, immature NP cell phenotype.

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AUTHOR CONTRIBUTIONS

B. V. Fearing designed and performed research, analyzed data, and wrote the paper; L. Jing, M. N. Barcellona, and S. E. Witte performed research and analyzed data; A. Pathak contributed to data collection; L. A. Setton contributed to data analysis and interpretation; J. M. E. Witte performed research and analyzed data, and wrote the paper; L. Jing, M. N. Barcellona, and S. E. Witte contributed to data collection; L. A. Setton contributed to the study conception and design and data interpretation; and all authors were involved in written revisions to the manuscript and approval of the final version for publication.

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