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Softening Substrates Promote Chondrocytes Phenotype via RhoA/ROCK Pathway

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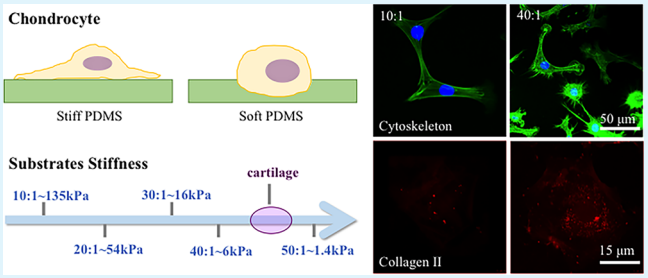
Supporting Information

ABSTRACT: Due to its evascular, aneural, and alymphatic conditions, articular cartilage shows extremely poor regenerative ability. Thus, directing chondrocyte toward a desired location and function by utilizing the mechanical cues of biomaterials is a promising approach for effective tissue regeneration. However, chondrocytes cultured on Petri dish will lose their typical phenotype which may lead to compromised results. Therefore, we fabricated polydimethylsiloxane (PDMS) materials with various stiffness as culture substrates. Cell morphology and focal adhesion of chondrocytes displayed significant changes. The cytoskeletal tension of the adherent cells observed by average myosin IIA fluorescent intensity increased as stiffness of the underlying substrates decreased, consistent with the alteration of chondrocyte phenotype in our study. Immunofluorescent images and q-PCR results revealed that chondrocyte cultured on soft substrates showed better chondrocyte functionalization by more type II collagen and aggrecan expression, related to the lowest mRNA level of Rac-1, RhoA, ROCK-1, and ROCK-2. Taken together, this work not only points out that matrix elasticity can regulate chondrocyte functionalization via RhoA/ROCK pathway, but also provides new prospect for biomechanical control of cell behavior in cell-based cartilage regeneration.



KEYWORDS: *chondrocytes, elastic substrates, phenotype, cytoskeleton, RhoA/ROCK pathway*

1. INTRODUCTION

Evascular, aneural, and alymphatic conditions, the most typical features of articular cartilage, may result in its extremely poor regenerative ability.[1] Therefore, cell-based tissue engineering either encapsulation of stem cells or chondrocytes, the sole cell type in cartilage, has been an attractive and effective alternative for the treatment of cartilage defect.[2,3] However, to govern stem cell fate determination is of great challenge due to wide aspects and serious obstacles involved.[4] Whereas chondrocytes-based tissue engineering dominates major advantages due to its specific function in the physiological state. Recently, biophysical properties, in spite of biochemical factors, revealed by following researches, were confirmed to play a critical role in the regulation of cell biology.[5−8] Accumulating evidence indicated that substrate elasticity or stiffness mimicking the microenvironment of different tissues, including neurons, adipose tissue, cartilage, and cancellous bone, being one of the hallmarks of biomechanics, were capable of influencing the differentiation of mesenchymal stem cells into various tissue cell end points[9] as well as the phenotype and functionalization of terminal cell types.[10] Furthermore, the recovery of chondrogenic phenotype can be achieved by restoring chondrocytes to their naturally round and three-dimensional morphologies via being encapsulated in a tuning elastic hydrogel and consequent rearrangement of F-actin cytoskeleton.[11] Fortunately, this result perfectly explains and solves the problem that chondrocytes will lose their typical phenotype and function which may lead to compromised results in cartilage tissue engineering during the monolayer expansion process *in vitro*.[12] Therefore, to soften substrates or even confirm the proper stiffness of biomaterials that mimic cartilage tissue microenvironments and further figure out its potential mechanism to facilitate stable phenotype and definite functionalization of chondrocytes in the application of regenerative medicine is of great importance as well as challenge.

Extracellular matrix (ECM), composed by different functional proteins, holds its specific biophysical and biochemical properties according to cell types and locations, and is reported to have a strong influence on cell biological behavior.[4] In the presence of soluble factors TGF-β1 or IL-1β, the response of chondrocytes cultured on various stiffness polyacrylamide gels differs. Synthesis of type II collagen and aggrecan, two prominent proteins of extracellular matrix, alters as the substrates stiffness increases from 1 to 90 kPa.[13] Further research demonstrated the elasticity of cartilage ECM itself also had been shown to regulate chondrocyte fate. Tensile modulus of nanofibrous scaffolds was noted to impact cell densities and the expression of chondrogenic markers, especially aggrecan.[14]Additionally,the increasing stiffness of cross-link chitosan also seemed to serve as an additional mechanical stimulus to promote chondrocyte proliferation.[15]

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Nevertheless, the mechanism of how ECM stiffness modulates chondrocytes behavior is still poorly understood.

To address this question, the key point is to figure out how living cells respond to the mechanical stimulations. Responding to physical stimulation, cells directly apply force to their surrounding ECM and then convert the stimuli into biological signals to intracellular and thus alter their cytoskeletons correspondingly. This process allows living cells to adjust themselves rapidly to accommodate the surrounding microenvironment. A recent study has shown chondrocytes cultured on 2D substrates surface or encapsulated in 3D hydrogels, displayed a better preservation of its morphology in response to stiff material.[16] However, the function of chondrocytes and the potential molecular regulative mechanism remain uncertain. Therefore, our study aims to figure it out via investigating changes of cell morphologies as well as potential pathways in chondrocytes cultured on various elastic substrates. In this study, varying elastomeric PDMS substrates coated by dopamine were designed to hold subcultured chondrocytes with stable adhesion. On soft substrate, chondrocytes displayed round morphology and remodeled their cytoskeleton to resemble *in situ* chondrocytes and expressed more type II collagen than those on stiff substrates. This technique suggested that pure elastic constraints can induce chondrogenic phenotype restoration on a 2D surface. Our data also provides a new insight into the way to precisely control microenvironment surrounding chondrocytes and therefore to unify the redifferentiation level of individual cell for cell-based cartilage regenerative strategies.

2. MATERIALS AND METHODS

2.1. Fabrication of the Various Elastic PDMS Substrates. Preweighed liquid oligomeric base and varying content of Sylgard184 (Corning, NY, USA) with different proportions (Sylgard184/oligomeric base =1:10, 1:20, 1:30, 1:40, and 1:50) were first mixed under stirring. Then, 1 ml mixture was cast on a single-well Petri dish (35 × 10 mm, Corning) and cross-linked in an oven at 60 °C for 24 h. Later, to promote cell adhesion, a self-polymerization of dopamine hydrochloride (BioKem) on PDMS surface was prepared by being socked in the tris(hydroxymethyl) aminomethane (Adamas) and dopamine solution (pH 8) for 24 h, twice.

2.2. Surface and Physical Characterization of PDMS Substrates. To avoid the topographic effect of PDMS substrates on cell behavior, we performed surface roughness using the tapping mode of the atomic force microscope (AFM, SPM5000, Benyuan, China). In the preparation process, PDMS samples were performed in 10 mm × 10 mm × 2 mm size. All of the samples were repeated five times (Figure S1). The elasticity of all substrates were measured by Universal Testing Machine and stiffness for each was evaluated by the following equation:

$$K = EA/L$$

where K is stiffness, E is Young modulus, A is cross sectional area (cm^2), and L is height.

In our study, the cross sectional area was 1 cm^2, and height of the sample was 0.8 cm.

2.3. Cell Culture. Primary articular chondrocytes were obtained from newborn rats (1−3 days old). After rats were sacrificed and sterilized, knee joint surface was exposed and the epidermis was stripped. Then the collected knee joints were minced into small pieces and trypsinized for 30 min in 0.25% protease solution dissolved in Dulbecco's modified eagle medium (high-glucose DMEM, 0.1 mM non essential amino acids, 4 mM L-glutamine, 1% penicillin-streptomycin solution, Hyclone, Logan, UT, USA), and then the solution was replaced by 0.5% type II collagenase for 3 h. After that, fresh 10% heat-activated fetal bovine serum (FBS) DMEM by 1:1 (v/v) was added to cease the trypsinization reaction and centrifuged at 179 × g for 5 min. After removing the supernatant, fully supplemented medium consisting of 10% FBS and 1% penicillin-streptomycin was mixed with the cells and tissues and then seeded into plates under the standard humidified atmosphere of 5% CO_2 at 37 °C. When fusing to 80−90%, chondrocytes were plated on different elastic substrates after being presterilized by ultraviolet light for 1h.

2.4. Scanning Electron Microscope (SEM). Cell adhesion and substrate surface were observed by SEM. After 24 h incubation, cell culture medium was removed and replaced by 2.5% glutaraldehyde for fixing overnight. Then chondrocytes were dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90%, 95%, and 100%) for 15 min at each level. The specimens were then dried in an exhaust hood at room temperature. The processed specimens were mounted on specimen holders, coated with a thin layer of gold, and then examined by SEM.

2.5. Immunofluorescence and Confocal Laser Scanning Microscope (CLSM). After 3 days incubation, chondrocytes on different elastic substrates were washed three times and fixed by 4% cold paraformaldehyde for 15 min, followed by three rinses with PBS. Then chondrocytes were permeabilized by 0.5% Triton X-100 for 10 min, washed with PBS, and then blocked by 5% sheep serum at room temperature for 1 h. After being rinsed 3 times for 15 min each, cells were incubated with respective antibodies of vinculin (mouse monoclonal, diluted 1:200, Abcam, ab18058), RhoA (rabbit monoclonal, diluted 1:150, Abcam, ab187027), nonmuscle myosin IIA (rabbit polyclonal, diluted 1:200, Abcam, ab24762), and type II collagen (mouse monoclonal, diluted 1:150, Abcam, ab185430) overnight at 4 °C. After being rinsed in PBS, samples were immunolabeled with the fluorescent secondary antibodies at a concentration of 1:500 for 1 h at 37 °C: Alexa Fluor 594 donkey antirabbit (A21207, Invitrogen, CA, USA) and Alexa Fluor 594 donkey antimouse (A21203,Invitrogen, CA, USA). Then cells were stained with 50 μg/mL fluorescent Phalloidin (Sigma-Aldrich, St. Louis, MO, USA) conjugate solution in PBS at room temperature for 1 h, and counterstained with 10ug/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, then visualized with CLSM (Leica TCS SP8 Germany).

2.6. Quantitative Real-Time PCR. Briefly, chondrocyte mRNA was collected at Day 3 and purified using the RNeasy Plus Mini Kit (Qiagen, Shanghai, China) and genomic DNA eliminator. The isolated mRNA was dissolved in RNase-free water and quantified by measuring the absorbance at 260 nm with a spectrophotometer. Then cDNA was prepared in a final volume of 20 μL using a synthesis kit (Mbi). The gene expression levels of Aggrecan, type II collagen (COL II), rasrelatedC3 botulinum toxin substrate 1 (Rac-1), ras homologue family member A (RhoA), Rho associated coiled coil containing protein kinase 1(ROCK-1), and Rho associated coiled coil containing protein kinase 2 (ROCK-2) (sequences of forward and reverse primers of mRNA were shown in Table 1) in chondrocytes were performed in a final 20 μL volume containing 2 μL cDNA by using PrimeScript RT-PCR Kit (Takara, Tokyo, Japan) and ABI 7300 (Applied Biosystems, Shanghai, China). RT-PCR amplification process was as follows: Denaturation for 30 s at 94 °C, followed by 40 cycles, consisting 5 s at 94 °C and 34 s at 60

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Table 1. Sequences of Forward and Reverse Primers of Selected Genes Designed for q-PCR

mRNA	product length	primer pairs	
GAPDH	233 bp	forward	ACAGCAACAGGGTGGTGGAC
		reverse	TTTGAGGGTGCAGCGAACTT
COL II	116 bp	forward	TCAAGTCGCTGAACAACCAG
		reverse	G TCTCCGCTCTTCCACTCTG
Aggrecan	137 bp	forward	GCAGCACAGACACTTCAGGA
		reverse	CCCACTTTCTACAGGCAAGC
Rac-1	200 bp	forward	GAGAGTACATCCCCACCGTC
		reverse	AACACGTCTGTTTGCGGGTA
RhoA	132 bp	forward	AACAGGATTGGCGCTTTTGGGAT
		reverse	GAGGCACCCCGACTTTT
ROCK-1	108 bp	forward	TTGGTTGGGACGTACAGTAAAA
		reverse	CGTAAGGAAGGCACAAATGAGA
ROCK-2	135 bp	forward	GACATTGAACAGCTTCGGTCGGA
		reverse	CATTGAACAGCTTCGGTCG

°C. For each reaction, a melting curve was generated to test the primer dimmer formation and false priming.

2.7. Statistic Analysis. All experiments were performed at least three separate times. Mean data of each group collected from RT-PCR was first quantified to GAPDH, and then normalized to data of 10:1 group. All statistical analysis was performed with SPSS 21.0 using one-way ANOVA. Data were considered significantly different if the two-tailed p value was <0.05.

3. RESULTS AND DISCUSSION

3.1. Mechanical Properties of Tunable Stiffness Substrates. For cartilage tissue engineering, chondrocytes play a critical role in the determination of regenerative quality. A consisting view has been set that on traditional culture dishes with the stiffness of gigapascal range, chondrocyte loses its chondrogenic phenotype during the expansion process,[17] indicating that substrate stiffness should be considered seriously for better functionalization of chondrocytes. To figure out the proper stiffness for chondrocytes living and to investigate the subsequent response to this physical stimulation, we fabricated tunable elasticity polydimethylsiloxane (PDMS) substrates to explore the potential mechanosensing mechanism for the maintenance of chondrocytes phenotype. Here, PDMS substrates with varying stiffness falling within the range reported for articular cartilage were fabricated via thermal cross-link with different proportions of monomer and initiator, respectively equaling to volume ratio of 10:1, 20:1, 30:1, 40:1, and 50:1 (v/v). Then physical properties of the well cross-linked PDMS substrates were tested. Stress-train curve obtained by universal testing machine demonstrated that with the decreasing proportion of initiator greater deformation of substrates were observed in response to the same applied stress (Figure 1a). Stiffness of different substrates with proportions from 10:1 to 50:1 showed a gradual decreased trend, consisting with Young modulus evaluation (Figure 1b,c).

The successful fabrication of the tuning stiffness substrates laid the foundation for the following investigation. For cell adhesion, PDMS was refined with dopamine solution treatment twice to attain a hydrophilic surface. The water contact angle of material surface was altered from 110° to 65°, giving its optimum for cell attachment (Table S1). Studies focused on the interactions between integrins, and extracellular matrix composition

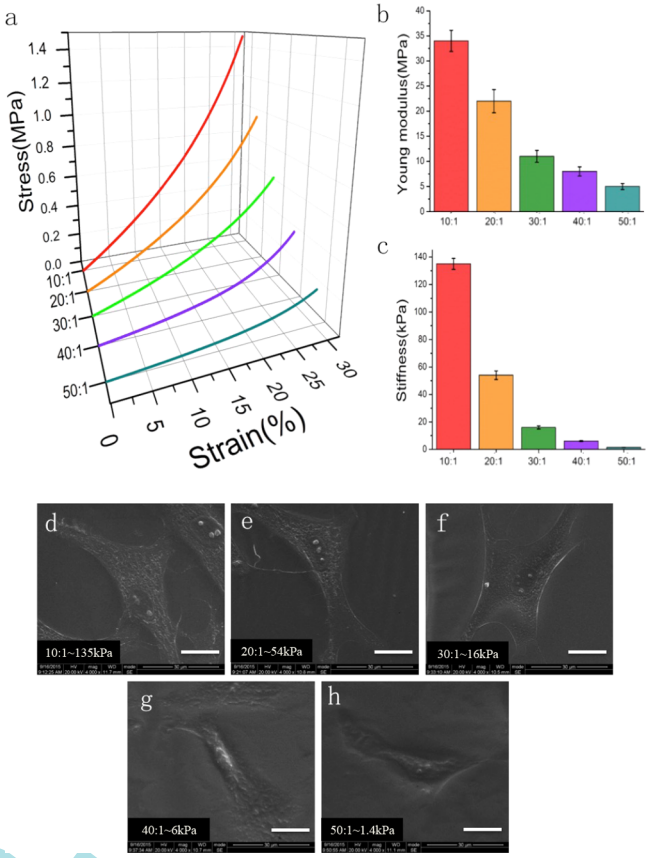

Figure 1. Mechanical properties of tunable elastic substrates. Stress−strain curve (a), Young modulus (b), and stiffness (c) of PDMS substrates tested by universal testing machine, three different samples as indicated. Scanning electron micrographs of chondrocytes plated on PDMS of the indicated stiffness (d−h). Scale bars are 15 μm.

demonstrated different ECM proteins had a strong effect on cell behavior, including cell proliferation and differentiation.[18,19] Based on its perfect hydrophilic properties, here we chose dopamine solution instead of other ECM proteins. Besides stiffness, other physical properties like topography contribute to the alteration of its overlying cells.[20,21] Therefore, surface roughness was also observed by AFM. Results showed that substrates with stiffness from 135 kPa, 54 kPa, 16 kPa, 6 kPa to 1.4 kPa were performed with roughness from 55.67 nm, 53.38 nm, 50.95 nm, 47.32 nm to 42.50 nm, respectively (Figure S1). Statistical differences were observed between every two samples (p < 0.05). However, previous studies indicated that no differences of cell behavior between those cultured on substrates with surface roughness of 45 and 21 nm or 80 nm. Differences of height in substrates nanotopography less than 20 nm had little effect on cell mechanosensitivity.[22,23] Then chondrocytes isolated from articular cartilage were plated onto the well-established materials. Dramatical alterations of chondrocyte morphology were observed after 1 day incubation. On stiff materials (10:1−20:1), cells spread more widely with a thinner layer, while on soft ones (40:1−50:1), chondrocytes scrunched and partly sank into the material resulting in a thicker layer, consisting of the typical cell shape of native chondrocyte. It can be explained that chondrocytes with a comparative round, thick, and more stereoscopic shape lie in the cartilage lacunae and arrange in clusters paralleling to the axis of stress transmission to perform better. Therefore, in our study, soft substrates (40:1−

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50:1) seem to be more suitable for the maintenance of chondrocyte morphology (Figure 1d–h).

3.2. Cell Morphology and Cytoskeleton Organization Are Controlled by Tuning Stiffness Substrates. The well-established elastic materials with dopamine coating allowed stable cell adhesion to explore cell morphology changes of chondrocytes response to the intrinsic physical property of the underlying substrates. In our study, phalloidin staining for cell cytoskeleton indicated apparent differences of F-actin distribution on varying stiffness matrix. On rigid substrates (10:1–20:1), chondrocytes displayed wide spreads and polygonal shapes with prominent, highly organized paralleled actin fibers. Chondrocytes on a compromised one (30:1) led to anomalous polygonal shape along with comparative fuzzy cytoskeleton. While on soft substrates (40:1–50:1), the vast majority of chondrocytes yield remarkably small and round shapes with disorganized actin filaments and sectional extensions, probably resulting from the contract force against the dramatically soft materials (Figure 2a).

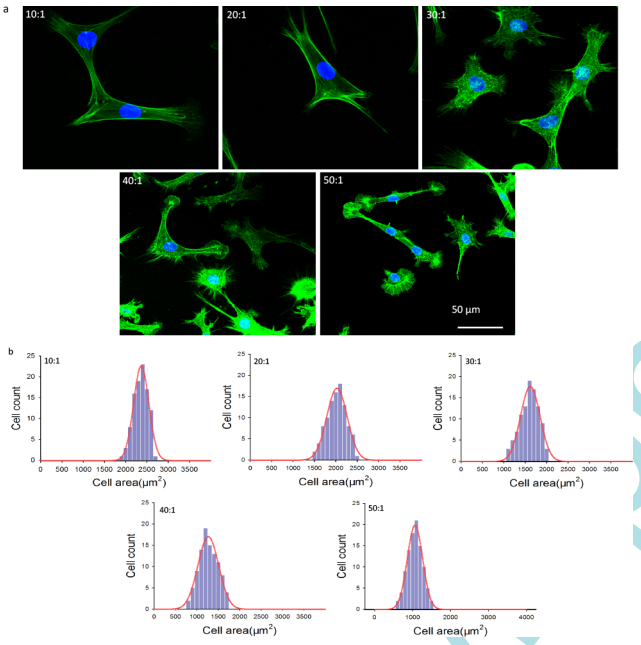

Figure 2. Quantitative analysis of cell morphology of chondrocytes on elastically tunable substrates. (a) Fluorescent images of cytoskeleton in chondrocytes (phalloidin staining of F-actin) on substrates with various stiffness (10:1, 20:1, 30:1, 40:1, 50:1, stiffness respectively equal to 135 kPa, 54 kPa, 16 kPa, 6 kPa, 1.4 kPa). Scale bars are 50 μm. (b) Distributions of cell area for chondrocytes plated on PDMS substrates with different stiffness.

In the mechanosensing process, a dynamic balance will be achieved between the contraction tension of cells and applied force of extracellular matrix. On elastic material, cells will contract their actin fibers to form force on ECM. When the resistance of a cell is equal to the applied force by the deformation of ECM, which is directly affected by the material stiffness, stable morphology will be maintained. In our study, chondrocytes preferred round morphologies on 40:1 substrates with stiffness of 6 kPa, which is equal to that of cartilage *in vivo*. Consistent with previous studies, analyses of cell areas demonstrated that substrate stiffness had a positive correlation to cell spreading[24] (Figure 2b). Additionally, cytoskeleton is composed of actin microfilaments, tubulin microtubules, and vimentin intermediate filaments. Disruption of any filaments will definitely affect the mechanosensitivity of chondrocytes[25] as well as the stabilization of cell phenotype and functionalization.

Interestingly, we found the formation of lamellipodio-like structures in leading edge of chondrocytes cultured on soft matrix. Lamellipodia are believed to be a characteristic feature of motile cells, which act as initiators to pull cells forward in their migration process. Down-regulation of RhoA activity and its downstream ROCK-MLC2 (myosin light chain 2) signaling pathway promoted lamellipodia formation thus inducing normal or tumor cells migration via Smurf1 modulation,[26,27] which may explain our results. However, studies focused on cancer aggressiveness indicated the extracellular matrix rigidity promoted the formation and activation of invadopodia via myosin II-FAK/Cas pathway.[28,29] Therefore, to point out the detailed mechanism of lamellipodia formation in chondrocytes on soft PDMS substrates, further investigation including chondrocyte migration and its relationship to cell phenotype and relevant matrix stiffness should be performed.

3.3. Total Area and Numbers of Focal Adhesion Differ as the Substrate Stiffness Alters. After observing cell morphology alteration in response to elastic substrates, a question of how physical stimulus affects and consequent signals transmit into intracellular to make cells adjusted to surrounding microenvironment has been raised. First, sensing physical stimulations, cytoplasmic tails of trans-membrane proteins integrin cluster, and contact to the adhesion structures called focal adhesions (FAs), composed of vinculin, talin, paxillin, and α-actin, the direct mechanical linkers to cytoskeleton.[30] Vinculin, being an essential member of focal adhesions, will reorganize and further influence the cytoskeleton structure to mediate cell biology, including cell attachment, spreading, migration, differentiation as well as stabilization of cell morphology.[31] Therefore, our study focused on the expression of vinculin in chondrocytes after being plated on different stiffness matrices. Immunostaining image of vinculin on stiff substrates (10:1–20:1) clearly showed widespreading along cell margins leading to a broader area per cell (Figure 3a, b), in accordance to morphology alteration of chondrocytes shown in Figure 2. More interestingly, vinculin was rearranged parallel with the axial of cytoskeleton on stiff PDMS

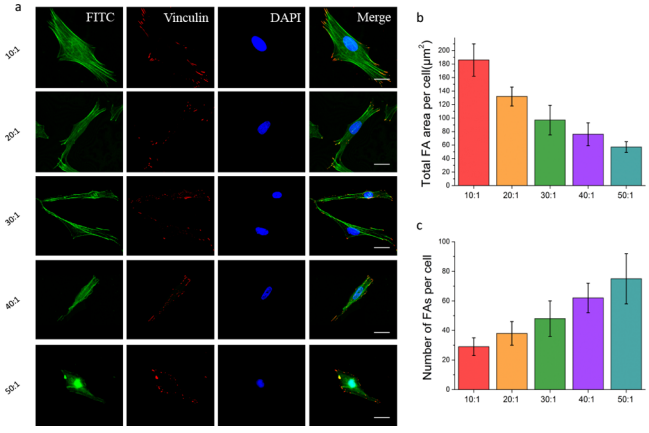

Figure 3. Total area and numbers of focal adhesions differ as the substrate elasticity alters. (a) Immunofluorescent images of vinculin in chondrocytes plated on different elastic substrates (from 10:1 to 50:1, stiffness respectively equal to 135 kPa to 1.4 kPa). Scale bars are 10 μm. (b,c) Total FA area (b) and total number of FA sites (c) per chondrocyte cultured on different elastic PDMS, for three different samples as indicated.

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(Figure 3a), which may be explained by under greater mechanical stimuli, binding of vinculin will be promoted via the higher tension applied to talin, a protein that links integrins to cell cytoskeleton, suggesting the importance of talin−vinculin system for force transduction.[32] Additionally, the interaction between vinculin and α-actinin leads to the bonding of integrin with the actin filaments cytoskeleton, further enhancing the relationship between focal adhesion and cytoskeleton.[33] As the substrate stiffness decreases (40:1−50:1), smaller and denser splattering distribution of vinculin was observed resulting in the numbers of vinculin per cell area increasing (Figure 3c). Along with the analysis of cell spreading area and the FAs, a strong correlation between cell spreading and focal adhesions can also be revealed, despite substrate stiffness.[34]

3.4. Distribution of Nonmuscle Myosin IIA in Chondrocytes Differs on the Varying Elastic Substrates. Immunofluorescent staining of nonmuscle myosin IIA, the candidate of acknowledged and potent cytoskeletal motors that is responsible for the regulation of cytoskeleton tension, was performed for chondrocytes adherent to varying elastic substrates. On rigid materials (10:1−20:1), myosin IIA was expressed homogeneous in chondrocytes. However, soft substrates induced heterogeneous expression of myosin IIA-intracellular expression slightly lower with substrate stiffness decreasing, whereas, a higher degree of myosin IIA along cell margins was observed on soft ones (40:1−50:1) (Figure 4), probably resulting from the asymmetrical force with a higher level at the periphery circle of cell−substrates interaction. This phenomenon may be explained by the study of geometric cues for stem cell differentiation, in which fluorescent heat-maps of stained myosin II in star shape showed higher cell contractility via denser expression at the edges that overlap in adhesive regions with lower vinculin expression, resulting in osteogenic differentiation.[35] Hence, a stable and proper high level of contractility is extremely important and the precondition for stress-bear cells in physiological condition.

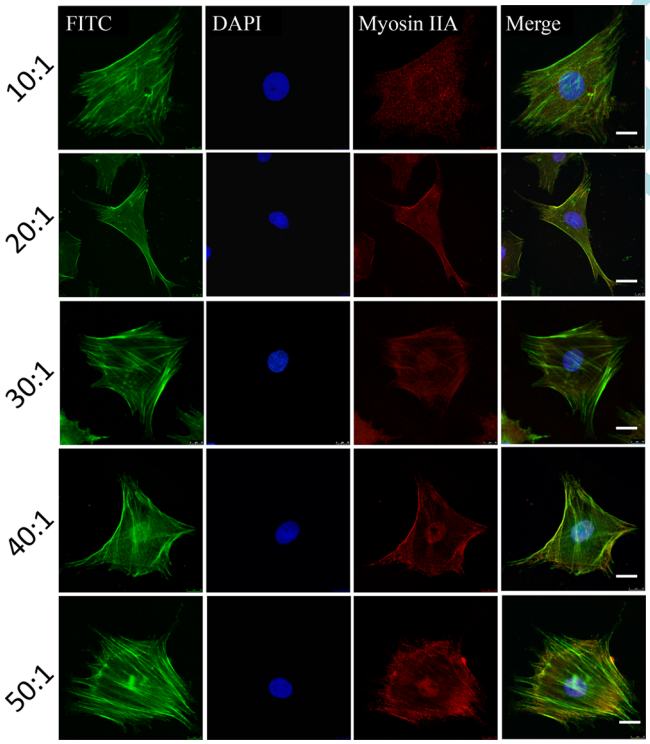

Figure 4. Distribution of nonmuscle myosin IIA expression in chondrocytes differs on the varying elastic substrates. Immunostaining images of nonmuscle myosin IIA for chondrocytes plated on PDMS substrates with different stiffness (from 10:1 to 50:1, stiffness respectively equal to 135 kPa to 1.4 kPa). Myosin IIA (red), cytoskeleton (green), nucleus (blue). Scale bars are 10 μm.

3.5. Softening materials induce more type II Collagen expression of chondrocytes. For different types of living cells, cell morphology is a critical factor to their function. For the maximal lipid storage in adipose tissue, round and spherical shapes are required for adipocytes. Also, broad spreading area allows osteoblasts to deposit more mineralized matrix in bone remodeling process.[36] Therefore, we also investigated specific proteins for chondrocytes in different stiffness groups. As a terminal type of cell, chondrocytes have a specific function in cartilage formation and bear a lot from the superficial to the deep zone of articular joint. Type II collagen, secreted by chondrocytes as the fundamental component of surrounding matrix, is usually regarded as the basic standard for the diagnosis of its phenotype and function. As supposed from above data, immunofluorescent images showed different expressions of type II collagen on various substrates (Figure 5a). As substrate stiffness decreased, elevated expression of type II collagen was observed. To confirm, we also investigated the gene expression of chondrocytes markers. On the third day, gene expression of aggrecan and type II collagen showed an obvious increase and the strongest expression was observed on 40:1 substrate (Figure 5b,c).

3.6. RhoA/ROCK Pathway May Regulate the Mechanotransduction of Chondrocytes Cultured on Various Stiffness Substrates. Here, we had found that cell morphology of chondrocytes had been better maintained as well as more type II collagen was expressed on 40:1 substrates. Next we investigate the potential signal pathway involved in a cell-matrix mechanotransduction process. RhoA, a small GTPase protein of Rho family, has been studied as the prominent molecular mediator in cytoskeleton regulation as well as actomyosin contractility.[37−40] In our study, immunofluorescent staining of RhoA displayed a more potent and homogeneous expression on stiff substrates (10:1−20:1), while with the substrates became softer, dramatically decreased expression that existed especially on 40:1 substrate (Figure 6a). On stiff materials, mechanical signals were transmitted via integrin and its increased recruited receptors involving vinculin to activate the downstream cascade like RhoA/ROCK pathway. For further confirmation, we also assessed mRNA levels of Rac-1, RhoA, ROCK-1, and ROCK-2. Interestingly, we found the lowest gene expression also showed on the 40:1 group (Figure 6b−e), consistent with the group having the strongest expression of type II collagen and aggrecan, which can be explained as RhoA/ROCK signaling suppressed chondrogenesis.[41] Together with previous studies on the relationship between chondrocyte phenotype and cytoskeleton,[41−43] our study indicated substrate with stiffness of 6 kPa facilitated round and proper morphology with more type II collagen expression for phenotypic maintenance of chondrocytes via Rho/ROCK signaling pathway.

As chondrocytes adhere to the surface of underlying materials and sense different stiffness stimulations, cells apply pulling force through its focal adhesions and then send mechanotransduction signals intracellular to make adjustments of actin fiber bundling and stress fiber contractility. The contractility of actin-myosin system and its downstream RhoA established a feedback loop. As greater stimuli exist, cells display high cytoskeleton tension by more myosin IIA generation, followed by a higher level of active

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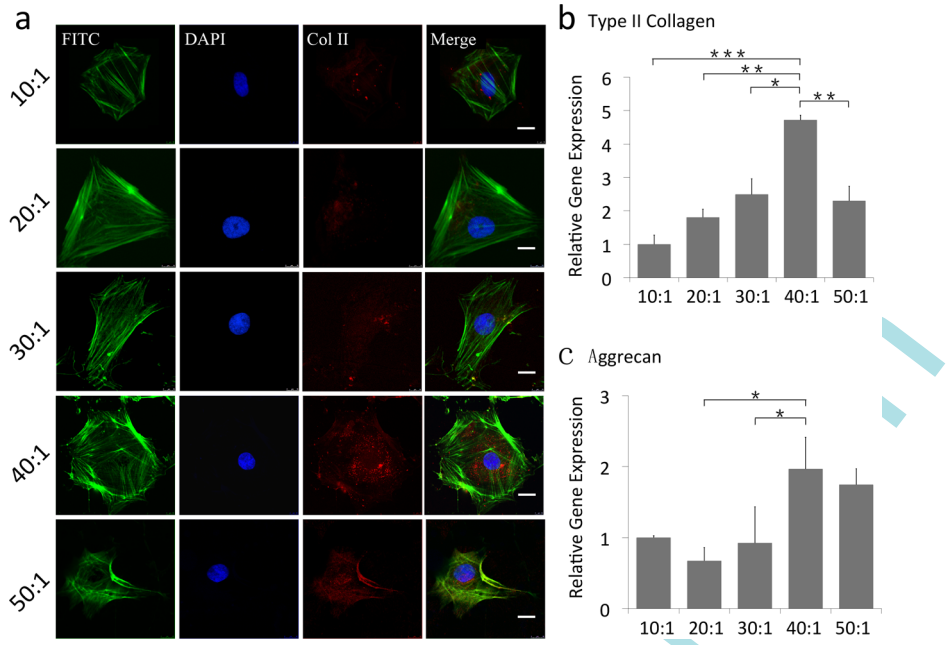

Figure 5. Softening materials induce more type II collagen expression of chondrocytes. (a) Immunostaining images of type II collagen for chondrocytes after 3 days plated on PDMS substrates with different stiffness (from 10:1 to 50:1, stiffness respectively equals to 135 kPa to 1.4 kPa). Type II collagen (red), cytoskeleton (green), nucleus (blue). Scale bars are 10 μm. (b,c) Type II collagen and Aggrecan gene expression in chondrocytes measured on different stiffness substrates. *GAPDH* levels set as internal normalized control. The samples were collected on Day 3. The results shown are representative of three different experiments (n = 3). Data presented as means ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.

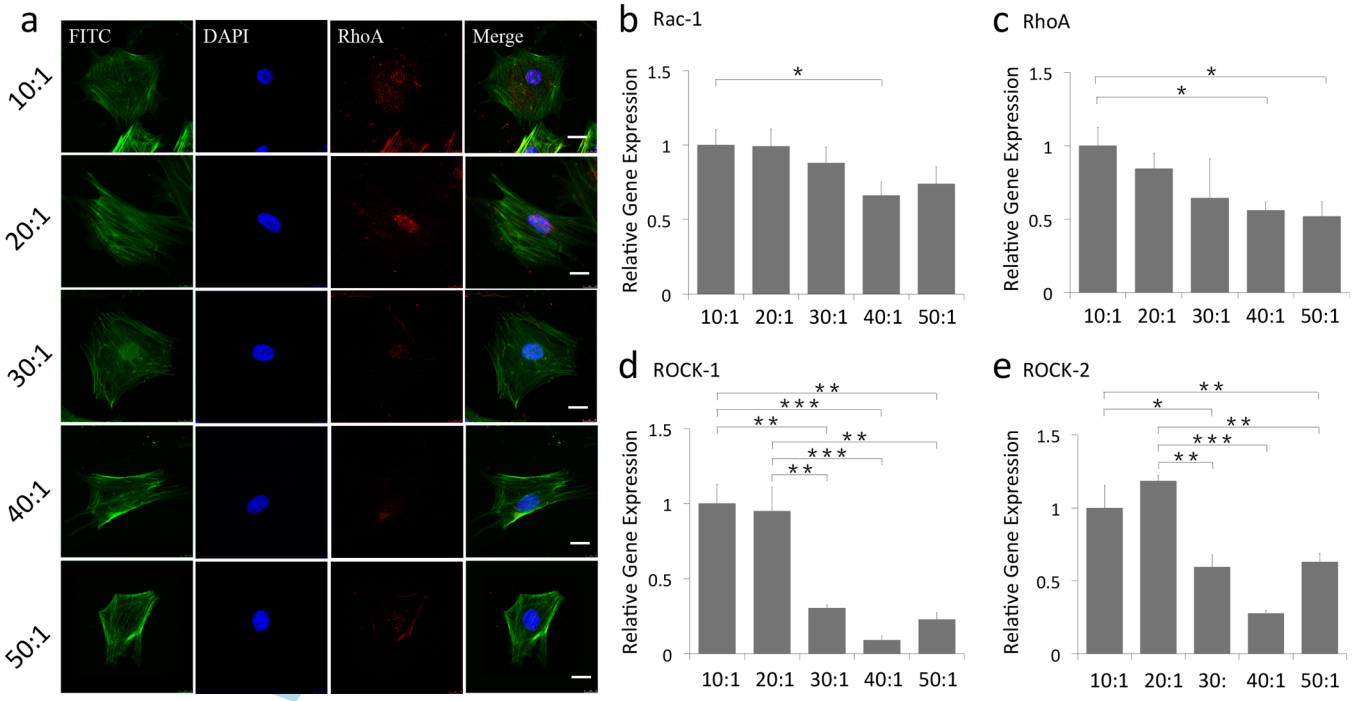

Figure 6. RhoA/ROCK pathway may regulate the mechanotransduction of chondrocytes cultured on various stiffness substrates. (a) Immunofluorescent images of RhoA observed by confocal laser scanning microscope in chondrocytes after 1 day incubation on different elastic PDMS (from 10:1 to 50:1, stiffness respectively equals to 135 kPa to 1.4 kPa). RhoA (red), cytoskeleton (green), nucleus (blue). Scale bars are 10 μm. (b–e) *Rac-1*, *RhoA*, *ROCK-1*, and *ROCK-2* gene expression in chondrocytes measured on different stiffness substrates. *GAPDH* levels set as internal normalized control. The samples were collected on Day 3. The results shown are representative of three different experiments (n = 3). Data presented as means ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.

RhoA.[44] In turn, RhoA activates ROCK leading to the remodeling of actin cytoskeleton in cells. When balance is achieved between expression of RhoA and cytoskeleton tension, proper cell morphology stabilizes. Computational model has shown that cellular microtubule compression forces go beyond the range of natural sensitivity of *in situ* cells, stress fibers will be enhanced or interrupted by reassembly or decreasing actin filament bundling.[45] Therefore, with approximately 6 kPa as its

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specific stiffness of ECM, chondrocytes cultured on high stiffness materials (>16 kPa) displayed a large spreading size and well-established actin fibers resulted in high cytoskeleton tension. Whereas, soft material (6 kPa) gave chondrocytes a proper living circumstance, leading to a round morphology, compromised cytoskeleton tension, and high expression of type II collagen. Previous studies found that pericellular matrix of human cartilage ranged from 17 to 200 kPa,[46−48] while cartilage stiffness may differ from human to other species like rat. To the best of our knowledge, few studies investigated the matrix stiffness of rat cartilage. However, substrate stiffness approximating to elastic modulus of 3 kPa directed stromal cells into chondrogenic lineage,[49] similar to the stiffness (40:1 PDMS with stiffness of 6 kPa, corresponding to the elastic modulus 4.8 kPa) in our study, also consisting to other previous studies.[16,50] For more precise controlling and further application in tissue engineering, chondrocytes isolated from human cartilage should be studied. Lastly, in our study, a conclusion can be drawn that the best stiffness for maintenance and stabilization of chondrocytes morphology and phenotype suggested in our study is 40:1, equal to 6 kPa.

4. CONCLUSIONS

In the cell-based tissue engineering, chondrocytes are still promising cell sources for cartilage repair. However, due to the de-differentiation and phenotypic loss of chondrocytes during monolayer culture *in vitro*, physical cues, like substrate stiffness, should be considered in biomaterial design. The tunable elastic PDMS substrates were coated by dopamine direct chondrocytes to display dramatically different morphologies and distinct alteration of major mechanosensitivity factors. Substrates with a stiffness of 6 kPa drive chondrocytes to a better phenotype and functionalization resulting from a round morphology and proper cytoskeleton tension conforming to physiological conditions via RhoA/ROCK pathway. Hence, our result highlighted the need to establish such a platform for probing the interaction between chondrocyte and its microenvironmental niches and demonstrated strongly the requirement of proper substrate stiffness in the design and selection of biomaterials. Furthermore, RhoA/ROCK signaling pathway may be a specific target in the regulation of chondrocytes function and cartilage repair. To sum up, this study provides new prospect for biomechanical control of cell behavior in cell-based clinical application for cartilage regeneration treatment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b07097.

Surface roughness and water contact angle of the elastic PDMS substrates (PDF)

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Notes

The authors declare no competing financial interest.

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