Published in final edited form as:

ACS Appl Mater Interfaces. 2018 October 03; 10(39): 33022–33031. doi:10.1021/acsami.8b11449.

Nanofibers Regulate Single Bone Marrow Stem Cell Osteogenesis via FAK/RhoA/YAP1 Pathway

Bei Chang, Chi Ma, and Xiaohua Liu*

Department of Biomedical Sciences, Texas A&M University College of Dentistry, Dallas, Texas 75246, United States

Abstract

Understanding cell-material interactions is a prerequisite for the development of bio-inspired materials for tissue regeneration. While nanofibrous biomaterials have been widely used in tissue regeneration, the effects of nanofibrous architecture on stem cell behaviors are largely ambiguous because the previous biomaterial systems used for nanofiber-cell interactions could not exclude the interference of cell-cell interactions. In this study, we developed a unique micropatterning technology to confine one single stem cell in a microisland of the nanofibrous micropatterned matrix; therefore, eliminating any potential intercellular communications. The nanofibrous micropatterned matrix, which mimicked both the physical architecture and chemical composition of natural extracellular matrix, was fabricated by a combination of electrospinning, chemical crosslinking, and UV-initiated photolithography. Compared to the non-nanofibrous architecture, a bone marrow mesenchymal stem cell (BMSC) cultured on the nanofibrous microisland exhibited a more in vivo-like morphology, a smaller spreading area, less focal adhesion, and fewer stress fibers. The BMSC cultured on the nanofibrous microisland also had higher alkaline phosphatase activity, indicating nanofibrous architecture promoted BMSC differentiation. A mechanistic study reveals that nanofibers regulate single BMSC osteogenesis via the FAK/RhoA/YAP1 pathway. The nanofibrous micropatterned matrix developed in this study is an excellent platform to promote the fundamental understanding of cell-matrix interactions, ultimately provide valuable insights for the development of novel bio-inspired scaffolds for tissue regeneration.

Graphical Abstract

*Corresponding Author: xliu@tamhsc.edu. Phone: 214-370-7007. Fax: 214-874-4538.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b11449.

Nanofibers decrease ROCK expression of a single BMSC (PDF)

Keywords

nanofiber; micropattern; cell-material interaction; signaling pathway; bone marrow mesenchymal stem cell

1. INTRODUCTION

Nanofibrous architecture of natural extracellular matrices (ECM) has long been considered to play pivotal roles in a variety of cellular behaviors.^{1–4} Using synthetic nanofibrous substrates as platforms, many efforts have been devoted to elucidate how this nanotopographic feature regulates stem cell fates, including cell shape, cell tension, integrin-mediated adhesion, migration, proliferation, and differentiation.^{5–9} However, none of the studies on those nanofibrous substrates could exclude cell–cell interactions, which contribute to almost all processes of the development and function of multicellular organisms. Consequently, the effects of nanofibrous architecture on stem cells based on those studies were actually the overall effects of nanofiber-cell and cell–cell interactions. To date, how a single stem cell interacts with nanofibers and the underlying mechanism remains largely ambiguous.

Micropatterning is a powerful technology that is capable of confining a single cell at sub-cellular scales with the deprivation of intercellular communications.¹⁰ Therefore, a micropatterned substrate is an excellent platform to explore cell-material interactions and excludes the influence of cell–cell communications. A number of micropatterning methods, such as photolithography, ink-jet printing, micro-contact printing, soft lithography, and self-assemble, have been developed in recent years.^{11–14} However, all of those methods usually adopt smooth surfaces for micropatterning. Therefore, none of them can directly generate micropatterned surfaces with nanofibrous architecture. Since electrospinning is a well-documented technique to fabricate nanofibers, the combination of micropatterning with electrospinning seems a feasible approach to incorporating nanofibers into micropatterned substrates.^{15–18} However, due to technical challenges, a micropatterned substrate that is capable of confining one single cell within a nanofibrous microisland has yet to be achieved.

In this work, we report a unique approach to fabricating a nanofibrous micropatterned (NF-MP) matrix that controls one single stem cell in a nanofibrous microisland. Our bio-inspired micropatterned matrix was fabricated by a process that combines electrospinning, chemical crosslinking, and UV-initiated photolithography. We selected gelatin as the micropatterned substrate because gelatin is a derivative of collagen, the major component of natural ECM. Therefore, we provided the first example of creating nanofibrous micropatterned artificial matrix that mimics both the physical architecture and chemical composition of natural ECM. We chose bone marrow mesenchymal stem cells (BMSCs) as the model cells in this study because of their broad potential in tissue engineering and regenerative medicine. Using this synthetic micropatterned matrix as a platform, we examined the effects of nanofibers on BMSC adhesion and differentiation, and successfully identified that the nanofibrous architecture modulates BMSC differentiation through a FAK/RhoA/YAP1 pathway.

2. RESULTS AND DISCUSSION

2.1. Preparation and Characterization of Nanofibrous Micropatterned (NF-MP) and Flat Film Micropatterned (FF-MP) Matrices.

Nanofibrous gelatin micropatterns were fabricated through a three-step process. First, nanofibrous gelatin matrix was prepared via electrospinning methacryloyl-modified gelatin (GelMA) that possesses double bonds in the main chain to allow the chemical reaction with polyethylene (glycol) diacrylate (PEGDA) during the micropatterning formation. Next, the nanofibrous gelatin matrix was crosslinked in a H₂O/acetone (5:95) mixture to stabilize the nanofibrous architecture. In the final step, a PEGDA solution was cast onto the surface of gelatin matrix and a UV-induced photolithography process was performed to create micropatterns. Figure 1a–c shows the scanning electron microscopy (SEM) images of a NFMP gelatin matrix which had a Young's modulus of 85 ± 3 MPa under wet conditions. Each microisland of the matrix was composed of gelatin nanofibers with the diameter of 250 ± 80 nm, which is at the same scale as the collagen fibers of natural ECM.¹⁹ In addition, each circular microisland was surrounded by a smooth surface that was covered with cell adhesion-resistant poly(ethylene glycol) (PEG). Therefore, cells could only adhere on the nanofibrous microislands. The size and shape of each microisland were controlled by the photomask used during photolithography. For comparison, micropatterned gelatin matrices of the same mechanical strength but with smooth surfaces on microislands were also fabricated (Figure 1d–f).

When BMSCs were seeded onto the two micropatterned matrices, cell adhesion was limited to the microislands, confirming the success of confining cells on the designated areas (Figure 2). In addition, most of the microislands were occupied by the BMSCs, indicating that the micropatterned gelatin matrices had excellent biocompatibility. To control the number of cells on a microisland, the size of the microisland was crucial. A small microisland is desired to limit one single cell on a microisland. However, a microisland that is too small will obstruct cell spreading and influence lineage commitments of stem cells.²⁰ We found that the average full spreading area of a BMSC 24 h after seeded on a petri dish was $2398 \pm 765 \mu\text{m}^2$. To exclude the cell size effect, we chose to use the microisland with a surface area of $3600 \mu\text{m}^2$ for our experiments. Under this condition, BMSCs on the FF-MP had a higher cell density than on the NF-MP (Figure 2e). However, the single cell ratio on the NF-MP was significantly higher than that on the FF-MP (Figure 2f), suggesting that a low cell density is favorable for a high single cell ratio on the micropattern. We selected those single cells in the NF-MP and FF-MP microislands and used them for the rest of the experiments.

2.2. BMSCs Morphologies on the NF-MP and FF-MP Matrices.

On the nanofibrous microisland, the BMSC exhibited a narrow, irregular shape with multiple cellular processes extending toward the margin of the microisland (Figure 3a,b). In contrast, the BMSC on a FF-MP had a flattened, spread shape without any obvious cellular processes toward the margin of the microisland (Figure 3c,d). We further tracked BMSC adhesion processes from initial seeding (1 h) to a stable attachment formation (24 h) on both the FF-MP and the NF-MP (Figure 3e). Overall, the spreading speed of the BMSC on the NF-MP

was slower than that on the FF-MP. Four hours after cell seeding, the BMSC on the NF-MP displayed a significantly smaller area than that on the FF-MP. At 24 h, the BMSC on the NF-MP had a stable adhesion area of $1928 \mu\text{m}^2$, while that number was increased to $2453 \mu\text{m}^2$ on the FF-MP. Besides, a BMSC on the NF-MP had a relatively smaller nucleus size than on the FF-MP (128 vs $148 \mu\text{m}^2$).

Two indexes were measured to quantify the cell morphology. The circularity index ($\text{CI} = 4\pi A/L^2$) represents the circularity of a cell, where A is the area of the cell, and L is the perimeter of the cell, with $\text{CI} = 1$ representing a perfect circle. The aspect ratio (AR) is calculated as a ratio of the major cell axis length to the minor cell axis length, which represents the symmetry of a cell, with $\text{AR} = 1$ indicating an absolute symmetry.²¹ The BMSCs on the NF-MP had a smaller CI value (0.31 vs 0.65) and a larger AR value (1.94 vs 1.14) compared to those on the FF-MP, suggesting the nanofibrous architecture modulated BMSC to form an in vivo-like morphology (Figure 3f,g).

The actin organization on the NF-MP and the FF-MP was also very different (Figure 3b,d). The majority of the actin filaments of the BMSC on the FF-MP formed well-organized stress fibers, and the actin cortex under plasma membrane was not obvious. In contrast, the stress fibers of the BMSC on the NF-MP were scarce, and the majority of actin filaments formed actin cortex underlying the plasma membrane. Quantitative analyses further indicated that only 33.6% of BMSCs on the NFMP displayed organized stress fibers, while about 84.3% of BMSCs on the FF-MP displayed apparent stress fibers (Figure 3h). Strikingly, the stress fiber intensity of the BMSC on the FF-MP was 8 times higher than that on the NF-MP (Figure 3i).

Cell attachment is a process that involves a cell continuously stretching out multiple filopodia or lamellipodia to explore the surrounding matrix. Once the filopodia or lamellipodia detect and anchor to stable anchoring sites, they rapidly recruit attachment-related molecules and form initial focal adhesions. Focal adhesions act as a link between actin fibers and integrins. The formation and maturation of focal adhesions rely on the feedback from both the actin cytoskeleton and integrin-based exterior signal transduction. Mature focal adhesions are linear vinculin patches associated with the termini of stress fibers localized at the cell periphery. In this work, vinculin immunofluorescence staining was used to show the difference in focal adhesion formation on both the NF-MP and the FF-MP (Figure 4). Typical focal adhesions on the FF-MP matrix, indicated by linear vinculin patches, were observed at the tips of stress fibers at the lamellipodia margins. However, such linear vinculin patches were barely seen on the NF-MP matrix, and the majority of the vinculin molecules accumulated into clusters underlying plasma membrane, consistent with previous studies that nano-topography impaired the formation and maturation of focal adhesions.²² Semi-quantitative analyses show that the focal adhesion number and focal adhesion area on the NF-MP were less than 1/11, and 1/14 to those on the FF-MP, respectively (Figure 4c,d). It should be noted that the typical focal adhesions are commonly artificial results when culturing cells on flat tissue culture polystyrene in vitro, and no significant focal adhesion formation were observed in vivo.²³ Therefore, these results suggest that nanofibrous architecture provides a more in vivo-like microenvironment for BMSC adhesion than the smooth surface.

2.3. Comparison of BMSC Osteogenic Differentiation on NF-MP and FF-MP.

After cultured in differentiation medium for 3 and 7 days, a stronger alkaline phosphatase (ALP) staining of the BMSC was detected on the NF-MP than on the FF-MP, suggesting nanofibrous architecture promoted BMSC osteogenesis (Figure 5a). At three days, 49.7% of the single cells on the NF-MP were positive for the ALP staining, while that number was 39.5% on the FF-MP (Figure 5b). More importantly, the relative ALP activity of the BMSC on the NF-MP was significantly higher than that on the FF-MP. After culturing for 7 days, the ALP positive ratio and relative activity on the NF-MP were still significantly higher than those on the FF-MP, confirming that nanofibrous structure enhances BMSC differentiation (Figure 5c).

2.4. Morphology and Differentiation of the BMSC on NF-MP and FF-MP were Modulated by RhoA/ROCK Pathway.

Previous studies had verified the relationship between stress fibers and osteogenesis.²⁴ RhoA, a member of small GTPase family, is a critical regulator of actin cytoskeleton. ROCK is the effector of RhoA which links to myosin II, the other major component of stress fiber besides F-actin to regulate actin contractility. To explore the regulatory role of RhoA/ROCK in BMSC behaviors on the NF-MP and the FF-MP, we first examined the expression of ROCK in BMSC on NF-MP and FFMP. It was observed that the ROCK expression level was significantly higher on the FF-MP than on the NF-MP (Supporting Information Figure S1), which not only corresponded the relatively high amount of stress fiber on FF-MP, but also confirmed the involvement of RhoA/ROCK signaling pathway in mediating the distinct behaviors of BMSCs on nanofibrous and flat surfaces. Moreover, Y-27632, an inhibitor of ROCK, was added into the culture medium to further identify the effect of RhoA/ROCK signaling pathway. The size of the BMSC cultured in the presence of Y-27632 decreased significantly from 1072 to 864 μm^2 on the NF-MP (Figure 6a,b). Similarly, the size of the BMSC on the FF-MP became smaller after the Y-27632 was added into the culture medium. Apart from the cell area, the cell morphology, the cell attachment ratio and the single cell ratio remained unchanged on both micropatterns after the addition of Y-27632. However, the inhibition of RhoA/ROCK signal pathway significantly impaired the osteogenic differentiation of the BMSC (Figure 6c,d). The ALP-positive ratio of the single cells was decreased from 48.0 to 32.2% on the NF-MP and from 39.9 to 33.3% on the FF-MP. In addition, the relative ALP activities on the NF-MP and FF-MP were reduced from 125.6 to 74.9, and from 69.6 to 32.4, respectively. These results revealed a critical role of actomyosin cytoskeleton in BMSC osteogenic differentiation on both the NF-MP and the FF-MP.

2.5. YAP1 and Runx2 were Involved in Mediating Osteogenic Differentiation of the BMSC on NF-MP.

YAP1 is critical in cell–matrix adhesion-mediated signaling and mechano-transduction,^{25–27} and is expressed both in the cytoplasm and in the nucleus. The cytoplasmic YAP1 is inactive while the nuclear YAP1 functions as a transcriptional co-activator. The shuffling of YAP1 between the nucleus and cytosol makes it as an indicator of ECM mechanical property and cellular tension. When cells are cultured on a stiff surface or forced to spread over a large area, YAP1 senses actin tensions and transfer its location to the nucleus. We examined the

YAP1 distribution within single BMSC on both the NF-MP and the FF-MP, and found that nuclear YAP1 expression accounted for 46% of the total YAP1 expression on the NF-MP, while for 62% on the FF-MP (Figure 7). Considering the relatively lower amount of stress fibers on the NF-MPs, this difference in YAP1 distribution was consistent with previous studies that YAP1 nuclear expression level is positively related to the cytoskeleton tension.²⁶ Moreover, the addition of ROCK inhibitor Y-27632 decreased the nuclear YAP1 expression on both the NF-MP and FF-MP, especially on the FF-MP although the total cellular YAP1 content did not change significantly. This result indicated that the expression of nuclear YAP1 was dependent on the RhoA/ROCK signaling pathway.²⁶ Since YAP1 nuclear translocation depends on actin network,²⁸ the decrease in nuclear YAP1 expression might be a result of an impaired actin polymerization by Y-27632.

In osteogenesis, YAP1 is expressed in immature osteoprogenitor cells and the osteogenesis is blocked by a high level of nuclear YAP1.²⁹ It has been documented that nuclear YAP1 suppresses the activity of Runx2 by forming the YAP1/Runx2 complex to restrict the effect of Runx2 that serves as a key transcriptional factor at bone-specific osteocalcin promoter.^{30,31} In our study, we found that Runx2 was expressed in the nucleus of single BMSC both on the NF-MP and FF-MP groups, and the expression level on the NF-MP was much stronger than that on the FF-MP (Figure 8), which further confirmed the pro-osteogenesis effect of the nanofibrous architecture. Moreover, the higher Runx2 expression and the cytoplasmic location of YAP1 on the NF-MP also indicated the negative relationship between the nuclear YAP1 content and the Runx2 activity. When the ROCK activity was inhibited by adding Y-27632, Runx2 expression was decreased on both the NF-MP and FF-MP. The Runx2 signal was hardly found on the FF-MP, while it was observed in both the nucleus and the cytoplasm on the NF-MP (Figure 8c,d). The decrease of Runx2 expression in nucleus further demonstrated that osteogenesis was dependent on the RhoA/ROCK signaling pathway.

Runx2 proteins are latent in the cytoplasm, and need to be transferred into the nucleus to play its role as the master transcriptional factor in osteogenesis.³² The cytoplasmic Runx2 expression has been found when microtubules are stabilized by taxol.³³ While in our study, the inhibition of RhoA/ROCK signaling pathway also induced a cytoplasmic Runx2 expression on the NF-MP. Considering that ROCK can regulate microtubule acetylation,³⁴ a post-translational modification that increased the stability of microtubules,³⁵ it is likely that this cytoplasmic expression of Runx2 was also owing to the dysfunction of microtubules, which might be induced by Y-27632. Therefore, the inhibiting of RhoA/ROCK not only decreased the synthesis of Runx2, but also hampered its translocation from the cytoplasm to the nucleus.

It should be noted that RhoA/ROCK is a ubiquitous signaling pathway, and the addition of Y-27632, a strong inhibitor of RhoA/ROCK, not only influences the YAP1 expression, but also affects many other downstream molecules related to osteogenesis. For example, MAPK pathway and CTGF pathway have also been demonstrated to mediate in RhoA/ROCK regulated osteogenesis.^{36,37} Therefore, it is possible to observe a decreased nuclear YAP1 expression and a decreased osteogenesis (e.g., lower ALP activity), owing to the extensive effect of Y-27632 on the many downstream signaling molecules of RhoA/ROCK.

Based on the above results, a possible molecular signaling pathway of how nanofibrous architecture interacts with BMSC is summarized as follows (Figure 9). When a BMSC initiates a contact with the nanofibers, the integrin receptors on the plasma membrane of the cell are activated, and subsequently fewer focal adhesions are formed compared to the flat surface. The fewer focal adhesion leads to a relatively low activity of RhoA/ROCK. Since ROCK positively regulates the formation of stress fibers via its downstream myosin, fewer stress fibers are formed on the nanofibers. Meanwhile, RhoA/ROCK regulates actin polymerization and this actin network is critical in the cytoplasm-to-nucleus translocation of YAP1, therefore; less nuclear YAP1 expression is observed on nanofibers. As a result, the restrain effect of YAP1 on Runx2 is partially released and the amount of functional Runx2 is increased on the nanofibers. As the master transcription factor in osteogenesis, Runx2 initiates the osteogenic differentiation of single BMSCs on the nanofibers and promotes the synthesis of ALP molecules, leading to enhanced differentiation of the BMSC on the nanofibrous architecture.

3. CONCLUSIONS

To explore the effect of nanofibrous architecture on stem cell behaviors, we developed a unique bioengineering approach that was capable of confining one single stem cell on a microisland of a nanofibrous micropatterned matrix, therefore, eliminating any potential intercellular communications. Bio-inspired nanofibrous micropatterned gelatin matrix, which mimics both the physical architecture and chemical composition of natural ECM, was fabricated by integrating a nano-fabrication technique, electrospinning, with a micro-fabrication process, photolithography. Using the nanofibrous micropatterned matrix as a platform, we examined how the nanofibrous architecture affects a single BMSC adhesion and differentiation. The BMSC on the nanofibrous microisland exhibited a more in vivo-like morphology, a smaller spreading area, less focal adhesion, fewer stress fibers, and higher ALP activity than on a nonnanofibrous surface. A mechanistic study indicated that the nanofibrous architecture enhances the BMSC differentiation via the FAK/RhoA/YAP1 pathway. The single-cell based in vitro platform provides an in-depth understanding of nanofiber-cell interactions and confirms the effect of nanofibrous architecture in promoting osteogenesis. The mechanistic study provides valuable insights for developing novel bio-inspired materials for tissue regeneration.

4. EXPERIMENTAL SECTION

4.1. Materials.

Gelatin (Cat #G9382), methacrylic anhydride (Cat #276685), poly(ethylene glycol) methyl ether methacrylate (Cat #447951), 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Cat #410896), *N*-hydroxysuccinimide (NHS) (Cat #130672), 2-(*N*-morpholino)ethanesulfonic acid (MES) (Cat #M3671), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Cat #E6383), and glycine (Cat #G8898) were purchased from Sigma-Aldrich (St. Louis, MO). Dialysis membrane (molecular weight 12–14 kD cutoff) was purchased from Spectrum Laboratories (Dallas, TX).

4.2. Synthesis of Gelatin Methacryloyl (GelMA).

Methacrylic anhydride (10 mL) was dropwise added to 100 mL of 10% gelatin in phosphate buffered saline (PBS) at 40 °C. After stirring at a speed of 100 rpm for 3 h, the polymer solution was diluted to a concentration of 2% and dialyzed against distilled water using 12–14 kDa cutoff dialysis tubing at 40 °C. The distilled water was changed every 12 h for 1 week. The obtained end product GelMA was lyophilized and stored at –20 °C for future use.

4.3. Fabrication of Nanofibrous Matrix.

GelMA was dissolved in a solvent mixture composed of hexafluoroisopropanol/acetic acid/ethyl acetate/water (5:2.5:1.5:1) to form a concentration of 20% (w/v). The solution was centrifuged at 15 000 rpm and the supernatant was collected for electrospinning. The electrospinning was carried out in an electrospinning system (Spraybase platform, Ireland) at room temperature through a metallic spray tip (22G). A power supply (Gamma High Voltage) was used to control the voltage at 15 kV. A feeding rate of 0.5 mL/h was controlled using a digital controlled infusion pump (Cole Plamer Inc). The electrospun nanofibers were collected onto a rotated drum collector that was covered with aluminum foil and had a width of 30 cm and a rotation speed of 80 rpm. The distance between the drum collector and the spray tip was 10 cm.

The obtained nanofibrous matrix was incubated in a pre-cooled medium composed of 5% crosslinking aqueous solution and 95% acetone for 12 h. The crosslinking aqueous solution contained 2.5 mM MES, 3 mM EDC, and 0.5 mM NHS. The crosslinked nanofibrous matrix was treated with 50 mM glycine aqueous solution at room temperature for 1 h to neutralize the unreacted EDC. Next, the crosslinked nanofibrous matrix was washed three times by distilled water (15 min each time) and dehydrated in ethanol. The dehydrated samples were dried in a vacuum oven and stored in –20 °C for future use.

4.4. Fabrication of GelMA Flat Film.

GelMA aqueous solution (0.2% w/v) was prepared by dissolving 1.0 mg of GelMA in 50 mL of deionized water at 40 °C. An ImmEdge Hydrophobic Barrier Pen (Vector Laboratories, H-4000) was used to define a 1.5 cm × 1.5 cm cubic area on glass slides. Twenty microliter of the GelMA solution was uniformly dripped into the pre-defined area, and was allowed to dry at room temperature within 4 h. The slides were then incubated at 4 °C for 30 min to ensure the GelMA gelation. Next, 150 μ L of crosslinking aqueous solution containing 2.5 mM MES, 3 mM EDC, and 0.5 mM NHS was dripped onto the GelMA gel and crosslinked at 4 °C for 12 h. The GelMA slides were immersed in 50 mM glycine aqueous solution for 1 h at room temperature to neutralize the unreacted EDC, followed by washing with deionized water for three times (15 min each time). The samples were dried in a vacuum oven and stored in –20 °C for future use.

4.5. UV-Initiated Photolithography of Nanofibrous Micro-patterns.

The micropattern was generated by a photolithography process. First, the crosslinked GelMA nanofibrous matrix was cut into 1 cm × 1 cm cubic sheets and soaked in a photo-crosslinking solution containing 20% poly(ethylene glycol) methyl ether methacrylate and 1% initiator Irgacure D-2959. A pre-designed photomask was gently settled onto the matrix

which was crosslinked at 40 mW/cm² under a UV light (CS2010, Thorlabs, Inc.) for 60 s. After peeling off the photomask, nanofibrous micropatterns (NF-MP) were obtained. For comparison, flat film micropatterns (FF-MP) were prepared from GelMA flat films using the same process.

4.6. Mechanical Test of the Micropatterned Matrix.

The NF-MP and FF-MP ($n = 5$) with a strip shape of 1 cm $\times$ 4 cm were used to test the Young's modules using Instron with 1 kN sensor (Instron calibration Laboratory) as we reported previously.³⁸

4.7. Rat BMSCs Isolation and Culture.

Primary rat BMSCs were isolated from rat bone marrows following a standard protocol.³⁹ The animal surgical procedures were approved by the University Committee on the Use and Care of Animals of the Texas A&M University College of Dentistry. Briefly, 5 week-old SD rats were sacrificed after anesthesia, and both femora and tibia were aseptically removed. Bone marrow was flushed down by a syringe filled with α -modified essential medium (α -MEM) (Gibco, A1049001) supplemented with 10% (v/v) FBS (Gibco, #26140079) and 1% penicillin/streptomycin (Sigma, #P333). The released cells were collected and cultured in a 75 cm² culture flasks and maintained in a 37 °C incubator. Cells were allowed to attach for 72 h and non-adherent cells were removed. The remaining cells were labeled as Passage 0 (P0). BMSCs of P3–P5 were used in this study. To prevent cell proliferation within micropatterns, aphidicolin (0.5 mg/mL, Sigma, #A0781) was added in culture medium 24 h after cell seeding, and the medium was changed every 3 days. For ROCK inhibition assay, 2 μ M Y-27632 (Calbiochem, #688000) was added to the culture medium. For BMSCs differentiation, 1 mM dexamethasone, 50 mM ascorbic acid-2-phosphate, and 10 mM β -glycerophosphate were added to the medium and changed every 3 days.

4.8. BMSC Seeding on NF-MP and FF-MP.

Slides loaded with NF-MP and FF-MP were cut to a size that fits into a 6-well plate. An ImmEdge hydrophobic barrier pen was used to define a 12 mm $\times$ 12 mm cubic area around NF-MP and FF-MP. The slides were immersed in 70% of ethanol for 30 min for sterilization, followed by washing with sterile PBS for three times. After trypsin digestion and resuspension, 100 μ L cell suspension that contained 2×10^4 BMSCs was evenly dripped within the cubic area of the slide. The 6-well plate was incubated at 37 °C for 30 min. Next, the slides in the plate were gently washed with culture medium for several times until no cells were detected outside the micropattern under an inverted differential interference contrast microscopy. Each well of the plate was pipetted with 3 mL of culture medium and was cultured in the incubator.

4.9. ALP Staining.

ALP staining was operated by following manufacturer's guidance (Sigma, #85L2). Briefly, NF-MP or FF-MP with cells were washed with PBS and fixed with citrate buffered acetone (60%) for 5 min, followed by washing with PBS for three times (5 min each time). Next, the NF-MP or FF-MP were immersed in an ALP staining solution for 30 min at room

temperature. The nuclei were stained with 1 $\mu\text{g}/\text{mL}$ Hoechst 33342 (Thermo Scientific, #62249) for 20 min. Samples were scanned under both bright field channel and fluorescence channel. Single ALP-positive cells were screenshot and counted. At least 30 ALP-positive cells on each matrix were analyzed using ImageJ following the protocol reported in the reference.⁴⁰

4.10. Immunofluorescence Staining.

NF-MP or FF-MP were fixed with 4% paraformaldehyde for 30 min and permeated with 0.3% Triton-100 for 10 min. After being blocked with 5% goat serum (Gibco, #16210064) for 4 h at room temperature, the NF-MP or FF-MP were stained with the anti-vinculin antibody (1:150, Abcam, ab129002), anti-ROCK1 antibody (1:200, Abcam, ab45171) or anti-YAP1 antibody (1:500, Abcam, ab39361), or anti-Runx2 antibody (1:150, Abcam, ab23981) overnight together with CF633 phalloidin (10 U/mL, Biotium, 00046) at 4 °C. The samples were stained with Alexa Fluor Plus 555 secondary antibody (1:200, Invitrogen, A32732) for 2 h at room temperature, followed by 1 $\mu\text{g}/\text{mL}$ Hoechst 33342 for 20 min. The NF-MP or FF-MP was placed on a glass slide and mounted for observation under a confocal laser scan microscope (TCS SP5, Leica, Buffalo). For cellular processes analysis, we consider a cytoplasmic extension as a cellular process when the distance between its distal end and the cell body is more than 5 μm .

4.11. Statistical Analysis.

Apart from cell attachment ratio and single cell ratio analysis, microislands occupied with only one single cell in a microisland were selected and analyzed in all experiments. Semiquantitative data from all images were analyzed with the ImageJ software, including cell area, circularity index, aspect ratio, fluorescence intensity and ALP density. For quantitative analysis, six regions of interest and at least 30 cells were selected for each group. All experiences were repeated twice. Data were analyzed with t-test, and statistical significance was set at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This study was supported by NIH/NIDCR R01DE024979 (X.L.).

REFERENCES

- (1). Hynes RO The Extracellular Matrix: Not Just Pretty Fibrils. *Science* 2009, 326, 1216–1219. [PubMed: 19965464]
- (2). Trappmann B; Gautrot JE; Connelly JT; Strange DGT; Li Y; Oyen ML; Stuart MAC; Boehm H; Li BJ; Vogel V; Spatz JP; Watt FM; Huck WTS Extracellular-matrix Tethering Regulates Stem-Cell Fate. *Nat. Mater* 2012, 11, 642–649. [PubMed: 22635042]

- (3). Silva GA; Czeisler C; Niece KL; Beniash E; Harrington DA; Kessler JA; Stupp SI Selective Differentiation of Neural Progenitor Cells by High-Epitope Density Nanofibers. *Science* 2004, 303, 1352–1355. [PubMed: 14739465]
- (4). Liu X; Smith LA; Hu J; Ma PX Biomimetic Nanofibrous Gelatin/Apatite Composite Scaffolds for Bone Tissue Engineering. *Biomaterials* 2009, 30, 2252–2258. [PubMed: 19152974]
- (5). Hu J; Liu XH; Ma PX Induction of Osteoblast Differentiation Phenotype on Poly(L-Lactic Acid) Nanofibrous Matrix. *Biomaterials* 2008, 29, 3815–3821. [PubMed: 18617260]
- (6). Christopherson GT; Song H; Mao HQ The Influence of Fiber Diameter of Electrospun Substrates on Neural Stem Cell Differentiation and Proliferation. *Biomaterials* 2009, 30, 556–564. [PubMed: 18977025]
- (7). Lim SH; Liu XY; Song HJ; Yarema KJ; Mao HQ The Effect of Nanofiber-Guided Cell Alignment on the Preferential Differentiation of Neural Stem Cells. *Biomaterials* 2010, 31, 9031–9039. [PubMed: 20797783]
- (8). Nur-E-Kamal A; Ahmed I; Kamal J; Schindler M; Meiners S Three-dimensional Nanofibrillar Surfaces Promote Self-Renewal in Mouse Embryonic Stem Cells. *Stem Cells* 2006, 24, 426–433. [PubMed: 16150921]
- (9). Yin Z; Chen X; Chen JL; Shen WL; Nguyen TMH; Gao L; Ouyang HW The Regulation of Tendon Stem Cell Differentiation by the Alignment of Nanofibers. *Biomaterials* 2010, 31, 2163–2175. [PubMed: 19995669]
- (10). Théry M Micropatterning As a Tool to Decipher Cell Morphogenesis and Functions. *J. Cell Sci* 2010, 123, 4201–4213. [PubMed: 21123618]
- (11). Xia YN; Whitesides GM Soft Lithography. *Angew. Chem., Int. Ed* 1998, 37, 550–575.
- (12). Bernard A; Renault JP; Michel B; Bosshard HR; Delamarche E Microcontact Printing of Proteins. *Adv. Mater* 2000, 12, 1067–1070.
- (13). Kumar A; Biebuyck HA; Whitesides GM Patterning SelfAssembling Monolayers-Applications in Materials Science. *Langmuir* 1994, 10, 1498–1511.
- (14). Gauvreau V; Laroche G Micropattern Printing of Adhesion, Spreading, and Migration Peptides on Poly(tetrafluoroethylene) Films to Promote Endothelialization. *Bioconjug. Chem* 2005, 16, 1088–1097. [PubMed: 16173784]
- (15). Lee HJ; Koh W-G Hydrogel Micropattern-Incorporated Fibrous Scaffolds Capable of Sequential Growth Factor Delivery for Enhanced Osteogenesis of hMSCs. *ACS Appl. Mater. Interfaces* 2014, 6, 9338–9348. [PubMed: 24915062]
- (16). Lee J; Lee SY; Jang J; Jeong YH; Cho D-W Fabrication of Patterned Nanofibrous Mats Using Direct-Write Electrospinning. *Langmuir* 2012, 28, 7267–7275. [PubMed: 22512407]
- (17). Jiang L; Zhang M; Li J; Wen W; Qin J Simple Localization of Nanofiber Scaffolds via SU-8 Photoresist and Their Use for Parallel 3D Cellular Assays. *Adv. Mater* 2012, 24, 2191–2195. [PubMed: 22431134]
- (18). Lee HJ; Nam SH; Son KJ; Koh W-G Micropatterned Fibrous Scaffolds Fabricated Using Electrospinning and Hydrogel Lithography: New Platforms to Create Cellular Micropatterns. *Sens. Actuators, B* 2010, 148, 504–510.
- (19). Liu X; Ma PX Polymeric Scaffolds for Bone Tissue Engineering. *Ann. Biomed. Eng* 2004, 32, 477–486. [PubMed: 15095822]
- (20). Peng R; Yao X; Cao B; Tang J; Ding JD The Effect of Culture Conditions on the Adipogenic and Osteogenic Inductions of Mesenchymal Stem Cells on Micropatterned Surfaces. *Biomaterials* 2012, 33, 6008–6019. [PubMed: 22681981]
- (21). Lomakin AJ; Lee K-C; Han SJ; Bui DA; Davidson M; Mogilner A; Danuser G Competition of Two Distinct Actin Networks for Actin Defines A Bistable Switch for Cell Polarization. *Nat. Cell Biol* 2015, 17, 1435–1445. [PubMed: 26414403]
- (22). Wang X; Nakamoto T; Duli ska-Molak I; Kawazoe N; Chen G Regulating The Stemness of Mesenchymal Stem Cells by Tuning Micropattern Features. *J. Mater. Chem. B* 2016, 4, 37–45.
- (23). Cukierman E; Pankov R; Stevens DR; Yamada KM Taking Cell-Matrix Adhesions to The Third Dimension. *Science* 2001, 294, 1708–1712. [PubMed: 11721053]

- (24). McBeath R; Pirone DM; Nelson CM; Bhadriraju K; Chen CS Cell Shape, Cytoskeletal Tension, and RhoA Regulate Stem Cell Lineage Commitment. *Dev. Cell* 2004, 6, 483–495. [PubMed: 15068789]
- (25). Dupont S Role of YAP/TAZ in Cell-Matrix Adhesion-Mediated Signalling and Mechanotransduction. *Exp. Cell Res* 2016, 343, 42–53. [PubMed: 26524510]
- (26). Dupont S; Morsut L; Aragona M; Enzo E; Giulitti S; Cordenonsi M; Zanconato F; Le Digabel J; Forcato M; Bicciato S; et al. Role of YAP/TAZ in Mechanotransduction. *Nature* 2011, 474, 179–183. [PubMed: 21654799]
- (27). Yang C; Tibbitt MW; Basta L; Anseth KS Mechanical Memory and Dosing Influence Stem Cell Fate. *Nat. Mater* 2014, 13, 645–652. [PubMed: 24633344]
- (28). Sen B; Xie Z; Uzer G; Thompson WR; Styner M; Wu X; Rubin J Intracellular Actin Regulates Osteogenesis. *Stem Cells* 2015, 33, 3065–3076. [PubMed: 26140478]
- (29). Seo E; Basu-Roy U; Gunaratne PH; Coarfa C; Lim D-S; Basilico C; Mansukhani A SOX2 regulates YAP1 to Maintain Stemness And Determine Cell Fate in The Osteo-Adipo Lineage. *Cell Rep.* 2013, 3, 2075–2087. [PubMed: 23791527]
- (30). Zaidi SK; Sullivan AJ; Medina R; Ito Y; Van Wijnen AJ; Stein JL; Lian JB; Stein GS Tyrosine Phosphorylation Controls Runx2-Mediated Subnuclear Targeting of YAP to Repress Transcription. *EMBO J.* 2004, 23, 790–799. [PubMed: 14765127]
- (31). Zhang H; Cooper LF; Zhang X; Zhang Y; Deng F; Song J; Yang S Titanium Nanotubes Induce Osteogenic Differentiation Through The FAK/Rhoa/YAP Cascade. *RSC Adv.* 2016, 6, 44062–44069.
- (32). Kim S; Koga T; Isobe M; Kern BE; Yokochi T; Chin YE; Karsenty G; Taniguchi T; Takayanagi H Stat1 Functions As a Cytoplasmic Attenuator of Runx2 in The Transcriptional Program of Osteoblast Differentiation. *Genes Dev.* 2003, 17, 1979–1991. [PubMed: 12923053]
- (33). Uemura M; Nagasawa A; Terai K Yap/Taz Transcriptional Activity in Endothelial Cells Promotes Intramembranous Ossification Via The BMP Pathway. *Sci. Rep* 2016, 6, No. 27473. [PubMed: 27273480]
- (34). Schofield AV; Steel R; Bernard O Rho-associated coiled-coil kinase (ROCK) Protein Controls Microtubule Dynamics in a Novel Signaling Pathway That Regulates Cell Migration. *J. Biol. Chem* 2012, 287, 43620–43629. [PubMed: 23093407]
- (35). Westermann S; Weber K Post-translational Modifications Regulate Microtubule Function. *Nat. Rev. Mol. Cell Biol* 2003, 4, 938–947. [PubMed: 14685172]
- (36). Khattiwala CB; Kim PD; Peyton SR; Putnam AJ ECM Compliance Regulates Osteogenesis by Influencing MAPK Signaling Downstream of RhoA and ROCK. *J. Bone Miner. Res* 2009, 24, 886–898. [PubMed: 19113908]
- (37). Xu Y; Wagner DR; Bekerman E; Chiou M; James AW; Carter D; Longaker MT Connective Tissue Growth Factor in Regulation of RhoA Mediated Cytoskeletal Tension Associated Osteogenesis of Mouse Adipose-Derived Stromal Cells. *PLoS One* 2010, 5, No. e11279. [PubMed: 20585662]
- (38). Ma C; Qu T; Chang B; Jing Y; Feng JQ; Liu X 3D Maskless Micropatterning for Regeneration of Highly Organized Tubular Tissues. *Adv. Healthcare Mater* 2018, 7, No. 1700738.
- (39). Zhang L; Chan C Isolation and Enrichment of Rat Mesenchymal Stem Cells (MSCs) and Separation of Single-Colony Derived MSCs. *J. Visualized Exp* 2010, No. 1852.
- (40). Tang J; Peng R; Ding J The Regulation of Stem Cell Differentiation by cell–cell Contact on Micropatterned Material Surfaces. *Biomaterials* 2010, 31, 2470–2476. [PubMed: 20022630]

Figure 1. SEM images of NF-MP (a–c) and FF-MP (d–f). (b, e) are a representative nanofibrous microisland and flat surface microisland, respectively. (c) is the high magnification of (b), showing the nanofibrous matrix covers the NF-MP microisland; and (f) is the high magnification of (e), showing the smooth surface covers the FF-MP microisland.

Figure 2.

(a–d) Confocal images of BMSCs adhesion on the NF-MP and FF-MP microislands. (e) Cell density of BMSCs on the NF-MP and FF-MP microislands. (f) Single cell ratio on the NF-MP and FF-MP microislands. Scale bar: 100 μ m.

Figure 3.

Nanofibers guide a single BMSC to form an in vivo-like morphology. (a–d) The morphology of a single BMSC adhered on the NF-MP and FF-MP microislands, separately. (e) The spreading rate of a single BMSC on the NF-MP and FF-MP microislands, separately. (f) The circularity index (CI) of a single BMSC on the NF-MP and FF-MP microislands, separately. (g) The aspect ratio (AR) of a single BMSC on the NF-MP and FF-MP microislands, separately. (h) Stress fiber ratio of a single BMSC on the NF-MP and FF-MP microislands, separately. (i) Stress fiber strength of a single BMSC on the NF-MP and FF-MP microislands, separately. Scale bar: 10 μm .

Figure 4.

Nanofibers decrease focal adhesion formation of a single BMSC. Immunofluorescence staining of vinculin of a single BMSC on the NF-MP (a) and FF-MP (b) microislands, separately. (c, d) Semi-quantitative data of focal adhesion number (c) and area (d) of a single BMSC on the NF-MP and FF-MP microislands, separately. Scale bar: 10 μ m.

Figure 5. Nanofibers promote osteogenic differentiation of a single BMSC. (a) ALP staining of a single BMSC on the NF-MP and FF-MP microislands, separately. (b, c) Semi-quantitative data of ALP activities of a single BMSC on the NF-MP and FF-MP microislands, separately. Scale bar: 20 μ m.

Figure 6.

Morphology and differentiation of a single BMSC on the nanofibers were modulated by a RhoA/ROCK pathway. (a) ALP staining of a single BMSC with the addition of ROCK inhibitor Y-27632 on the NF-MP and FF-MP microislands, separately. (b) Cell area of a single BMSC with the addition of Y-27632 on the NF-MP and FF-MP microislands, separately. (c, d) The effect of Y-27632 on a single BMSC osteogenic differentiation on the NF-MP and FF-MP microislands. Scale bar: 20 μm .

Figure 7. Nanofibers promote YAP1 cytoplasmic localization of a single BMSC. (a–d) The immunofluorescence staining of YAP1 of a single BMSC on the NF-MP and FF-MP microisland with the addition of Y-27632, separately. (e) The distribution of YAP1 of a single BMSC on the NF-MP and FF-MP microislands, separately. (f) The influence of Y-27632 on the expression of nuclear YAP1 on NF-MP and FF-MP microislands. Scale bar: 10 μ m.

Figure 8.

Nanofibers promote Runx2 expression of a single BMSC. (a–d) The immunofluorescence staining of Runx2 of a single BMSC on the NFMP and FF-MP microislands with and without Y-27632, separately. (e) The relative amount of Runx2 on NF-MP and FF-MP with and without Y-27632. Scale bar: 10 μ m.

Figure 9. Schematic of the proposed model for how nanofibers regulate single bone marrow stem cell osteogenesis via FAK/RhoA/YAP1 Pathway.