Relationship of Matrix Stiffness and Cell Morphology in Regulation of Osteo Genesis and Adipogenesis of BMSCs

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Abstract

Backgrounds: Both matrix stiffness and cell morphology have been found as important factors directing MSCs (mesenchymal stem cells) differentiation, but cells also spontaneously adapt their morphology under matrix stiffness stimulation. This study aimed to investigate the interplay of cell morphology and matrix stiffness on osteogenesis and adipogenesis of rBMSCs(rat bone BMSCs) on 2D substrates.

Methods and Results: First, we modulated MSCs morphology through different fibronectin (FN) concentrations on tissue culture plates (TCPs). We found FN promoted and osteogenesis of BMSCs while suppressing adipogenesis, mediated by FN-induced F-actin polymerization and cell spreading. Based on these findings, we modulated BMSCs morphology on 0.5 kPa and 32 kPa CytoSoft® plates through FN concentrations. We found BMSCs on 0.5 kPa substrates coated with 300µg/ml of FN manifested similarly spreading morphology with cells on 32 kPa substrates coated with 100 µg/ml of FN, and cells in both groups dominantly commit osteogenesis. On the other hand, BMSCs on 32 kPa substrates coated with 30µg/ml of FN manifested similarly restricted morphology with cells in on 0.5 kPa substrates with 100µg/ml of FN, and in both groups cells mainly commit adipogenesis. Immunofluorescence staining indicated YAP/TAZ mainly located in cytoplasm when cells exhibited restricted morphology on stiff matrices, while exhibiting significant nuclear translocation when cells spread on soft matrices.

Conclusions: Cell morphology overrode effects of matrix stiffness on BMSCs differentiation through more robust regulation of YAP/TAZ. Matrix stiffness depended on cell morphology to regulate osteogenesis and adipogenesis of BMSCs.

1 Introduction

Tissue engineering requires precise control of stem cell fate decisions, which remains a challenge in the status quo. For both research and clinical application, bone mesenchymal stem cells (BMSCs) are one of the most widely applied stem cells for their multi-differentiation potential, easy accessibility and little ethical concern [1].

In the last decade, biophysical cues represented by matrix stiffness have been verified to regulate BMSCs differentiation. Anchoring integrin to surrounding matrix, BMSCs exert traction force through cytoskeleton system to perceive their mechanical microenvironment, and matrix stiffness determines the resistance they receive [2]. A rigid substrate of 25-40 kPa produces high resistant force to BMSCs and induces FAs (focal adhesions) assembly and F-actin polymerization that promotes nuclear translocation of transcriptional regulators YAP/TAZ, thereby promoting osteogenesis of BMSCs, while soft substrates of 0.5-5 kPa inhibit the mechanotransduction process above and favor adipogenic differentiation [2-4]. On flat substrates, cytoskeleton dynamics induced by matrix stiffness simultaneously results in cell morphology change: cells spread to large areas with high cytoskeletal tension on rigid substrates while rounding up with reduced cytoskeletal tension on soft substrates [5].

However, independent of external mechanical stimulus, cell morphology has also been demonstrated as an important regulator of BMSCs differentiation [6], as under osteogenic-adipogenic co-induction, spreading morphology directly induced BMSCs into osteogenesis while restricted morphology promoted adipogenesis of BMSCs. These findings raised a question that cell morphology whether an accompanied effect or actually the downstream mediator of matrix-stiffness-induced BMSCs differentiation. Recent studies in 3D culture systems showed that matrix stiffness regulated osteogenesis and adipogenesis of BMSCs through adhesive ligand clustering without cell morphology change [7]. As cell mechanobiology has been found distinctive in 3D and 2D systems, and mechanical stimuli in 3D culture system are less controllable and more intertwined than in 2D systems [8], the decoupling of matrix stiffness and cell morphology in the 3D system needs to be reexamined on 2D substrates.

In our study, we investigated the relationship of matrix stiffness and cell morphology in the regulation of osteogenesis and adipogenesis of BMSCs. We applied different concentrations of adhesive protein fibronectin (FN) to modulate cell morphology on substrates of different stiffness to study their interplay on osteogenesis and adipogenesis of BMSCs and YAP/TAZ activity. We believe this study could help elucidate the intricate mechanotransduction process of stem cells and facilitate tissue engineering in the future.

2 Methods & Materials

2.1. Isolation and culture of rBMSCs

We isolated rat bone mesenchymal stem cells from 3-week-old male Sprague–Dawley (SD) rats. Isolated cells were cultured in alpha-modified essential medium (α -MEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). Non-adherent cells on tissue culture plates were removed. The cells were passaged after reaching 70% to 80% confluence and collected with 0.1% trypsin/EDTA solution. We used the second-passage BMSCs in the following study.

2.2 Adipogenic-osteogenic co-induction of BMSCs under different concentrations of fibronectin on tissue culture plates

First, fibronectin (FN) (Corning, USA) was diluted with PBS into a concentration gradient of 0, 5, 10, 20 $\mu\text{g/ml}$, and then coated onto tissue culture plates (Corning, USA) before BMSCs were seeded. BMSCs of passage two were plated onto substrates at 5×10^4 cells/ml under adipogenic-osteogenic co-induction (Cyagen Biosciences, China).

Second, to investigate the role of the cytoskeleton under effects of FN on BMSCs differentiation, we set three experiment groups: BMSCs plated on TCPs with 0 $\mu\text{g/ml}$ of FN (group FN), BMSCs plated on TCPs with 1 μM of cytochalasin D (Apexbio, USA) and without FN (group FN0+CD), and BMSCs plated on TCPs

with 10 µg/ml of FN and 1 µM cytochalasin D (group FN10+CD). BMSCs in all groups were cultured in the complete culture medium of adipogenic-osteogenic co-induction (Cyagen Biosciences, China). Three independent experiments were repeated for each group.

2.3 Adipogenic-osteogenic co-induction of BMSCs on 0.5 kPa and 32 kPa CytoSoft® culture plates coated with different concentrations of FN

The CytoSoft® culture plate (Advanced Biomatrix, USA) is a product covered with a thin layer of uniform and biocompatible silicone with standard elastic moduli. The silicone gels are not susceptible to hydrolysis. We selected 0.5 kPa and 32 kPa CytoSoft® culture plates for experiments after ultraviolet sterilization for 1 hour. First, we coated the standard FN concentration (100 µg/ml) the producer recommended on CytoSoft® plates. After preliminary experiments, we found cells on 32 kPa plates coated with 30 µg/ml of FN exhibited similar morphology with 0.5 kPa plates coated with 100 µg/ml of FN. Also, we found cells on 0.5 kPa plates coated with 300 µg/ml of FN showed similarly restricted morphology with cells on 32 kPa plates coated with 100 µg/ml of FN. Then we investigated BMSCs differentiation and YAP/TAZ activity in these groups.

BMSCs in the α-MEM medium of passage 2 were plated onto 0.5kPa and 32kPa CytoSoft® culture plates at densities of 4×10^4 /ml. After 12 hours, cells were exposed to complete medium of adipogenic-osteogenic co-induction (Cyagen Biosciences, China) following protocol in previous study [9]. The adipogenic and osteogenic co-induction medium was replenished every 3 days.

2.4 Osteogenesis and adipogenesis analysis

After 15 days of co-induction, cells were fixed by 4% paraformaldehyde and stained with Fast blue and Oil Red O staining (Solarbio, China) which respectively indicated osteogenesis and adipogenesis of BMSCs. Fast Blue RR/naphthol was applied to stain ALP in cells and followed by Milli-Q water (Millipore, USA) rinsing, and then we rinsed cells by 60% isopropanol and applied Oil Red O to stain fat vacuoles. A Nikon E600 microscope with a color camera (Japan) was used for imaging.

2.5 Immunofluorescence analysis

After 15 days of adipogenic/osteogenic co-induction, cells were rinsed with PBS and then fixed with 4% paraformaldehyde for 30 min. Then cells were permeabilized with 0.1% Triton X-100 (Amresco, USA) for 5 min and then rinsed with PBS 2-3 times. Then cells were incubated with mouse monoclonal YAP (Abnova, Taiwan) and TAZ (Abcam, USA) antibody solutions with 1% BSA overnight, and removed at the second day. Then we rinsed cells with PBS for several times before cells were incubated with rabbit anti

mouse secondary antibodies (invitrogen, Thermofisher, USA) for several hours. Later, we rinsed cells with PBS for 3-5 times, and then incubated cells with phalloidin-Rhodamine (Yeasen, China) for 30 min to label filamentous actin cytoskeleton, and then cells were rinsed with PBS for 3 times before treated with 5 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, China) for 1 min to stain nuclei. All the samples were rinsed with Milli-Q water before fluorescence microscope observation (Leica, Germany). We applied ImageJ software to measure cell spreading area on immunofluorescence-staining pictures of F-actin.

2.6 Real-time Rt-PCR

After 15 days of adipogenic-osteogenic co-induction, total RNA in cells was extracted with Trizol reagent (Invitrogen, USA) and cDNA was synthesized with PrimeScript RT Reagent Kit (TaKaRa, Japan) according to the manufacturer’s instructions. The quantitative real-time RT-PCR was then performed using an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, USA). The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was concurrently amplified in each sample as control and was used for normalization. The primers for genes we tested were designed according to NCBI GenBank and are listed in Table 1.

Table 1

Real-Time RT-PCR primers used in our experiments

Target gene	Primer sequences
RUNX2	F: 5'-cctgaactcagcaccaagtcct-3'
	R:5'-tcagaggtggcagtgcatca-3'
OCN	F:5'-accctctctctgctcactctgct-3'
	R:5'-gctccaactccattgttgaggtag-3'
CEBP-α	F:5'-gccaagaagtcggtggataaga-3'
	R:5'-ggcggtcattgtcactggtc-3'
PPARγ-2	F: 5'-gtctcacaatgccatcaggttt -3'
	R:5'-agggggggtgatatgtttgaact-3'
GAPDH*	PF: 5'-gcaagttcaacggcacag-3'
	PR: 5'-gccagtagactccacgaca-3'

*GAPDH, glyceraldehyde-3-phosphate dehydrogenase

2.7 Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) test and SNK-q test was used to perform statistical analysis using SPSS17.0. Data were collected from three independent experiments with at least 50 cells for each calculation. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Increased cell spreading area promoted osteogenesis and inhibited adipogenesis of BMSCs through F-actin assembly

We modulated cell spreading area through different concentrations of fibronectin (FN) at 5×10^4 /ml of MSCs on tissue culture plates (TCPs). FN is the adhesive protein exists ubiquitously in ECM that cells attach to integrin and actin cytoskeleton (Kilian et al, 2012), so we explored whether FN could regulate cell morphology and BMSCs differentiation on TCPs, which are stiffer more than several MPas.

For BMSCs differentiation, initially with 0 $\mu\text{g/ml}$ of FN, cells exhibited both fat vacuoles and ALP staining, and as FN concentration increased to 5,10,20 $\mu\text{g/ml}$, more pronounced ALP staining and fewer fat vacuoles were exhibited, indicating BMSCs commit higher extent of osteogenesis and lower adipogenesis (Fig 1A). Also, Rt-PCR illustrated an increase of osteogenic markers (RUNX2 and OCN) and a decrease of adipogenic markers (PPAR- γ 2 and CEBP- α) along with higher FN concentration (Fig 1B). These results indicated that larger spreading area directed BMSCs into higher extent of osteogenesis and lower adipogenesis. Meanwhile, immunofluorescence staining indicated that an increase of FN concentration significantly promoted filamentous actin assembly and cell spreading area (Fig 2).

Then, we investigated the underlying role of F-actin in BMSCs differentiation induced by FN. On TCPs with 0 $\mu\text{g/ml}$ of FN at a seeding density of 5×10^4 /ml (group FN0), after 14 days of adipo-osteo co-induction, cells exhibited both fat vacuoles and ALP deposition (Fig 3). Cytochalasin D is an inhibitor of F-actin polymerization and elongation. In the group that BMSCs cultured with actin polymerization inhibitor cytochalasin D and 0 $\mu\text{g/ml}$ of FN (group FN0+CD), we found cells exhibited mainly fat vacuoles and less ALP deposition (Fig 3A) and PPAR- γ 2 and CEBP- α exhibited the highest levels in all groups, indicating osteogenesis of BMSCs was inhibited by CD. In the group that BMSCs cultured with 10 $\mu\text{g/ml}$ of FN and CD (group FN10+CD), FN rescued osteogenesis of BMSCs and inhibited adipogenesis compared with group FN0+CD, as more ALP and fewer fat vacuoles were exhibited and RUNX2 and OCN mRNA levels were higher than group FN0+CD (Fig 3). These results illustrated that FN increased cell spreading area and promoted osteogenesis of BMSCs through promoting F-actin assembly.

3.2 Interplay of effects of FN and matrix stiffness on BMSCs differentiation

Cytosoft® plates with elasticity moduli of 0.5 kPa and 32 kPa were chosen for they respectively resembled rigidity of adipose tissue and pre-calcified bone and have been proven to induce BMSCs into adipogenesis and osteogenesis [10]. As we have verified the effects of FN in regulation of cell spreading area, we seeded BMSCs on soft and stiff substrates coated with different concentrations of FN to modulate cell spreading areas.

First, we found increase of FN concentrations on both 0.5 kPa and 32 kPa substrates induced higher osteogenesis and lower adipogenesis of BMSCs (Fig 4). Cells exhibited more pronounced ALP deposition and fewer oil droplets as FN concentration increased from 100 to 300 µg/ml on 0.5 kPa substrates, and from 50 to 100 µg/ml on 32 kPa substrates (Fig 4A). Also, PCR results showed OCN and RUNX2 levels increased while CEBP-α and PPAR-γ levels decreased when FN concentrations increased on both substrates (Fig 4B). Meanwhile, immunostaining results showed cells manifested more spreading morphology as FN concentrations increased on both substrates (Fig 5A).

Second, we found when cell spreading area reached similar levels on 0.5 kPa and 32 kPa substrates through different concentrations of FN, cell differentiation of BMSCs didn't manifest significant difference either. We coated 300 µg/ml of FN on 0.5 kPa substrates (FN300/0.5 kPa group) to achieve similar cell spreading areas with cells on 32 kPa substrates coated with 100 µg/ml of FN (FN100/32 kPa group), and also we coated 30 µg/ml of FN on 32 kPa (FN30/32 kPa group) to reach similar cell spreading areas with cells on 0.5 kPa substrates coated with 100 µg/ml of FN (FN100/0.5 kPa group), as shown in immunostaining of F-actin in Fig 5A. Then we found when cell spreading areas reached similar levels, cells showed similar extent of osteogenesis and adipogenesis on substrates of different stiffness (Fig 4). Cells in both FN300/0.5 kPa group and FN100/32 kPa group showed mainly ALP staining and few fat vacuoles, indicating BMSCs mainly commit osteogenesis (Fig 4A). PCR results showed consistent results, as OCN and RUNX2 mRNA showed similarly high levels in both groups without significant difference, and CEBP-α and PPAR-γ levels showed similarly low levels (Fig 4B). On the contrary, cells in both FN100/0.5 kPa group and FN30/32 kPa group showed little ALP deposition and many fat vacuoles (Fig 4A), and OCN and RUNX2 mRNA showed similarly low levels in both groups without significant difference, and CEBP-α and PPAR-γ levels showed similarly high levels (Fig 4B). These results indicated when cell morphology and matrix stiffness exerted antagonistic effects, the former took over. Also, when cell spreading area was set, increase of matrix stiffness failed to exert significant effects on osteogenesis and adipogenesis of BMSCs.

Finally, we found matrix stiffness could exert effects on osteogenesis and adipogenesis of BMSCs only when cell morphology simultaneously adapted. Coated with the same FN concentration of 100 µg/ml, matrix stiffening from 0.5 kPa to 32 kPa still prominently promoted osteogenesis and suppressed adipogenesis, as more ALP and fewer fat vacuoles were exhibited (Fig 4A). Also, PCR results indicated significantly higher OCN, RUNX2 and lower CEBP-α and PPAR-γ levels as matrix stiffening (Fig 4B). More importantly, cells simultaneously spread to larger areas when matrix stiffness increased, as indicated in immunostaining of F-actin (Fig 5A).

3.3 YAP/TAZ activity of BMSCs under regulation of FN and matrix stiffness

First, we found increase of FN concentration on both 0.5 kPa and 32 kPa substrates induced nuclear translocation of YAP/TAZ of BMSCs. As shown in Fig 5B, YAP/TAZ mainly localized in cytoplasm on 0.5 kPa substrates coated with 100 µg/ml of FN and on 32 kPa coated with 30 µg/ml of FN, while YAP/TAZ translocated to nuclei on 0.5 kPa substrates coated with 300 µg/ml of FN and on 32 kPa coated with 10 µg/ml of FN.

Second, when cell spreading area reached similar levels on 0.5 kPa and 32 kPa substrates through respectively 300 µg/ml and 30 µg/ml of FN, YAP/TAZ immunostaining also manifested similar intensity and localization in both nuclei and cytoplasm. As shown in Fig 5B, in both FN300/0.5 kPa group and FN100/32 kPa group, YAP/TAZ mainly localized in nuclei, while in both FN100/0.5 kPa group and FN30/32 kPa group, YAP/TAZ mainly localized in cytoplasm.

Finally, increase of matrix stiffness promoted YAP/TAZ nuclear translocation of BMSCs when cell morphology could simultaneously adapt. As shown in Fig 5B, on substrates coated with 100 µg/ml of FN, increase of matrix stiffness from 0.5 kPa to 32 kPa significantly promoted YAP/TAZ nuclear translocation.

4 Discussion

BMSCs adapt their morphology under stimulation of surrounding matrix stiffness through cytoskeleton reorganization, while cell morphology is also an independent factor that induces cytoskeletal dynamics and regulates BMSCs differentiation [11-12]. Also, both cell morphology and matrix stiffness have been found to regulate MSCs differentiation through YAP/TAZ activity [13]. Therefore, the relationship of cell morphology and matrix stiffness was complicated but worth exploring.

First, we verified increased FN concentration promoted BMSCs spreading on TCPs, and osteogenesis of BMSCs was thereby promoted and adipogenesis suppressed. Cytochalasin D is an inhibitor of F-actin assembly, and we found CD abrogated effects of FN, while higher FN concentration compensated effects of CD. Therefore, FN promoted cell spreading through F-actin polymerization. The process of cell spreading on 2D substrates have been well-documented with FAs assembly and cytoskeleton polymerization and elongation [14]. Therefore, FN induced cell spreading could be regarded as equivalent to natural cell spreading.

Based on these findings, we used various FN concentrations to alter BMSCs morphology on stiff and soft matrix. Again, we verified FN concentrations could regulate cell morphology on Cytosoft® plates. Further, to unravel the relationship of cell morphology and matrix stiffness, we managed to achieve similar cell

spreading areas on 0.5 kPa and 32 kPa substrates through different FN concentrations. As effects of FN have been equivalent with cell spreading, we could focus on effects of matrix stiffness when cell morphology was set in these situations. Interestingly, we found matrix stiffening from 0.5 to 32 kPa failed to significantly affect MSCs differentiation when cell spreading area was set, and matrix stiffness only exerted significant effects when cell morphology could be simultaneously altered. When cells manifested spreading morphology on both soft and stiff plates, BMSCs commit mainly osteogenesis, while when cells were restricted from spreading regardless on soft and stiff plates, osteogenesis of BMSCs were significantly suppressed while adipogenesis was promoted. These results indicated that matrix stiffness failed to regulate MSCs differentiation when cell morphology didn't exert consistent effects, indicating that matrix stiffness depended on cell morphology to modulate osteogenesis and adipogenesis of BMSCs.

YAP/TAZ are well-documented transcription co-activators that relay biophysical cues and regulate osteogenesis and adipogenesis of BMSCs [16-18]. In our study, we found cell morphology overrode effects of matrix stiffness on YAP/TAZ activity. Cells on soft substrates with spreading morphology induced more prominent YAP/TAZ nuclear translocation than cells on stiff substrates with restricted spreading area, indicating YAP/TAZ was more responsive to cell morphology than matrix stiffness.

Altogether, we found matrix stiffness couldn't exert its effects on BMSCs differentiation without corresponding cell morphology, and cell morphology could be the downstream mechanism of matrix-stiffness-induced BMSCs differentiation instead of an accompanying effect through regulation of F-actin assembly and YAP/TAZ activity.

Similar with our study, Harris et al [19] manipulated cell morphology through the Micropattern technique and found stiff matrix didn't promote osteogenesis of BMSCs set with a small area of $1000\ \mu\text{m}^2$ [19]. Their result indicated matrix stiffness depended on proper cell morphology to regulate BMSCs differentiation. Previous studies found stiff matrix induced cytoskeletal contraction and F-actin elongation [14], while the former alone could be insufficient to significantly affect BMSCs differentiation. In our study, F-actin stress fibers massively assembled in FN-induced BMSCs spreading but didn't significantly assemble in cells on stiff matrix with low FN concentration. Also, Tee et al. found an increase of substrate rigidity barely enhanced cell stiffness when cell morphology was restricted to a small area on Micropattern [20]. Besides, FAs formation and F-actin elongation could be a more important regulator of YAP activity than cytoskeletal contractility, as YAP nuclear localization was reported to be only partially affected by cytoskeletal contraction but predominantly modulated by F-actin assembly [13,16]. These studies all implied that F-actin polymerization underlying cell morphology was indispensable for matrix-stiffness-induced BMSCs differentiation.

Recent researches in 3D systems decoupled cell morphology with effects of matrix stiffness on the regulation of BMSCs differentiation, as researchers found clustering of adhesive ligands on adjacent polymer chains and activation of BMSCs-ECM adhesion was sufficient to initiate downstream pathways and affect BMSCs differentiation in the absence of cell spreading [18,20]. However, on 2D flat substrates

where adhesive ligands were coated on the substrate surface, BMSCs could only increase BMSCs-ECM adhesion through higher cell-matrix contact i.e. cell spreading, and this could be part of the reason why matrix stiffness depended on cell spreading to affect BMSCs differentiation in the 2D culture system.

5 Conclusion

In conclusion, our research showed that cell morphology was a downstream mediator of the effects of matrix stiffness on osteogenesis and adipogenesis of BMSCs on 2D substrates through regulation of F-actin assembly and YAP/TAZ activity. We believe our findings could help elucidate the complicated control system cells constructed in response to mechanical cues and inspire future studies in this field.

Abbreviations

BMSCs Mesenchymal stem cells

FN fibronectin

Declarations

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Study design: J.L; Experimentation: Y.G, Y.Q; Data Collection & Analysis: F.J, C.Y, S.Q; Manuscript writing: Y.G.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Ethical statement

I certify that the current submission is not previously published or under consideration elsewhere and that all submitted images are in compliance with our image manipulation policy.

All animal procedures performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, with approval from Research Ethics Committee of West China Hospital of Stomatology.

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Figures

A

B

Figure 1

Osteogenesis and adipogenesis of BMSCs with 0,5,10,20 $\mu\text{g/ml}$ of FN on TCPs. (A) After 15 days of co-induction, cells on TCPs were stained by Fast Blue-Oil Red O concerning ALP (blue) and fat vacuoles (red) ($\times 100$, $\times 200$). Scale bars, 50 μm . Cells exhibited more ALP deposition and fewer fat vacuoles as FN concentration increased. (B) Real time Rt-PCR results of osteogenic markers (OCN, RUNX2) and adipogenic markers (PPAR- γ 2, CEBP- α) after 7-day co-induction. Cells showed significantly higher ALP and RUNX2 and lower PPAR- γ 2 and CEBP- α as FN density increased. * $p < 0.05$.

Figure 2

BMSCs morphology and cytoskeleton system with 0,5,10,20 $\mu\text{g/ml}$ of FN. (A) Immunofluorescent images of BMSCs on TCPs after 15 days of adipogenic-osteogenic co-induction with different FN densities. Red: F-actin, blue: nuclei ($\times 100$, $\times 200$). Scale bars, 50 μm . Cells manifested more F-actin stress fibers and spread to a larger area with an increase of FN density. (B) Average spreading area of BMSCs calculated by ImageJ software. The average spreading area of BMSCs was measured by ImageJ software. BMSCs were selected randomly in 10 microscopic fields ($N > 50$). When difficult to isolate individual BMSC at high

density, we calculated the average projected area as the total area of cell aggregates dividing cell number (measured by the number of cell nucleus). The cell spreading area significantly increased as FN density rose. *p<0.05.

Figure 3

Cytoskeleton dynamics underlying the regulation of FN on BMSCs differentiation. A. After 15 days of adipogenic-osteogenic co-induction, cells at a density of 5 x10⁴/ml on TCPs were stained with Fast Blue-Oil Red O concerning ALP (blue) and fat vacuoles (red) (×40, ×100; scale bars,200 μm). On substrates

without FN or other adhesive proteins (group FN0), cells exhibited mainly aggregated fat vacuoles and sparse ALP staining. Cells cultured with CD and no FN (group FN0+CD) exhibited mainly fat vacuoles and little ALP staining. Cultured with 10μg/ml FN and CD (FN10+CD), cells showed pronounced ALP deposition and sparse fat vacuoles. B. OCN, RUNX2, PPAR- γ2, CEBP- α mRNA levels of group FN0, group FN0+CD, group FN10+CD after 15 days of osteo-adipo co-induction. *p<0.05.

Figure 4

BMSCs differentiation with different concentrations of FN on soft and stiff substrates. A. After 15 days of adipogenic-osteogenic co-induction, cells on 0.5 and 32 kPa Cytosoft® plates coated with different FN concentrations were stained with Fast Blue-Oil Red O concerning ALP (blue) and fat vacuoles (red) (×40, ×100; scale bars, 200 μm). B. OCN, RUNX2, PPAR- γ2, CEBP- α mRNA levels of after 15 days of osteo-adipo co-induction. *p<0.05.

Figure 5

Immufluorescence staining of F-actin and YAP/TAZ. (A) Immuno-staining of F-actin of BMSCs on 0.5 kPa and 32 kPa substrates after 15 days of adipogenic-osteogenic co-induction. Red: F-actin, blue: nuclei ($\times 100$, $\times 200$). Scale bars, 75 μm . (B) Immuno-staining of YAP/TAZ of BMSCs on 0.5 kPa and 32 kPa substrates. YAP/TAZ manifested nuclear translocation in 0.5 kPa substrates coated with 300 $\mu\text{g/ml}$ of FN and 32 kPa substrates coated with 100 $\mu\text{g/ml}$ of FN, while YAP/TAZ was located in cytoplasm in the other two groups.