Relationship of matrix stiffness and cell morphology in regulation of osteogenesis and adipogenesis of BMSCs

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Abstract

Backgrounds Matrix stiffness has been found to regulate cell morphology, while both cell morphology and matrix stiffness are verified as important factors directing BMSCs (bone marrow mesenchymal stem cells) differentiation. This study aimed to investigate whether matrix stiffness depended on cell morphology to regulate osteogenesis and adipogenesis of BMSCs on 2D substrates.

Methods and results First, we seeded BMSCs on tissue culture plates (TCPs) with different fibronectin (FN) concentrations and cytoskeleton inhibitor cytochalasin D, and FN was found to promote cell spreading and osteogenesis while inhibiting adipogenesis of BMSCs through F-actin reorganization. Based on these, we modulated BMSCs morphology on 0.5 kPa and 32 kPa CytoSoft® substrates through FN. High concentration of FN (300 μg/ml) coated on 0.5 kPa substrates promoted cell spreading to similar levels with 32 kPa substrates coated with 100 μg/ml of FN, and cells in both groups dominantly commit osteogenesis. On the other hand, low FN concentration (30 μg/ml) on 32 kPa substrates induced restricted cell morphology similar with 0.5 kPa substrates coated with 100 μg/ml of FN, and cells in both groups mainly commit adipogenesis. Immunofluorescence indicated nuclear translocation and higher intensity of YAP/TAZ when cells spread to larger areas, regardless of matrix stiffness. However, when cell spreading areas were fixed as similar levels, matrix stiffness didn’t significantly affect YAP/TAZ intensity or location.

Conclusions Matrix stiffness failed to regulate BMSCs differentiation and YAP/TAZ activity without corresponding cell morphology. Cell spreading area could mediate effects of matrix stiffness on osteogenesis and adipogenesis of BMSCs.

Keywords Matrix stiffness · Cell morphology · Osteogenesis · Adipogenesis · BMSCs

Abbreviations

BMSCs Bone marrow mesenchymal stem cells
ECM Extracellular matrix
FA Focal adhesion
F-actin Filamentous actin
FN Fibronectin
TCP Tissue culture plate

FBS Fetal bovine serum
CD Cytochalasin D
DAPI 4′,6-Diamidino-2-phenylindole
qRT-PCR Quantitative real-time polymerase chain reaction

Introduction

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

In the last decade, biophysical cues including matrix stiffness have been found to regulate BMSCs differentiation. BMSCs anchor integrin to extracellular matrix (ECM) and exert traction force from cytoskeleton, and matrix stiffness...
determines the resistance they receive [2]. Rigid substrates of 25–40 kPa produce high resistant force to BMSCs and induce FAs (focal adhesions) assembly, F-actin (filamentous actin) polymerization, and osteogenic differentiation. However, soft substrates of 0.5–5 kPa inhibit F-actin assembly and favor adipogenic differentiation [2–4]. For downstream mechanism, transcription regulators YAP/TAZ of Hippo pathway have been found to relay mechanical cues and interact with transcription factors including RUNX2 to regulate BMSCs differentiation. Stiff matrix induces activation and nuclear translocation of YAP/TAZ promoting osteogenesis of BMSCs, while soft matrix results in YAP/TAZ inhibition and cytoplasmic localization which induces adipogenic differentiation [4].

On flat substrates, cytoskeleton dynamics induced by matrix stiffness result in cell morphology change: on rigid substrates cells spread to large areas with high cytoskeletal tension while rounding up to small areas on soft substrates [5]. However, independent of external mechanical stimuli, cell morphology has been found to directly regulate BMSCs differentiation and YAP/TAZ activity [6]. Spreading morphology activates YAP/TAZ and promotes osteogenesis of BMSCs, while restricted morphology inhibits YAP/TAZ and induces adipogenesis [7, 8]. These findings raised a question that whether cell morphology an accompanied effect or the downstream mediator of BMSCs differentiation induced by matrix stiffness.

In our study, we investigated the relationship of matrix stiffness and cell morphology in 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 and analyzed cell differentiation. FN is one of the most ubiquitous extracellular matrix proteins that cells attach to integrin and actin cytoskeleton and has been found to promote ECM-MSCs adhesion and F-actin elongation, of similar mechanism with cell spreading [5, 7, 9–11]. We believe this study could help elucidate the intricate mechano-transduction process of stem cells and facilitate the development of tissue engineering.

Methods and materials

Isolation and culture of BMSCs

We harvested tibias and femurs from male 2-week-old Sprague–Dawley rats to isolate BMSCs, according to protocols of previous studies [12, 13]. A 5 ml syringe containing 4 ml of growth medium was used to flush out the bone marrow under sterile condition. The growth medium was composed of α-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). Cells were collected by centrifugation of the bone marrow (200 g, 10 min) and then re-suspended in growth medium. After 2 days of culture at 37 °C with a humidified atmosphere of 5% CO2, cells failing to adhere were removed by changing medium. After 5–7 days of primary culture, each flask was passaged into 2–3 flasks every 3–4 days. The same SD rat donor was applied in a single experiment.

Osteogenic-adipogenic co-induction of BMSCs under different concentrations of fibronectin on tissue culture plates

First, FN (Corning, USA) was diluted with PBS in a concentration gradient of 0, 5, 10, 20 μg/ml and then added to tissue culture plates (TCPs) (Corning, USA). After FN coated on TCPs for 1 h on horizontal rotator. BMSCs of passage two were plated onto substrates at 5 × 10^3 cells/cm² in growth medium. After 24 h of adhesion, cells were treated with osteogenic-adipogenic co-induction medium for 15 days, and the medium was changed every 3 days. The osteogenic medium contained 50 μM ascorbic acid, 10 mM b-glycerophosphate, and 10 nM dexamethasone and growth medium [3]. Adipogenic medium contained 250 mM IBMX, 25 mM indomethacin, 5 mg/mL insulin, 25 mg/mL ascorbic acid, 100 nM dexamethasone, 5 mM β-glycerophosphate and growth medium (all from Sigma, USA). Adipogenic and osteogenic medium were mixed at 1:1 (v:v) as co-inductive medium [14].

Osteogenic-adipogenic co-induction of BMSCs with fibronectin and cytochalasin-D on tissue culture plates

BMSCs of passage 2 in growth medium were seeded at 5000 cells/cm² on TCPs without FN (group FN0) or with 10 μg/ml of FN. Cytochalasin D (CD) is an inhibitor of F-actin polymerization [10]. 1 μM of CD (Apexbio, USA) was added to BMSCs at day 2 on TCPs without FN (group FN0 + CD) and TCPs with 10 μg/ml of FN (group FN10 + CD) and replenished every 3 days. Osteogenic-adipogenic co-induction medium replaced growth medium 24 h after cell seeding in all the three groups and the medium was changed every 3 days during 15 days of co-induction.

Osteogenic-adipogenic co-induction of BMSCs on 0.5 kPa and 32 kPa CytoSoft® culture substrates coated with different concentrations of FN

CytoSoft® culture substrate (Advanced Biomatrix, USA) is covered with a thin layer of uniform and biocompatible silicone gel with different elastic moduli, and cells were unable to directly adhere. We selected 0.5 kPa and 32 kPa CytoSoft® substrates because they respectively simulated
stiffness of adipose and cross-linked collagen of osteoid [15]. After sterilization of CytoSoft® substrates with ultraviolet for 1 h, we coated FN diluted in PBS onto CytoSoft® substrates to modulate cell spreading area. 100 μg/ml (manufacturer recommended) and 30 μg/ml of FN were respectively coated onto 32 kPa substrates (group 32 kPa/FN100; group 32 kPa/FN30); 100 μg/ml and 300 μg/ml of FN were coated onto 0.5 kPa substrates (group 0.5 kPa/FN100; group 0.5 kPa/FN300). Then the substrates were put on horizontal rotator for 1 h to let FN sufficiently adhere to silicone gels. After FN adhesion, PBS in wells were quickly replaced by cell suspension of BMSCs of passage 2 in growth medium at densities of 5 × 10^3/cm^2. After 24 h of cell adhesion, cells were exposed to osteogenic-adipogenic co-induction for 15 days with medium changed every 3 days.

**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 60% isopropanol and Oil Red O was applied to stain fat vacuoles. A Nikon E600 microscope with a color camera (Japan) was used for imaging.

To quantify cell differentiation on Cytosoft® substrates, the percentage of cells underwent osteogenesis and adipogenesis was respectively analyzed by counting the number of ALP-stained and Oil Red O positive cells in three randomly selected fields (10 × magnification) [3].

**Immunofluorescence analysis**

After 15 days of osteogenic-adipogenic co-induction, cells were rinsed with PBS and then fixed with 4% paraformaldehyde for 20 min. Then cells were permeabilized with 0.1% Triton X-100 (Amresco, USA) for 5 min and blocked with 5% bovine serum albumin (Sigma, USA) for 30 min and then rinsed with PBS. Cells were incubated with mouse monoclonal YAP (Abnova, Taiwan) and mouse TAZ antibody (Abcam, USA) solutions (1:1) overnight at 4 °C. Cells were rinsed with PBS for 3–5 times before incubation with goat anti-mouse Alexa488 conjugated IgG (Invitrogen, USA) for 2 h at room temperature. Rhodamine-Phalloidin (Yeasen, China) was used to stain F-actin for 30 min and cell nuclei were stained by DAPI (Beyotime, China). Fluorescence staining of cells were observed with a confocal laser scanning microscope (Olympus, FV3000, Japan) and fluorescence microscope (Leica DMi8, Germany).

Finally, images were analyzed by ImageJ software. Average cell spreading areas were analyzed with all cells in 3 randomly selected microscopic fields of Rhodamine-Phalloidin images (10× magnification), and about 200 cells on TCPs and 80–90 cells on Cytosoft® plates were included for analysis in every field. YAP/TAZ nuclear:cytoplasmic fluorescence intensity ratio and YAP/TAZ intensity normalized to DAPI were analyzed with all cells in 3 randomly selected microscopic fields (20× magnification), and 30–40 cells were included for analysis in every field.

**qRT-PCR analysis**

After 15 days of co-induction, qRT-PCR was applied to quantify mRNA expression levels. Total RNA in cells was extracted with Trizol reagent (Invitrogen, USA) and cDNA was synthesized with PrimeScript RT Reagent Kit (TaKaRa, Japan) and analyzed with SYBR Premix Ex Taq (Takara, Japan) in an ABI PRISM 7300 Real-time PCR System (Applied Biosystems, USA). The primers for genes were designed according to NCBI GenBank shown in Table S1. Relative mRNA expression was calculated using a 2^−ΔΔCt method by normalizing with GAPDH housekeeping gene expression and presented as fold increase relative to control.

**Statistical analysis**

Data were presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) test and SNK-q test was used to perform statistical analysis using SPSS17.0 (Chicago, USA). Data were collected from three independent experiments. P < 0.05 was considered statistically significant.

**Results**

**FN promoted cell spreading and osteogenesis of BMSCs through F-actin assembly**

We explored whether FN could regulate cell morphology and BMSCs differentiation on TCPs, which are stiffer than several MPa [16]. Immunofluorescence staining indicated that increased FN concentration significantly increased F-actin assembly and cell spreading area (Fig. 1). For BMSCs differentiation, initially with 0 μg/ml of FN, cells exhibited both fat vacuoles and ALP, and as FN concentration increased, more pronounced ALP and fewer fat vacuoles were shown, indicating BMSCs commit higher extent of osteogenesis and lower adipogenesis (Fig. 2A). Also, qRT-PCR results illustrated an increase of osteogenic markers (RUNX2 and OCN) and a decrease of adipogenic markers (PPAR-γ2 and CEBP-α) with higher FN concentrations (Fig. 2B).
Then, we investigated the underlying role of F-actin in BMSCs differentiation and cell morphology induced by FN. In group FN0, after 15 days of osteogenic-adipogenic co-induction, cells exhibited both fat vacuoles and ALP deposition (Fig. 3). In group FN0 + CD, cells exhibited mainly fat vacuoles and little ALP deposition (Fig. 3A). Also, OCN and RUNX2 showed lower levels while PPAR-γ and CEBP-α exhibited higher levels in group FN0 + CD than group FN0, indicating osteogenesis of BMSCs was significantly inhibited while adipogenesis was promoted by CD. In group FN10 + CD, more ALP and fewer fat vacuoles, higher RUNX2 and OCN mRNA levels were shown compared with group FN0 + CD, indicating FN rescued osteogenesis of BMSCs (Fig. 3A). Meanwhile, CD significantly inhibited cell spreading and cells mainly manifested round shape (Fig. 3B). However, administration of FN rescued cell spreading, and more prominent F-actin were shown in group FN10 + CD (Fig. 3B). These results indicated that FN increased cell spreading area and osteogenesis of BMSCs through F-actin assembly.

**Interplay of effects of FN and matrix stiffness on BMSCs spreading area and differentiation**

As we have verified the effects of FN on cell spreading area, we seeded BMSCs on soft and stiff substrates coated with different concentrations of FN to modulate cell morphology. After preliminary experiments, we found BMSCs on 32 kPa substrates coated with low FN concentration (30 µg/ml) exhibited similar spreading area with 0.5 kPa substrates coated with 100 µg/ml of FN, and cells on 0.5 kPa substrates coated with high concentration of FN (300 µg/ml) showed similarly spreading morphology with cells on 32 kPa substrates with 100 µg/ml of FN (Fig. 4A). Then we investigated BMSCs differentiation and YAP/TAZ activity in these groups.
We found when cell spreading area reached similar levels on 0.5 kPa and 32 kPa substrates, cell differentiation directions were highly similar (Fig. 4B, C). Cells exhibited more ALP deposition and fewer fat vacuoles as FN concentration increased. B qRT-PCR results of osteogenic markers (OCN, RUNX2) and adipogenic markers (PPAR-γ, CEBP-α) after 15-day co-induction. Cells showed significantly higher ALP and RUNX2 and lower PPAR-γ and CEBP-α as FN density increased. *p < 0.05. (Color figure online)

Fig. 2 Osteogenesis and adipogenesis of BMSCs with different concentrations of FN on TCPs. A After 15 days of osteogenic-adipogenic co-induction, cells on TCPs were stained by Fast Blue-Oil Red O concerning ALP (blue) and fat vacuoles (red) (×100, ×200). Scale bars, 100 µm. Cells exhibited more ALP deposition and fewer fat vacuoles as FN concentration increased. B qRT-PCR results of osteogenic markers (OCN, RUNX2) and adipogenic markers (PPAR-γ, CEBP-α) after 15-day co-induction. Cells showed significantly higher ALP and RUNX2 and lower PPAR-γ and CEBP-α as FN density increased. *p < 0.05. (Color figure online)

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

First, we found when cell spreading area reached similar levels on 0.5 kPa and 32 kPa substrates, YAP/TAZ also manifested similar intensity and localization. As shown in Fig. 5, in both 0.5 kPa/FN300 group and 32 kPa/FN100 groups, YAP/TAZ mainly localized in nuclei with high intensity, while in both 0.5 kPa/FN100 group and 32 kPa/FN30 groups, YAP/TAZ mainly localized in cytoplasm with lower intensity than the former 2 groups.

Second, we found increase of FN concentration on both soft and stiff substrates induced activated YAP/TAZ. YAP/ TAZ exhibited higher intensity and nuclear-cytoplasm ratio in 0.5 kPa/FN300 group than 0.5 kPa/FN100 group, and also in 32 kPa/FN100 group than 32 kPa/FN30 group (Fig. 5).
Discussion

Both matrix stiffness and cell morphology have been found as paramount biophysical regulators of BMSCs differentiation. However, it remains unclear whether cell morphology is an accompanied effect or the downstream mediator of matrix-stiffness-induced BMSCs differentiation.

In this study, we verified FN promoted both cell spreading area and osteogenesis of BMSCs differentiation through F-actin reorganization. Similar to our results, researchers found FN promoted cell adhesion to substrates which further induced stress fiber alignment and cell spreading in many systems, including plastic dishes, hydroxyapatite surfaces and 3D microenvironment [17–19]. Based on these results, we applied FN to manipulate cell spreading area on 0.5 kPa and 32 kPa substrates and unravel the relationship of cell morphology and matrix stiffness. Interestingly, when cell spreading areas reached similar levels under different FN concentrations, matrix stiffening from 0.5 to 32 kPa failed to significantly alter BMSCs differentiation. However, cell spreading area induced by FN showed robust effects on osteogenesis and adipogenesis of BMSCs on both soft and stiff substrates. These results indicated that matrix stiffness failed to regulate BMSCs differentiation when cell morphology didn’t exert consistent effects. Furthermore, transcription co-factors YAP/TAZ [20–22] activity corresponded to cell differentiation, as matrix stiffening from 0.5 to 32 kPa didn’t significantly alter YAP/TAZ intensity and localization when cell morphology was fixed, while when cells became more spreading, YAP/TAZ was activated and showed nuclear accumulation regardless of matrix stiffness.

Altogether, matrix stiffness didn’t exert classic effects on BMSCs differentiation without corresponding cell morphology adaption. Cell morphology could be the downstream mediator of matrix-stiffness-induced BMSCs differentiation through F-actin reorganization and YAP/TAZ activity.
Similar with our study, Harris et al. [23] manipulated cell morphology through FN islands of different sizes and found BMSCs mainly commit adipogenesis instead of osteogenesis with small spreading areas on stiff substrates, indicating the dependence of effects of matrix stiffness on cell morphology. For downstream cytoskeletal dynamics, studies found cell spreading promoted F-actin assembly and stress fiber elongation, and stiff matrix induced both cytoskeletal contraction and F-actin elongation within cells. Tee et al. [24] found increase of matrix stiffness barely enhanced cell stiffness when cell morphology was restricted to a small area, and cytoskeletal contraction alone was found to be insufficient to induce BMSCs differentiation [20]. Also, F-actin assembly more significantly modulate YAP activity than cytoskeletal contraction [25, 26]. These studies implied that F-actin assembly underlying cell spreading could be indispensable for BMSCs differentiation and YAP/TAZ activity.

However, recent researches of 3D hydrogels showed different results with 2D systems. Some studies decoupled cell morphology with hydrogel stiffness in regulation of BMSCs differentiation, as researchers found clustering of adhesive ligands on hydrogel polymers was sufficient to affect BMSCs differentiation without cell spreading [22, 27]. However, on flat substrates where adhesive ligands were coated on
surface, BMSCs could only increase cell-ECM adhesion through larger spreading areas, and this may account for the dependence of matrix stiffness on cell spreading area to affect BMSCs differentiation on 2D systems.

**Conclusions**

In conclusion, our research showed that cell spreading area overrode effects of matrix stiffness on YAP/TAZ activity and osteogenesis and adipogenesis of BMSCs, and matrix stiffness could depend on cell spreading area to regulate BMSCs differentiation.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-07075-5.

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

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**Availability of data and materials** The datasets during and/or analyzed during the current study available from the corresponding author on reasonable request.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

**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. Grant number: WCHSIRB-D-2019–120.
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