Designing Elastic Modulus of Cell Culture Substrate to Regulate YAP and RUNX2 Localization for Controlling Differentiation of Human Mesenchymal Stem Cells

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To establish a guideline for the design of cell culture substrates to control human mesenchymal stem cell (MSC) differentiation, we quantitatively characterized the heterogeneity in the responsiveness of MSCs to the elastic modulus of culture substrates. We analyzed the elastic modulus-dependent dynamics of a mechanotransducer, YAP, and an osteogenic differentiation factor, RUNX2, in three different MSC lots using a styrenated gelatin gel with controllable elastic modulus. The percentage of cells with YAP in the nucleus increased linearly with increases in the elastic modulus, reaching a plateau at 10 kPa for all the lots analyzed. The increase in the percentage with the substrate elastic modulus was described by the same linear function. The percentage of cells with RUNX2 nuclear localization also increased linearly with increases in the substrate elastic modulus, plateauing at 5 kPa, although the regression lines to the linearly increasing regions varied between lots. These similarities and differences in YAP and RUNX2 dynamics among cell populations are basis to design the substrate elastic modulus to manipulate YAP and RUNX2 localizations.

Keywords Mesenchymal stem cell, styrenated gelatin gel, elastic modulus, mechanotransduction, heterogeneity

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substrates. The resulting StG (33.3 wt.%) was dissolved in phosphate-buffered saline (PBS). The StG solution was mixed with PBS containing 2,2’-azobis[2-(2-imidazolin-2-yl)propane]-dihydrochloride (VA-044, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to prepare 30 wt.% StG sol solution, including 0.032, 0.06, or 0.1 wt.% VA-044. Thirty microliters of the StG sol solution were placed on a glass coverslip (0.12 – 0.17 mm in thickness, 18 mm in diameter; Matsunami, Osaka, Japan) coated with poly(N-isopropylacrylamide) (PNIPAAm) (KOHJIN, Tokyo, Japan). Then, a vinyl-silanized coverslip was placed on the StG sol solution to spread the solution between the glass coverslips. The solution was then incubated at 45°C to gelation for 270 or 300 min. Lastly, the StG gel was detached from the coverslip coated with PNIPAAm and washed with PBS. The surface elastic modulus of the StG gel was regulated by a combination of VA-044 concentration and thermal crosslinking time (Table S1, Supporting Information).

The surface elastic modulus of the StG gel was measured by using an atomic force microscope (JPK NanoWizard 4, JPK Instruments, Bruker Nano GmbH, Germany). A commercial silicon-nitride cantilever with a nominal spring constant of 0.03 – 0.09 N/m was used (qp-BioAC-CI CB3, Nansensors, Neuchatel, Switzerland) for force-indentation analysis. Young’s modulus of the surface was evaluated from force-indentation curves by nonlinear least-square fitting to the Hertz model in the case of a parabolic indenter (tip radius of curvature 30 nm; Poisson ratio: 0.5). The surface elastic moduli were measured at the center of the gel, and at a randomly selected point 2.5 and 5.5-mm away from the center, and were averaged to obtain the surface elastic modulus of the StG gel (Table S1, Supporting Information). For all experiments, the StG gels were used as cell culture substrates after the measurement of the surface elastic modulus.

Cells
Three lots of human bone marrow derived MSCs were used: #70011720 from American Type Culture Collection (Manassas, Virginia), and #471980 and #00003525 from Lonza Japan (Tokyo, Japan), denoted as lots 1, 2, and 3, respectively. These cells were expanded in the mesenchymal stem cell growth medium (MSCGM) (Lonza Japan) on tissue culture polystyrene surfaces (Corning Japan, Tokyo, Japan) at 37°C in a humidified atmosphere with 5% CO2. MSCs at passage 4 were used for all experiments.

Osteogenic differentiation assay
Mesenchymal stem cells were seeded on a 12-well polystyrene tissue culture plate coated with collagen I (Corning Japan, Osaka, Japan) at 3100 cells/cm² and incubated in MSCGM at 37°C in a humidified atmosphere with 5% CO2. After 24 h, the cells were cultured in an osteogenic induction medium (Lonza Japan) for 4 days, replacing the medium every 2 days. After 4 days, the MSCs were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) for 15 min, then permeabilized and blocked with PBS containing 0.5% Triton X-100 (Sigma Aldrich), 10% donkey serum (Sigma Aldrich), and 1% albumin from bovine serum (FUJIFILM Wako Pure Chemical Corporation) for 45 min. The cells were incubated with a primary antibody, rabbit anti-YAP (1:100, Cell Signaling Technology Japan, Tokyo, Japan) or goat anti-RUNX2 (1:15, R&D Systems, Minneapolis, Minnesota) for Y AP and RUNX2 labeling, respectively, at 4°C overnight. Subsequently, the cells were incubated with a secondary antibody for 60 min at 37°C. As the secondary antibody, anti-rabbit IgG conjugated with Alexa Fluor 488 (1:1000; Cell Signaling Technology Japan) was used for YAP labeling, and anti-goat IgG conjugated with Alexa Fluor 564 (1:1000; Cell Signaling Technology Japan) was used for RUNX2 labeling. Finally, the cells were immersed in PBS containing DAPI (5 μg/mL; Cell Signaling Technology Japan) to label DNA.

Fluorescent microscopy
For the evaluation of YAP and RUNX2 localization, the MSCs were detached from the tissue culture polystyrene surfaces and seeded onto a StG gel at 2500 cells/cm² and cultured in MSCGM for 4 days, replacing the medium every 2 days. After 4 days, the MSCs were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) for 15 min, then permeabilized and blocked with PBS containing 0.5% Triton X-100 (Sigma Aldrich), 10% donkey serum (Sigma Aldrich), and 1% albumin from bovine serum (FUJIFILM Wako Pure Chemical Corporation) for 45 min. The cells were incubated with a primary antibody, rabbit anti-YAP (1:100, Cell Signaling Technology Japan, Tokyo, Japan) or goat anti-RUNX2 (1:15, R&D Systems, Minneapolis, Minnesota) for YAP and RUNX2 labeling, respectively, at 4°C overnight. Subsequently, the cells were incubated with a secondary antibody for 60 min at 37°C. As the secondary antibody, anti-rabbit IgG conjugated with Alexa Fluor 488 (1:1000; Cell Signaling Technology Japan) was used for YAP labeling, and anti-goat IgG conjugated with Alexa Fluor 564 (1:1000; Cell Signaling Technology Japan) was used for RUNX2 labeling. Finally, the cells were immersed in PBS containing DAPI (5 μg/mL; Cell Signaling Technology Japan) to label DNA.

The fluorescent images were acquired with a laser scanning confocal microscope (FV 3000, Olympus, Tokyo, Japan) with a UPLFLN 10x (numerical aperture (NA), 0.3) objective lens (Olympus). The z-slice images were acquired with pin hole diameter of 151 μm, scanning rate of 2.0 μs/pixel, scanning size of 1086 × 1086 pixels at 1.24 μm pixel pitch, and z-section interval of 6.0 μm.

Adipogenic differentiation assay
Mesenchymal stem cells were seeded on a 24-well polystyrene tissue culture plate (AGC Techno Glass, Shizuoka, Japan) at 21000 cells/cm² and incubated in MSCGM at 37°C in a humidified atmosphere with 5% CO2. The MSCGM was replaced with fresh medium every 2 – 3 days until the cells reached confluence for 5 days. At confluence, adipogenic differentiation was induced with 3 cycles of induction/maintenance. Each cycle consisted of 3 days of culture with an adipogenesis induction medium (Lonza Japan) followed by 1 day of culture with an adipogenic maintenance medium (Lonza Japan). After the 3 cycles of induction/maintenance, the cells were cultured in the adipogenic maintenance medium for 7 days. During the maintenance culture, the medium was replaced with a fresh one every 2 – 3 days. To observe lipid vacuoles in the adipogenic induced cells, the cells were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) for 60 min, then immersed in 1.8 mg/mL Oil Red O (Sigma Aldrich) with 60% isopropanol (FUJIFILM Wako Pure Chemical Corporation) for 20 min at room temperature.

The samples were rinsed by distilled water and 60% isopropanol and photographed using a single-lens reflex camera (D7000, Nikon).
To observe the cells in suspension, the paraformaldehyde fixation was performed to cells attached to a poly-L-lysine (Sigma Aldrich) coated glass substrate by electrostatic interaction. MSCs were seeded onto a glass substrate coated with poly-L-lysine at 5000 cells/cm² in MSCGM for 10 min and immediately fixed with 4% paraformaldehyde before integrin-based cell adhesion occurred. The RUNX2, YAP, and DNA were stained with the same method applied to the adhered cells. The fluorescent images were acquired by using a laser scanning confocal microscope (FV 3000, Olympus) with a UPlanSApo 60× (NA, 1.42) oil-immersion objective lens (Olympus). The z-slice images were acquired with pin hole diameter of 202 μm, scanning rate of 2.0 μs/pixel, scanning size of 800 × 800 pixels, and z-section interval of 0.4 μm.

Quantitative image analysis of YAP and RUNX2 localization

A stack of the confocal images was z-projected at maximum intensity to calculate the nuclear to cytoplasmic ratios of YAP and RUNX2. The ratios were obtained by dividing the mean fluorescent intensity of the nucleus area, which was determined from the DAPI images, by the mean fluorescent intensity of randomly selected regions (10 × 10 pixels at 1.24 μm pixel pitch) of the cytoplasm around the nucleus on the same slice. For both YAP and RUNX2, the ratio of 1.6 or more was regarded as nuclear localization. This threshold value was set in reference to the results of imaging based evaluation for nuclear YAP and RUNX2 in previous studies.

To quantitatively characterize the YAP and RUNX2 dynamics in relation to the substrate elastic modulus, a regression line was calculated using the least-square method for a linearly increasing region in the scatter plot of the percentage of cells with nuclear YAP or RUNX2 against the substrate elastic modulus. Saturated values of the percentage of cells in the scatter plots were calculated by averaging the percentages deviated from the linear increasing region, specifically, at the points of 10 kPa or more for YAP, and at the points 5 kPa or more for RUNX2.

Results and Discussion

Lot-to-lot variation of differentiation potential

We first evaluated the heterogeneity of the differentiation potential of the three lots used for the analysis. All three MSC lots showed potential for differentiation into both osteoblast and adipocyte, although the balance of osteogenic and adipogenic differentiation potential varied. Alizarin red S staining showed the osteoblastic differentiation 14 days after the induction of differentiation (Fig. 1(a)). Calcium deposition detected by Alizarin red S staining was observed in all three lots, and the quantity of deposition varied. Specifically, lot 3 showed the highest deposition, followed by lot 2 and 1. Oil Red O staining showed the adipogenic differentiation 20 days after the induction of differentiation (Fig. 1(b)). Lipid droplets were detected by Oil Red O staining in all three lots, and the quantity of deposition was found to vary. Specifically, lot 3 showed a lower deposition than the other samples. These results suggested that each lot had different differentiation potential to osteoblasts and adipocyte. Differentiation into osteoblast or adipocyte is known to be competitively and delicately balanced in MSCs.

Initial localization of YAP and RUNX2 before exposure to mechanical cues from elastic StG gel

The responses of YAP and RUNX2 to substrate elastic modulus were assumed to vary among MSCs with different differentiation potentials. To evaluate the responsiveness of YAP and RUNX2, we first examined the initial localization of YAP and RUNX2 in the MSCs expanded on tissue culture polystyrene surfaces. Figure 2(a) shows an MSC in suspension, that is, before detecting substrate elastic modulus. As shown in the center of Fig. 2(a), YAP diffused between the nucleus and cytoplasm. In contrast, as shown in the bottom of Fig. 2(a), RUNX2 was localized in the nucleus. YAP diffusion and RUNX2 nuclear localization were the same tendency in all three lots (Fig. S1, Supporting Information). The nuclear localization of YAP is caused by actin development. Consistently, in the initial state of the MSCs in suspension, the actin filament was depolymerized, which may cause YAP diffusion. Although RUNX2 is not supposed to be
Differentiation. In osteogenic-biased MSCs, RUNX2 is retained localized in the nucleus in the initial state of the MSCs. Because structure and/or nucleoskeleton, regardless of actin disturbance.RUNX2 was released from the nucleus in MSCs exposed to mechanical cues from the stiff substrate (13.4 kPa) are provided in Fig. 2(b). In the MSCs cultured on the soft substrate, both YAP and RUNX2 were diffused in MSCs cultured on a substrate with an elastic modulus lower than 5 kPa, whereas localized in the nucleus in MSCs cultured on a substrate with an elastic modulus over 10 kPa. The nuclear localization of YAP requires the development of actin cytoskeleton, which is affected by the surface elasticity. Combined with these findings, our results, demonstrating the relationship between the elastic modulus of substrates and the percentages of cells with YAP in the nucleus, suggest that at least 10 kPa elastic modulus is required for the development of actin cytoskeleton well enough to induce the nuclear localization of YAP. As shown in Fig. 3(b), the percentage of cells with RUNX2 in the nucleus increased linearly with an increasing elastic modulus in the substrate up to approximately 5 kPa. The saturation values were 88, 83, and 80% in lots 1, 2, and 3, respectively. The rates of increase in the percentage of the cells with RUNX2 nuclear localization were 8.0, 8.8, and 10.3 in lots 1, 2, and 3, respectively. The extrapolated values of the percentage of the cells with RUNX2 nuclear localization at 0 kPa were 49, 32, and 20% in lots 1, 2, and 3, respectively. These results indicate that RUNX2 was robustly retained in the nucleus of cells, even when exposed to mechanical cues from substrates with an elastic modulus being just about 0 kPa. The extrapolated value at 0 kPa is likely to correspond to the baseline degree of the elastic modulus-dependent changes in the localization of YAP and RUNX2 in MSC populations. Next, we quantified the nuclear to cytoplasmic ratios of YAP and RUNX2, and the percentages of cells with nuclear YAP and RUNX2 depending on the substrate elastic modulus from 1 to 30 kPa (Fig. 3, Fig. S3 (Supporting Information)). As shown in Fig. 3(a), the percentage of the cells with YAP in the nucleus increased linearly with an increasing elastic modulus, reaching saturation at 90, 79, and 87% in lots 1, 2, and 3, respectively, with an elastic modulus of 10 kPa or higher. In Fig. 3(a), all cells that fall below the nuclear to cytoplasmic ratios of 1.6 were excluded from those with YAP nuclear localization, even though YAP to a certain extent was localized in the nucleus. As shown in Fig. S3, the nuclear to cytoplasmic ratio of YAP was saturated with an elastic modulus of 10 kPa or higher at far more than 1.0, i.e., around 2.5 in lot 1, and around 2.0 in lots 2 and 3, indicating that YAP localized in the nucleus in the majority of cells on the substrate with an elastic modulus of 10 kPa or higher in all three lots. For all the MSC lots analyzed, the relationship between the percentage of the cells with YAP in the nucleus and the elastic modulus up to 10 kPa was approximated by a linear function with a slope of 10 and an intercept at zero. The elastic modulus-dependent changes in the localization of YAP in the three MSC lots were consistent with previous reports: YAP is diffused in MSCs cultured on a substrate with an elastic modulus lower than 5 kPa, whereas localized in the nucleus in MSCs cultured on a substrate with an elastic modulus over 10 kPa. The nuclear localization of YAP requires the development of actin cytoskeleton, which is affected by the surface elasticity. Combined with these findings, our results, demonstrating the relationship between the elastic modulus of substrates and the percentages of cells with YAP in the nucleus, suggest that at least 10 kPa elastic modulus is required for the development of actin cytoskeleton well enough to induce the nuclear localization of YAP. As shown in Fig. 3(b), the percentage of cells with RUNX2 in the nucleus increased linearly with an increasing elastic modulus in the substrate up to approximately 5 kPa. The saturation values were 88, 83, and 80% in lots 1, 2, and 3, respectively. The rates of increase in the percentage of the cells with RUNX2 nuclear localization were 8.0, 8.8, and 10.3 in lots 1, 2, and 3, respectively. The extrapolated values of the percentage of the cells with RUNX2 nuclear localization at 0 kPa were 49, 32, and 20% in lots 1, 2, and 3, respectively. These results indicate that RUNX2 was robustly retained in the nucleus of cells, even when exposed to mechanical cues from substrates with an elastic modulus being just about 0 kPa. The extrapolated value at 0 kPa is likely to correspond to the baseline degree of the nuclear retentivity of RUNX2. There are conflicting reports on the effect of substrates with elastic modulus of 2 kPa on RUNX2 localization. Our results on the lot-to-lot variations in the elastic modulus-dependent changes in RUNX2 localization provide an insight into this inconsistency. In lot 1, the percentage of cells with RUNX2 in the nucleus was 78% for 2 kPa substrate, which was consistent with a previous report that found RUNX2 was retained in the nucleus of MSCs cultured on 2.0 kPa substrate. By contrast, in lots 2 and 3, the percentage of cells with nuclear RUNX2 was less than half for 2.0 kPa substrate. This was consistent with previous reports that RUNX2 was removed from the nucleus in MSCs with 2 kPa substrate. The contradiction of previous reports on RUNX2 localization for 2 kPa substrate may result from differences among the MSC population in baseline degree of RUNX2 nuclear retentivity and the rate of the
enhancement of nuclear RUNX2 with substrate elastic modulus. RUNX2 nuclear localization occurred at a lower elastic modulus than with YAP. The difference in the responsiveness of YAP and RUNX2 is probably due to differences in signal transduction pathway downstream of actin. Soft substrates lead to suppression of focal adhesion maturation and actin development. Actin filament depolymerization activates the pathway for YAP phosphorylation and resulting nuclear exclusion of YAP, probably, within the range of less than 10 kPa. On the other hand, RUNX2 localization in the nucleus is suggested to be associated with condensed chromatin. The structural change in actin cytoskeleton under the mechanical cues from soft (< 5 kPa) substrate may promote chromatin decondensation, resulting in a decreased nuclear retentivity of RUNX2.

Relationship between RUNX2 responsiveness to substrate elastic modulus and osteogenic differentiation potential of MSCs

YAP and RUNX2 are known to affect the expression of osteogenic-related genes and differentiation direction. Their responsiveness to extracellular mechanical cues are expected to be related to the differentiation potential of MSCs.

In our analysis using three different lots of MSCs with different osteogenic differentiation potentials (Fig. 1(a)), the elastic modulus-dependence percentages of cells with YAP in the nucleus were the same among all the experimental lots (Fig. 3(a)). The results indicated that YAP responsiveness to substrate elastic modulus was similar regardless of the difference in osteogenic differentiation potential among the MSC lots.

In contrast to YAP, the elastic modulus-dependence of the percentage of cells with RUNX2 in the nucleus was different lot-to-lot (Fig. 3(b)). In particular, the extrapolated values of the percentage of the cells with RUNX2 nuclear localization against 0 kPa, and the plateau values of the percentage in sufficiently stiff (> 5 kPa) substrate varied among the lots. These values, especially the extrapolated values, most likely indicate the baseline degree of nuclear retentivity of RUNX2. Notably, the baseline degree of nuclear retentivity of RUNX2 and the efficiency of osteogenic differentiation were found to be negatively correlated. RUNX2 is known to be a transcription factor which localizes in the nucleus and activates osteogenic differentiation-related gene expression in the early phase of osteogenic differentiation, while in the later phase, it suppresses the progress and maturation of osteogenic differentiation. Thus, the baseline degree of nuclear retentivity of RUNX2 may be correlated with the efficiency of RUNX2 release in the later phase of osteogenic differentiation, in MSCs with a sufficient potential of RUNX2 nuclear localization in the early stage. If this is the case, the efficiency of the transition to mature osteogenic differentiation will be low in a population of MSCs with a higher baseline of RUNX2 nuclear retentivity.

Fig. 3 Relationship between the elastic modulus of StG gel and the percentage of cells with (a) YAP or (b) RUNX2 in the nucleus. The percentage is the ratios of the number of cells with nuclear YAP and RUNX2 divided by total cell number.
RUNX2 and PPARγ activities will be regulated by their upstream factor as reported by previous studies.\textsuperscript{22,29}

A design of culture substrate to control YAP and RUNX2 localization in MSC population

MSCs are highly mechanosensitive and biased toward their osteoblastic lineage, losing their multipotency and their ability to self-renew on stiff substrates, such as conventional tissue culture polystyrene. Since the localization of YAP and RUNX2 to the nucleus triggers the early steps of osteoblast differentiation,\textsuperscript{6} cell culture conditions that suppress YAP and RUNX2 nuclear translocation can prevent unintended MSC osteoblastic differentiation.

The elastic modulus-dependent change in the percentage of cells with YAP localized to the nucleus was the same in different lots, that is, in different MSC populations. The relationship between the percentage of YAP nuclear localization and the elastic modulus (Fig. 3(a)) suggests that substrates higher than 10 kPa can promote YAP nuclear localization. Additionally, the substrate elastic modulus required to suppress YAP nuclear localization could be predicted based on the linear function, which describes the increase in the percentage of YAP nuclear localization below 10 kPa.

As for RUNX2, culturing on substrates with an elastic modulus higher than 5 kPa ensures the stability of RUNX2 nuclear retention (Fig. 3(b)). The elastic modulus-dependent increase in the percentage of cells with RUNX2 nuclear localization was different from lot to lot. Nevertheless, RUNX2 nuclear localization in a population of MSCs can be controlled by mechanical cues from a soft elastic culture substrate in a predictable way, based on our finding that the rate of RUNX2 nuclear localization increased linearly with the elastic modulus. By acquiring only two data, which are the percentage of cells with RUNX2 nuclear localization at 5 kPa and the percentage at less than 5 kPa, a regression line can be estimated for unknown MSCs. Based on the regression line, the substrate’s elastic modulus for controlling the percentage of cells with nuclear RUNX2 can be determined.

Differentiation of stem cells is known to be affected not only by substrate elastic modulus but also by chemical properties, such as composition, density and conformation of proteins coating the surface of the culture substrate.\textsuperscript{30-32} Polyacrylamide gels with chemically coupled collagen have been frequently used as the substrate with tunable elastic modulus to investigate the effect of substrate mechanical property on differentiation of MSCs.\textsuperscript{3} The collagen coated polyacrylamide gel involves chemically undefined factors, specifically conformational change in collagen and content of collagen triple helix. The conformation change and amount of the triple helix in chemically coupled collagen is difficult to measure due to sensitivity limitation of circular dichroism spectroscopy for thin collagen layer. The collagen triple helix includes GFOGER sequence, a binding site to integrin.\textsuperscript{31} Since the integrin is a biomechanical sensor that serves as hubs for transmitting signals between cells and the culture substrate,\textsuperscript{34} the uncertainty in the conformation and content of integrin binding site complicates the attempt to characterize and control MSCs. In contrast, cell adhesion to gelatin in StG gel is relatively simple and defined, since the cell adhesion on the gelatin is only via the adhesive ligands contained in culture medium. Chemical composition of the gelatin, denaturated form of collagen is almost the same as collagen, whereas, gelatin itself does not have collagen triple helix including the integrin binding GFOGER sequence. Gelatin-based StG gel that can clearly define the effect of the adhesion ligands enables reproducible analysis and control of differentiation of MSCs. Additionally, considering that clinical grade gelatin has also been developed, StG gel substrate for \textit{in vitro} MSC analysis and preparation is, in combination with \textit{in vivo} analytical technique such as \textit{in vivo} imaging,\textsuperscript{35} a great platform for MSCs studies and medical applications in the future.

Supporting Information

The surface elastic moduli of the StG gel obtained in conditions with different combinations of VA-044 concentration and thermal crosslinking time are summarized in Table S1. Intracellular localizations of YAP and RUNX2 in three different lots of MSCs before and four days after exposure of mechanical cues from the StG gel are shown in Figs. S1 and S2, respectively. Nuclear to cytoplasmic ratios of YAP and RUNX2 in three different lots of MSCs are shown in Fig. S3.

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