The effect of culture conditions on the adipogenic and osteogenic inductions of mesenchymal stem cells on micropatterned surfaces

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1. Introduction

Stem cells are regarded as an ideal type of seeding cells for tissue engineering and regenerative medicine due to their multipotential differentiation and self-renewal ability. The latest decade has witnessed much progress in discovery of various cues to determine the lineage commitments of stem cells [1–8]. While some material cues such as functional groups [9,10], substrate stiffness [11–13], and some cellular factors such as cell shape [14–17] have been realized to influence the differentiation of stem cells, predominant lineage commitments of stem cells reported so far have been induced by supplementary soluble factors added into the basic growth media.

As the induction media are concerned, most of differentiations of stem cells are made along a sole lineage commitment in the corresponding induction media [16–19], and some of in vitro differentiations have also been carried out in a mixed medium towards two potential lineage commitments [14,15]. Nevertheless, no report so far is focused upon a comparative study of the sole induction and co-induction of stem cells under comparable conditions. It is thus a bit ambiguous whether or not a conclusion about an effect obtained from the sole inductions could straightforwardly be applied in a co-induction, and vice versa.

The present study is aimed to make such a comparative study. We cultured bone marrow mesenchymal stem cells (MSCs) in an adipogenic or osteogenic induction medium and also in a mixed 1:1 medium with the same concentration of the key chemicals as those in the sole induction media. Effects of cell size and cell–cell contact will be employed as two demonstrations to check the possible similarity or difference between sole induction and co-induction.

The authors are aware of the cell size effects and cell–cell contact effects on differentiation of MSCs due to our investigation of the cell density effect. The cell density is a very common yet not fully understood factor, which also regulates the lineage commitment of stem cells [20–23]. For MSCs, a high cell density was found to enhance adipogenesis while a low cell density favored

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osteogenesis under an adipogenic-osteogenic mixed medium [14]. The group of CS Chen further indicates, by controlling the spreading of individual cells on squared microislands of length 32, 45 and 100 μm, that the cell spreading area is the underlying phenom-
nological factor to regulate the differentiation of stem cells, because a high plating density must lead to a less spread cell shape. (They used the term ‘shape’ effect to describe the spreading effect. We prefer to use the term ‘size’ to describe the spreading of a cell in two dimensional culture.) Besides cell size, cell—cell contact is also inevitably involved in the effect of cell density and closely coupled with the effects of cell size at different cell densities, as indicated in Fig. 1A. It is thus meaningful to investigate the corresponding effects of cell size and cell—cell contact in a decoupled way, and then to integrate these two factors into the cell density effect.

Herein, the spreading area and contact extent were controlled by a micropatterning material technique. An important fundamental topic in the fields of biomaterials and regenerative medicine is cell—material interactions and cell—cell interactions [24–26]. In this study, the cell—material interaction has been applied as well as investigated. While cell behaviors could be adjusted by both chemical and topographical patterns [27–34], we used chemical patterns with a significant contrast of cell adhesion on a substrate in the present study, as schematically shown in Fig. 1B. The pion-
neering work to localize individual cells on adhesive microislands was done by the groups of Ingber and Whitesides [35]. They used a microcontact printing to generate microislands of proteins such as fibronectin on the background of a self assembled monolayer of oligo(ethylene glycol) (OEG). In order to obtain a more potent and lasting non-fouling background for controlling cell localization in the long process of stem cell differentiation, we improved the micropatterning technique to use a poly(ethylene glycol) (PEG) hydrogel as the background substrate. We also employed an oligopeptide of arginine, glycine and aspartic acid (RGD) as the adhesive chemical. RGD is a sequence existing in some proteins in the extracellular matrix and has been well applied in cell studies and biomaterial modifications [36–40]. Since a PEG hydrogel is chemically inert, RGD cannot be directly linked to it. A transfer strategy has been suggested to prepare micropatterns on PEG hydrogels by us in cooperation with Spatz group [41]. By this approach, we can finally obtain cell-adhesive microislands on the persistent anti-adhesion background.

MSCs in this study were isolated from bone marrow of neonatal Sprague Dawley (SD) rats. Upon culture on micropatterned surfaces, cells are finally localized on those adhesive microislands. Those adherent cells cannot migrate from one microisland to another due to the strong and persistent anti-adhesion PEG hydrogel. So, we can observe cells of varied cell sizes and cell—cell contacts, which are fixed during cell differentiations once the cells adhere on the microislands. Under appropriate microisland areas and spacings as in the present studies, we can select those micro-

islands with given cell sizes and numbers, as schematically pre-

sented in Fig. 1C, which enables the examinations of the effects of the cell size and cell—cell contact on adipogenic and osteogenic differentiations on the level of individual cells.

With the increase of cell seeding density, even the concentration of soluble factors might be increased due to the paracrine of cells, and also concentrations of remained nutrients and metabolism products might be different. By using the micropatterning tech-
nique, we can achieve a series of local cell densities on adhesive microislands by one seeding density, as schematically presented in Fig. 1B and C, and thus the interference of the soluble factors could be completely ruled out. That is why we use the term ‘cell density’ instead of ‘seeding density’ to describe our results on micro-

patterned surfaces. Both co-induction and sole inductions of stem cells on the well-designed micropattern will be examined.

Fig. 1. A: Schematic presentation of the underlying effects of cell density on differentia-
tion of stem cells. B: Design of a micropatterned surface with cell adhesion contrast. Cells are localized on adhesive microislands on a background to resist cell adhesion strongly and persistently. One seeding density and thus the same concentration of paracrine soluble factors, but different cell sizes, cell—cell contacts and cell densities on different microislands. C: Schematic presentation of some microisland cases to examine the effects of cell density, cell size, and cell—cell contact on microislands in a decoupled way.
2. Materials and methods

2.1. Preparation of RGD micropatterns on PEG hydrogels

The micropattern was prepared based on a transfer strategy [17,41]. In brief, our preparation technique includes three stages: fabrication of gold microislands on glass, transfer lithography to move the pattern onto a PEG hydrogel, then converting the gold microislands to RGD microislands. The key stage is the second one. At this stage, we first grafted, in vacuum, a volatile linker allyl mercaptan (Fluka) to the gold microislands; then we polymerized poly(ethylene glycol) diacrylate (PEGDA) (MW 700, Sigma) via an ultraviolet irradiation initiated by 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropophenone (Sigma); the gold microislands on the PEG hydrogels were obtained after peeling the hydrogels off the glass slides. Finally, an aqueous solution of cyclic peptides c(-RGDfK-)-OEG-COCH2CH2SH (MW 836.4) (R: arginine, G: glycine, D: aspartic acid, f:D-phenylalanine, and K: lysine) (Peptides International, USA) was used (25 mM). The thiol-end ligand was easily grafted to gold via the S-Au covalent bond, and the amount of RGD was sufficiently high to guarantee the full coverage of gold microislands. We eventually obtained the RGD microislands on the PEG hydrogels. The sizes of adhesive microislands were well controlled by design of appropriate masks for photolithography at the first stage.

2.2. Isolation and culture of MSCs

We isolated MSCs from neonatal SD rats [42]. The marrow was cultured in low-glucose Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). After 3 days, non-adherent cells on tissue culture plates (TCPs) were removed. We used the second-passage MSCs in the following differentiation inductions.

2.3. Fluorescent staining of cell cytoskeleton and cell nuclei

Cells on micropatterns were rinsed carefully with phosphate buffer saline (PBS) after 1 day of incubation. All samples were fixed with 4% paraformaldehyde in PBS for 15 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min followed by triplicate rinsing with PBS for 5 min each time. Then cells were incubated with 1 μg/ml phalloidin-TRITC (Sigma) for 30 min at room temperature to label the filamentous actins. After rinsed with PBS for 5 min 3 times, cells were treated by 5 μg/ml 4’,6-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min to stain nuclei. Before microscopic observations, all the stained samples were rinsed with Milli-Q water thoroughly.

Fig. 2. Co-induction of adipogenic and osteogenic differentiations of MSCs on TCPs. A–B: Phase-contrast micrographs of MSCs (before induction) of the initial cell densities of 6580 (A) and 52,640 (B) cells per cm². C–D: Brightfield micrographs of MSCs of the initial cell densities of 6580 (C) and 52,640 (D) cells per cm² in the co-induction medium for 7 days. Cells were stained by both Fast Blue and Oil Red O with respect to ALP (blue) and fat vacuoles (red) as indicators of osteogenic differentiation and adipogenic differentiation, respectively. E: Fractions of the osteogenic and adipogenic differentiations of MSCs at the four initial cell densities in the mixed induction medium for 7 days. The p values of t tests of the data are listed in Supplementary Tables S1–3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.4. Co-induction of MSCs on TCPs with different initial cell densities

MSCs in the growth medium (low-glucose DMEM, 10% FBS) were seeded on TCPs with initial cell densities of 6580, 13,160, 52,640, 105,280 per cm². Two hours later, the stem cells were exposed to the adipogenic-osteogenic co-induction medium. Following the protocol reported by McBeath et al. [14], the mixed medium contained high glucose DMEM, 10% FBS, 1 μM dexamethasone, 200 μM indomethacin, 10 μg/ml insulin, 0.5 mM 3-mercaptopropionylglycine, 50 μM ascorbic acid-2-phosphate, and 10 μM β-glycerophosphate (Sigma). After 3 days, the medium was replaced by the adipogenic-osteogenic maintenance medium (high glucose DMEM, 10% FBS, 10 μg/ml insulin, 50 μM ascorbic acid-2-phosphate, 10 μM β-glycerophosphate, and 100 μM dexamethasone). Two days later, the cells experienced the co-induction medium for another 2 days. Aphidicolin (0.5 μg/ml, Sigma) was added chronically to inhibit proliferation.

2.5. Co-induction and sole inductions of MSCs on micropatterns

The PEG hydrogels with micropatterned surfaces were put in the 12-well TCPs (Corning) and MSCs in the growth medium (low-glucose DMEM, 10% FBS) were seeded at a density of 5 × 10⁴ cells per well, and the non-adherent cells were removed after 1.5 h. The stem cells started to be induced in the differentiation medium 6 h later. For the sole adipogenic differentiation, the stem cells were exposed in the adipogenic induction medium (high glucose DMEM, 10% FBS, 1 μM dexamethasone, 10 μg/ml insulin, 0.5 mM 3-mercaptopropionylglycine (Sigma)) for 3 days, in the adipogenic maintenance medium (high glucose DMEM, 10% FBS, 10 μg/ml Insulin) for 2 days, and in the induction medium for another 2 days. In the case of the osteogenic differentiation, MSCs were cultured in the osteogenic induction medium (high glucose DMEM, 10% FBS, 50 μM ascorbic acid-2-phosphate, 10 μM β-glycerophosphate and 100 μM dexamethasone (Sigma)) for 7 days. The cells experienced the same period of changing medium as the case of sole adipogenic induction.

For the co-induction tests on micropatterns, we used the mixed medium, the same as the co-induction of MSCs on TCPs. The induction lasted for 7 days. Again, the stem cells were exposed to aphidicolin chronically to inhibit MSCs proliferation. While the sole induction media follow our previous publications [17,19], the co-induction media was a 1:1 mixture of osteogenic and adipogenic media, as suggested by McBeath et al. [14].

2.6. Osteogenesis and adipogenesis analysis

We employed the marker of osteoblasts, alkaline phosphatase (ALP) to examine the efficacy of osteogenesis. Fast Blue RR/naphthol was used to stain ALP in cells. The cell nuclei were stained by DAPI, which enabled counting the number of cells on each microisland.

The adipogenesis was indicated by staining fat vacuoles of adipocytes. MSCs were fixed in 10% formalin, rinsed in Milli-Q water and 60% isopropanol, and then treated by Oil Red O (30 mg/ml, Sigma) in 60% isopropanol, and rinsed in water again. Cell nuclei were stained by DAPI.

For the co-induction samples, ALP was stained first, followed by rinsing in Milli-Q water. We then rinsed cells by 60% isopropanol, stained fat vacuoles by Oil Red O (30 mg/ml, Sigma) in 60% isopropanol. After cells were rinsed again by Milli-Q water, the nuclei were stained by DAPI.

The stained cells on micropatterns were photographed for later statistical analysis in an inverted microscope (Axiovert 200, Zeiss) mounted with a CCD (AxioCam HRC, Zeiss). A successful event of adipogenesis was counted when significant red fat vacuoles were seen in a cell on an adhesive microisland, and a successful event of osteogenesis was counted when the cell on an adhesive microisland was significantly blue upon ALP staining. The cells without any successful ALP and fat vacuole staining were non-differentiated cells.

It is important to examine the corresponding fluorescent images of nuclei to ensure the number of cells on adhesive microislands for statistics. There were at least 6000 microislands of 6 areas (177, 353, 707, 1413, 2826 and 5652 μm²) per sample. The statistics was made first for all of appropriate microislands with desired cell numbers (for instance, 1, 2, 4, and 8, as indicated later in section of Results) in one sample, and four samples were further averaged.

2.7. Data analysis

Independent experiments (n = 4) were averaged in the osteogenesis and adipogenesis analysis. A difference was regarded as significant when p < 0.05 in a t test.

3. Results

3.1. Differentiation of MSCs on TCPs of different initial cell densities

Cell density is an important parameter in experimental cell biology and tissue engineering. Before our micropatterning studies, we first confirmed the density effects of adipogenic and osteogenic differentiations of MSCs on TCPs, with the results shown in Fig. 2. The stem cells were incubated in the mixed osteogenic-adipogenic induction medium for 7 days. Then the cells were stained by both Fast Blue and Oil Red O, corresponding to the ALP expression as an indicator of osteogenic differentiation and to the fat vacuoles as an indicator of adipogenic differentiation, respectively. The fractions of osteogenic and adipogenic differentiations in Fig. 2E reflected a significant density effect. It is interesting that the cells seeded in a low density were inclined to an osteogenic lineage commitment.

![Fig. 3. A: Brightfield image of as-prepared micropattern with RGD-grafted gold microislands on the background of a PEG hydrogel. B: Fluorescent micrograph of cell nuclei of MSCs on the micropatterned surface. Dashed circles outline the microislands. The second microisland (from left) in the lower row was designed of diameter of 30 μm, with area 707 μm². The other circles had areas of 1/4, 1/2, 2, 4, and 8 folds of this microisland. C: Distribution of the numbers of adherent MSCs on microislands of 177, 353, 707, 1413, 2826 and 5652 μm², respectively.](image-url)
while the cells in a high density, an adipogenic lineage commitment. Our experiments are consistent with the reports of CS Chen et al. [14].

3.2. Localization of cells on microislands with varied local cell densities

Cells plated in a low density exhibited larger spreading sizes but less cell–cell contacts, while cells seeded in a high density exhibited more cell–cell contacts but smaller spreading sizes. We then used our micropatterning technique to decouple the size and contact effects, as illustrated in Fig. 1B. The micropattern was prepared based on a transfer strategy, and the brightfield micrograph of the microislands on the surface of PEG hydrogel is shown in Fig. 3A. MSCs in the growth medium were seeded at a density of $5 \times 10^4$ cells per sample and the non-adherent cells were removed after 1.5 h. MSCs were well localized on the microislands and the cell numbers were counted after fluorescent staining of cell nuclei, as shown in Fig. 3B. While the smallest two microislands had high probabilities without cell adhesion, the other four microislands were usually occupied by cells. The distribution of cell adhesion number on microislands of areas from 170 to 5600 $\mu m^2$ is summarized in Fig. 3C. So, different local cell densities as well as cell sizes and aggregation numbers could be achieved on adhesive microislands even under one seeding density. It seems worthy of indicating that the migration of cells out of adhesive microislands were prohibited or very less probable because the background of the PEG hydrogel resisted cell adhesion strongly and persistently. So, the local cell density on each microisland could be well kept in the process of later cell induction.

3.3. Differentiation of MSCs on microislands in induction media

After cell seeding on micropatterns, we performed the co-induction of MSCs on micropatterned surfaces as well as the sole osteogenic and adipogenic inductions. The stem cells were incubated in the corresponding induction media for 7 days. Then the cells were stained by both Fast Blue and Oil Red O. The case of single cells on the microislands upon co-induction is demonstrated in Fig. 4A. We here took “a”, “o” and “u” to represent a successful adipogenic differentiation towards adipocytes, a successful osteogenic differentiation towards osteoblasts, and an unsuccessful differentiation remaining the undifferentiated state, respectively. In the case of two cells, all of the six possible combinations of those three cell phenotypes were found, as listed in Fig. 4B. So, it is available for us to make statistics of differentiation fractions for all of desired cases with given cell numbers on microislands of given areas indicated as follows.

3.4. Sole induction and co-induction of MSCs on microislands with varied local cell densities

As illustrated in Fig. 1C, we could examine the effects of local cell density, cell size and cell–cell contact simply by selecting appropriate series of microislands. We first chose the series of varied cell numbers 1–10 on the microislands of 5652 $\mu m^2$ (Fig. 5A), and then carried out statistics of differentiation fractions for the co-induction (Fig. 5B), sole osteogenic induction (Fig. 5C) and the sole adipogenic induction (Fig. 5D). The case of 2 cells on a microisland of 5652 $\mu m^2$ reads $3.5 \times 10^4$ cells per $cm^2$, and thus even this case does not correspond to a low global density. Adipogenesis was increased very significantly with the local cell density in both sole adipogenic induction and co-induction under our experimental conditions and examined ranges. (The $p$ values are listed in Supplementary information.) Different behaviors of osteogenesis were, however, observed in the cases of sole induction and co-induction: while the fraction of osteogenic differentiation was significantly decreased with cell density under co-induction (Fig. 5B), the sole osteogenic induction did not result in a significant monotonic change with cell density (Fig. 5C). The role of cell density contains both the effect of cell size and that of cell–cell contact. So, we decoupled these two effects in the following analysis.

![Fig. 4. A: Typical micrographs of single cells on microislands of 707 $\mu m^2$. MSCs have been co-induced in the mixed medium for 7 days. Cells were stained by Fast Blue for ALP expression as an indicator of osteoblast, by Oil Red O for fat vacuoles as an indicator of adipocytes, and by DAPI as an indicator of cell nuclei. The corresponding fluorescent images of cell nuclei on the micropatterned PEG hydrogel, indicating a single cell on each microisland. B: Typical micrographs of two cells on microislands of 1413 $\mu m^2$ in the mixed induction medium for 7 days. The upper row shows representative brightfield images of two cells after the co-induction. The letters “a”, “o” and “u” stand for “adipocyte”, “osteoblast” and “undifferentiated MSC”, respectively. The lower row shows the corresponding fluorescent images of cell nuclei on the micropatterned PEG hydrogel, indicating two cells on each microisland.](image-url)
3.5. Analysis of differentiation of single MSCs on microislands of varied areas to reveal the size effect

Now we just paid attention upon single cells on RGD microislands of varied areas in order to reveal the size effect. Some representative fluorescent images of single MSCs on microislands of 177, 353, 707, 1413, 2826 and 5652 μm² are shown in Fig. 6A. The statistical percentages of adipogenesis and osteogenesis after the co-induction and the two sole inductions are summarized in Fig. 6B–D. Both co-induction and sole inductions exhibited significant monotonic trends of the effects of cell size: small and large cells prefer to adipogenic and osteogenic commitments, respectively.

![Different cell densities on microislands of given area](image)

**Fig. 5.** Differentiation of MSCs on microislands of 5652 μm² with different local cell densities. A: Fluorescent micrographs of cell nuclei stained by DAPI, indicating local cell numbers from 1 to 10. B: Percentages of adipogenesis, osteogenesis, and undifferentiation of MSCs on the microislands in the mixed differentiation medium for 7 days. C: Percentages of osteogenesis and undifferentiation of MSCs on the microislands under the sole osteogenic induction medium for 7 days. D: Percentages of adipogenesis and undifferentiation of MSCs on the microislands under the sole adipogenic induction medium for 7 days. The average was made over at least 1000 microislands on a sample, and four independent samples were averaged. The data with respect to the case of ‘10’ came from the statistics of microislands with the numbers of cells equal to or more than 10. The p values of t tests of the data are listed in Supplementary Tables S4–8.
3.6. Analysis of differentiation of MSCs with a given size but varied aggregation numbers on microislands to reveal the effect of cell–cell contact

We further made careful statistics of the cell–cell contact effects on microislands at three typical cell sizes, small, medium and large, as shown in Fig. 7. The statistical results of differentiations of cells with desired aggregation numbers under the co-induction and sole inductions are presented in Fig. 8. Cell–cell contact enhanced both adipogenic and osteogenic lineage commitments under the two sole induction conditions. However, contrary to the cell size effect as revealed in Fig. 6, the cell–cell contact effect under the co-induction was rather complicated: the osteogenesis exhibited a non-monotonic change for small cells while the adipogenesis kept increased with the cell–cell contact extent. Such a complication might arise from the competition between adipogenic and osteogenic lineage commitments.

The non-monotonic trend of osteogenesis was relatively less significant on medium and large microislands. It could be understood from relative loose contact of cells on medium and large

![Fig. 6. Differentiation of single MSCs on microislands of a series of sizes. A: Fluorescent micrographs of single MSCs on microislands of varied sizes. Red: F-actin, blue: nuclei. B: Percentages of adipogenesis, osteogenesis, and undifferentiation of single MSCs in the mixed induction medium for 7 days. C: Percentages of osteogenesis and undifferentiation of single MSCs in the sole osteogenic induction medium for 7 days. D: Percentages of adipogenesis and undifferentiation of single MSCs in the sole adipogenic induction medium for 7 days. Four independent samples were averaged. The p values of t tests are listed in Supplementary Tables S9–13. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
microislands than on small microislands with the same aggregation number. So, the three cases of small, medium and large microislands did not contradict with each other, but strengthened the importance of cell–cell contact and emphasized the difference of this effect on osteogenesis under the sole osteogenic induction and co-induction. In all of examined cases, the adipogenesis exhibited a monotonic increase with the aggregation number of cell clusters, let alone sole induction and co-induction.

4. Discussion

4.1. Co-induction versus sole induction of adipogenic and osteogenic differentiations: similar cell size effects but different cell–cell contact effects

While most of in vitro differentiations of stem cells are made along a sole lineage commitment in the corresponding induction medium, the in vivo differentiation of stem cells is frequently faced upon competition between different lineage commitments. Therefore, the examination of an in vitro differentiation towards two potential lineage commitments has its own right. While a sole lineage commitment in an induction medium and simultaneous two lineage commitments in a mixed medium have been reported in stem cell research, we herein make a comparative study of sole induction and co-induction, with the effects of cell size and cell–cell contact as two demonstrations.

Small MSCs were confirmed to favor adipogenesis, while large or more spread MSCs preferred osteogenesis under sole inductions; so, co-induction must lead to a monotonic and significant size effect, as schematically presented in the upper row of Fig. 9. The results of our co-induction of rat MSCs (Fig. 6B) are consistent with those reported by McBeath et al. for human MSCs on a different micropatterned surface [14]. The observations of sole inductions (Fig. 6C and D) also agree with the examinations of human MSCs on another micropatterned surfaces by Song et al. [18]. Our comparative study strengthens the cell size effect of MSC differentiation.

The cell–cell contact effect under co-induction is, however, complicated. Both the sole adipogenic and osteogenic differentiations of MSCs in contact were enhanced compared to single MSCs, as revealed in Fig. 8. Then which will be predominant, adipogenesis or osteogenesis, when stem cells in contact are co-induced in a mixed medium? As schematically illustrated in the lower row of Fig. 9, we indicate, for the first time, the possible competition between adipogenic differentiation and osteogenic differentiation under co-induction as the cell–cell contact effect of MSC differentiation is concerned. We also reveal that the adipogenesis is more sensitive to the cell–cell contact than osteogenesis, for adipogenesis in the co-induction kept increased with the cell–cell contact monotonically while the osteogenesis exhibited a complicated trend, as carefully checked by us and presented in Fig. 8 (especially in the case of small microislands, where the contact effect is more significant). Hence compared with sole inductions, co-induction of MSC differentiation leads to similar cell size effects but different cell–cell contact effects.

4.2. Cell size effect versus cell–cell contact effect with increase of cell density: cooperative for adipogenic differentiation but competitive for osteogenic differentiation

The effect of cell density is a combination of those of cell size and cell–cell contact, as indicated in Fig. 1A. With the increase of cell density, cell spreading sizes decreased and cell–cell contacts increased. The adipogenesis of MSCs increased significantly with cell density as indicated in Fig. 5, because both the size effect and contact effect are beneficial for the adipogenesis as schematically illustrated in the upper row of Fig. 10. The osteogenesis exhibited complicated effects of cell density. With the increase of cell density, the less cell spreading did not favor osteogenesis (Fig. 6C) while the more cell–cell contact enhanced osteogenesis (the middle row of Fig. 8). So, a competition between the cell size effect and cell–cell contact effect must exist, as schematically indicated in the lower row of Fig. 10. Our experiments also revealed that the density dependence was relatively

Fig. 7. Fluorescent micrographs of typical cases of MSCs with desired numbers on adherent microislands to examine cell–cell contact effects of indicated cell sizes. Cell aggregation numbers are (1, 2, 4, 8) for small cells, (1, 2, 4) for medium cells, and (1, 2) for large cells. Dashed circles outline the microislands. Red: F-actin, blue: nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
stronger for adipogenesis (Fig. 5D) than for osteogenesis (Fig. 5C). So, the present comparative study has revealed, for the first time, the interplays (cooperation or competition) between cell size effect and cell–cell contact effect (for adipogenic differentiation or osteogenic differentiation, respectively) with the increase of cell density.

The global density effect revealed by differentiation of MSCs on TCPs (Fig. 2) without patterning shows a trend similar to the local density effect (Fig. 5B), which strengthens the potential interest of patterning to investigate the role of cell density on the differentiation in this study. We would like to roughly divide the cell density $d$ into two regions by the critical contact density $d_{\text{contact}}$ as schematically indicated in Fig. 11. In spite of no strict formula for the critical value, $d_{\text{contact}}$ should be reversely proportional to the spreading areas of MSCs. At sufficiently low density, MSCs are isolated, and thus in region I the size, contact and density effects might be insignificant. Region II exhibits a significant density effect for adipogenesis yet relatively less significant effect for osteogenesis under sole inductions, as indicated in Fig. 11A. However, the co-induction experiments resulted in a significant density effect even for osteogenesis. In fact, while the osteoblast fraction in the case of 10 cells on a microisland of 5652 $\mu$m$^2$ was 72% under the sole osteogenic culture (Fig. 5C), the fraction dropped to only 20% under the co-induction (Fig. 5B). On some very crowded microislands, say, 8 cells on microisland of 1413 $\mu$m$^2$, no osteoblasts were observed and all of MSCs became adipocytes for the available cases in our present experiments. The significant difference of co-induction from sole induction could be interpreted by the competition between osteogenesis and adipogenesis, namely, a significant increase of adipogenesis with cell density indirectly made the osteogenesis decreased, as schematically illustrated in Fig. 11B.

Our comparative investigations of co-induction and sole inductions of MSCs on micropatterned surfaces reveal unambiguously that competition should be taken into consideration in co-
induction of stem cells. That is to say, one should be careful to extend a conclusion from an ideal sole induction to a practical case such as an in vivo application. Cell experiments are done most probably in region II, and thus the effect of cell density is very important for researchers in the fields of cell biology, medicine, and biomaterials.

4.3. Understanding of the geometry effect related to that of cell density

In our experiments, we did not consider the effect of soluble factors underlying the effect of cell density. All of our experimental examinations of the global density effect (Fig. 2), local density effect (Fig. 5), cell spreading effect (Fig. 6) and cell–cell contact effect (Fig. 8) support that cell geometry is very important in differentiation of stem cells. While a comprehensive interpretation is beyond this paper, we tried to preliminarily understand the geometry effects here. In order to check the inherent cell sizes, we measured the spreading areas of free MSCs on TCPs, and the corresponding adipocytes and osteoblasts after respective adipogenic and osteogenic differentiations were examined as well, resulting in $A_{(adipocyte)}: A_{(MSC)}: A_{(osteoblast)} = 1:4:8$. Compared to MSCs, adipocytes were smaller and osteoblasts were larger. This is consistent with our observations on TCPs and micropatterned surfaces: MSCs under a high seeding density or on a small adhesive microisland favored adipogenic differentiation, while the stem cells under a low density or on a large microisland favored osteogenesis. So, the size effect implied in the cell density effect might be due to an inherent trend of a differentiation commitment towards the preferred “natural” state.

As the cell–cell contact effect is concerned, the gap junction has been found to join in this effect according to our previous report in the cases of sole inductions of MSCs on micropatterned surfaces [19]. Discher et al. revealed that material stiffness afforded a potent cue of differentiation of stem cells [11], and Fu et al. illustrated that soft and hard matrices favored adipogenic and osteogenic differentiations, respectively [43]. Their findings stimulated us to understand the effect of cell–cell contact in our experiments as follows: If we take neighbor cells as a microenvironment or matrix of a cell in contact with them, such a matrix might usually be a soft one, which favors adipogenesis more than osteogenesis. So, in our co-induction of MSCs, adipogenesis was more sensitive to the cell–cell contact than osteogenesis. Other cell communication ways might also influence the cell differentiation, yet remain to be confirmed. The eventual interpretation on the molecular level is challenging. We could also understand why osteogenic cells appear to be relatively less sensitive to cell–cell contacts and more sensitive to the interactions with the substrate, by considering their fate in the tissues with more ECM secretion in bones, compared to adipocytes in fats.

We would like to indicate that our conclusions are drawn for cells adhering on two dimensional planes. Here, the so-called ‘size’ refers to the spreading area in two dimensions, which is closely related to cytoskeleton and intracellular stress. CS Chen et al. have

Fig. 9. Schematic presentation of comparison between co-induction and sole inductions as cell size effect (upper) and cell–cell contact effect (lower) on differentiation of stem cells are concerned.

Fig. 10. Schematic presentation of the underlying geometry effects of cell density on adipogenic (upper) and osteogenic (lower) differentiations of stem cells.
illustrated that cytoskeletal tension is the key to the spreading effect [14]. They used merely the effect of cell spreading (size) to interpret the effect of cell density [14]. Our present study reveals that cell–cell contact effect as another geometry factor underlying cell density could not be neglected. First, the difference between the density effects of adipogenesis and osteogenesis is, to a large extent, determined by the interplay between size and contact effects, as indicated by comparison of Fig. 5C and D, and schematically illustrated in Fig. 10. Second, in three dimensions, the cell–cell contact extent at high density must be higher than that on two-dimensional cultures due to more contact directions, and the three dimensional cell volumes might not be so sensitive to cell density as two dimensional spreading areas. So, we anticipate that the effect of cell–cell contact could be much more significant in three dimensional in vitro cultures (let along sole induction and co-induction), and most of in vivo processes. Our literature searching also output some reports mentioning that even osteogenesis was enhanced in three dimensional cell aggregates. For instance, Tobata group found that MSC aggregates loaded in gelatin microspheres produced more ALP than MSC monolayers [44].

Before ending discussion, we would like to mention that the microisland edge or shape can also influence cell spreading and differentiation [45, 46]. While it could not alter the basic conclusions about the effects of cell sizes and cell–cell contacts drawn in the present study based on our circular microislands, further investigation including more regulators might be helpful for more comprehensive understanding of the geometry cues of MSC differentiation on material surfaces. Examination of more differentiation markers besides ALP and lipid vacuoles and more time points in the future might also be very beneficial to better clarify competition between adipogenic and osteogenic lineage commitments in MSC differentiation.

5. Conclusions

This study has made the first comparative study of sole induction and co-induction of stem cells on micropatterned surfaces. We confirmed that cell density was important to influence cell differentiation. With employment of the unique micropatterning technique, we illustrated that cell size (spreading) and cell–cell contact (aggregation) were two implied geometry factors of cell density in regulating the stem cell differentiation. While cell size effects are likewise under sole induction and co-induction, different cell–cell contact and cell density effects have been revealed by us. Based upon our analyses, we have illustrated the competition between adipogenic differentiation and osteogenic differentiation under co-induction as the cell–cell contact effect is concerned. We have also indicated the interplay (cooperation or competition) between cell size and cell–cell contact effects with the increase of cell density (in adipogenesis or osteogenesis, respectively). Hence, the sole induction and co-induction are both very useful to reveal the science of stem cells, and the approach of the comparative study of the two induction ways is fruitful in stem cell research.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.05.010.

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