Effect of RGD nanospacing on differentiation of stem cells

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ABSTRACT

Nanopatterns of a cell-adhesive peptide arginine–glycine–aspartate (RGD) on a persistently non-fouling poly(ethylene glycol) hydrogel were prepared, and behaviours of mesenchymal stem cells (MSCs) on patterns of five RGD nanospacings from 37 to 124 nm were examined under a full level of serum for eight days. Besides cell adhesion, osteogenic and adipogenic inductions of MSCs from rat bone marrow were observed in corresponding media. We not only confirmed the nanospacing dependence of cell spreading previously reported in other cell types (non-stem cells) such as less spreading in the case of nanospacings larger than the critical 70 nm, but also found the effect of RGD nanospacing on lineage commitments of stem cells. Both osteogenic and adipogenic inductions resulted in higher differentiation extents on patterns of large nanospacings than of small nanospacings. Under co-induction in the mixed osteogenic/adipogenic media, osteogenesis was predominant over adipogenesis on patterns of large RGD nanospacings, although a less cell spreading itself was beneficial not for osteogenesis but for adipogenesis according to previous studies without nanopatterns. The effect of RGD nanospacing on lineage commitments of stem cells is unexpected and cannot be interpreted via the cell spreading effect. Thus, the differentiation of stem cells might be regulated inherently by nanospacing of bioactive ligands on the material surfaces.

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1. Introduction

Cell and material interaction is a very fundamental topic in regenerative medicine and biomaterials [1–6]. One of the most important cellular events on a material surface is cell adhesion. Specific cell adhesion is achieved via formation of focal adhesion and triggered by bioconjugation of some ligands in extracellular matrix (ECM) to the corresponding receptor, integrin [7]. The ligands contain some peptide sequences such as arginine–glycine–aspartate (RGD) [8]. The RGD-containing agents have been widely used in biomaterial modification [9–14]. Some regular RGD arrays have also been prepared by grafting RGD onto micropatterns or nanopatterns [15–19]. Considering that an integrin molecule is of about 8–12 nm [20], nanopatterns with RGD peptides grafted onto gold nanodots less than 10 nm are particularly interesting. The underlying nanopattern might lead to a well-defined connection between a single nanodot on the substrate and a single integrin in the cell membrane, and thus precisely design the spatial distribution of the eventual integrins on the molecular level. RGD nanospacing has been revealed as a regulator of specific cell adhesion. The critical nanospacing is around 70 nm [15], and it has been known that the RGD nanospacing within a local cluster is more essential than RGD density to determine cell adhesion [15,21–23]. A shorter nanospacing leads to a more significant cell spreading.

Cell differentiation is another very important cellular event. While there are many publications about material-related differentiation [24–30], the present study distinguishes itself as focusing upon the possibility of a direct nanospacing effect on differentiation of stem cells, as schematically indicated in Fig. 1. Previous studies based upon micropatterning techniques have revealed that cell differentiations of mesenchymal stem cells (MSCs) such as osteogenic and adipogenic lineage commitments are regulated by cell spreading [31,32], and studies on nanopatterns have illustrated that cells on substrates of varied RGD spacings spread quite differently [21,33,34]. It is thus not surprising that different differentiation extents might be observed if one cultured MSCs on RGD nanopatterns of varied nanospacings. It is, however, hard to predict whether or not the different differentiation behaviours come from simply the cell spreading effect or a direct nanospacing effect beyond cell spreading. The latter conclusion might be drawn if the differentiation outputs cannot be fully
interpreted from cell spreading and other existing knowledge. We herein address this question. To confirm or rule out the hypothesis of a nanospacing effect beyond cell spreading drove our present study.

The key to an appropriate RGD nanopattern for study of cell differentiation is a strong and persistent non-fouling background against cell adhesion in culture media with sufficient serum for a long time, and otherwise results of cell experiments might lead to an ambiguous or even wrong conclusion. As usual, one day is sufficient for study of cell adhesion, but one week at least is required for cell differentiation, and a full concentration of serum (usually 10 v% ) is normally used in cell culture for differentiation. So, most of present patterning techniques for study of cell adhesion cannot be simply extended into that of cell differentiation, considering the persistency of the pattern background. Poly(ethylene glycol) (PEG) is a perfect non-fouling molecule, but the non-fouling background generated classically via a self-assembly monolayer of PEG or of block copolymer containing PEG is not sufficiently persistent [35,36]. RGD nanopatterns on hydrogels of PEG possibly resolve this problem; yet, it is hard to fabricate regular RGD nanopatterns straightforwardly on a PEG hydrogel. A transfer lithography strategy was put forward to resolve this problem [37]. In general, this strategy contains three basic steps: preparation of a gold nanopattern on glass by block copolymer micelle nanolithography [38], transfer of the gold nanopattern to a PEG hydrogel surface [39], and grafting RGD agents to gold nanodots [40]. The transfer stage is assisted by a bifunctional linker, which covalently binds both gold and PEG hydrogel. The technique has been applied to prepare nanoscaled patterns to study cell adhesion [37]. Microscaled RGD patterns on PEG hydrogels have also been used by us to reveal the effects of cell adhesion on lineage commitments of MSCs [41].

In this study, we will employ this unique transfer lithography to prepare RGD nanopatterns on PEG hydrogels to study cell differentiation as well as cell adhesion of stem cells. We designed five nanospacings, one around, two below, and two above the critical nanospacing 70 nm. Nanospacings will be controlled by microfabrication conditions such as composition of block copolymers used in preparation of virgin gold nanopatterns on glass. MSCs from rat bone marrow will be cultured on the nanopatterns to examine the feasibility to extend the concept of critical adhesion nanospacing found in non-stem cells into the case of stem cells. Then, osteogenic and adipogenic inductions will be triggered in osteogenic, adipogenic or mixed induction media. If different differentiation extents on nanopatterns of varied nanospacings can be found, our emphasis will be focused upon checking whether or not the nanospacing effect on differentiation of stem cells are reflected simply via cell adhesion such as different spreading areas. The basic idea of the present study is schematically presented in Fig. 1.

2. Materials and methods

2.1. Preparation of RGD-grafted nanopatterns

First, we prepared gold nanopatterns on PEG hydrogels via block copolymer micelle nanolithography plus transfer nanolithography, as schematically presented in Fig. 2. Copolymers of polystyrene-block-poly(2-vinylpyridine) (PS-b-P2VP, Polymer Source) with varied block lengths as indicated in Table 1 were dissolved in toluene to form reverse micelles. Then HAuCl₄ was added into the reverse micelle solution to complex with the micellar core mainly composed of the poly(2-vinylpyridine) block. Subsequently, the solution was dip-coated onto a glass surface. Polymers were removed by an oxygen treatment. Meanwhile the gold acid was reduced to gold, and thus an Au nanopattern on glass was obtained. At the transfer stage, N,N'-bis(acryloyl) cystamine (Sigma) was used as the linker [42]. After treatment of gold nanopatterns with the linker solution, the mixture of macro-monomer PEG-DA (M₀, 700, Sigma) and initiator 2-hydroxy-4’-(2-hydroxyethoxy)-2-methylpropophenone (D2959, Sigma) was added onto the glass. A chemical gel was formed after polymerization triggered via UV illumination. The resultant PEG hydrogel could be peeled off from glass, and thus the Au nanopattern on the surface of the PEG hydrogel was obtained. The nanoparticles on the surface of the PEG hydrogel were characterized by a field-emission scanning electron microscope (FE-SEM, Ultra Plus, Zeiss).

Prior to cell culture, the PEG hydrogel was soaked in a 25 μM aqueous solution of c(-RGDfK-)-thiol ligands (f:D-phenylalanine, K:L-lysine; Peptides International) at 4 °C for 4 h. Due to the easy chemical reaction between the thiul group and gold, RGD peptides were grafted onto gold nanodots. An RGD nanopattern on the PEG hydrogel was eventually generated.
2.2. Isolation and culture of MSCs

Stem cells were isolated from bone marrows of 7-day-old newborn Sprague-Dawley rats. Cells were cultured on tissue culture plates in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco). The growth medium was exchanged and non-adherent cells were removed after 48 h. The second-passage MSCs were used in the following differentiation inductions on RGD nanopatterns. The seeding density in the following cell adhesion and differentiation tests was kept as 6600 cells per cm².

2.3. Immunofluorescent staining of cell cytoskeleton and cell nuclei

After 24 h or 8 days of culture, cells on nanopatterns were rinsed carefully with phosphate buffer saline (PBS) solutions. Samples were fixed with 4% paraformaldehyde for 15 min, followed by being permeabilized with 0.1% Triton X-100 in PBS for 5 min and then rinsed with PBS triplicate (5 min each time). Afterwards the fixed cells were incubated with 1 μg/ml phalloidin–TRITC (Sigma) at room temperature for 30 min to label the filamentous actins (F-actins), and rinsed again with PBS triplicate (5 min each time). To label vinculins, the cells were incubated with a 1:100 dilution of the primary antibody (mouse monoclonal anti-vinculin antibody, Sigma) for 2 h followed by triplicate rinsing with PBS, then the cells were incubated with a 1:100 dilution of the secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG, Invitrogen) for 2 h. Subsequently, cells were treated with 5 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) for 10 min to label cell nuclei. At last the samples were rinsed with Milli-Q water for 6 times (5 min each time). Microscopic observations of stained cells were made under an inverted microscope (Axiovert 200, Zeiss) mounted with a CCD (AxioCam HRC, Zeiss). The single cells were outlined using the software ImageJ (freely available at http://www.nih.gov) to measure cell spreading areas.

2.4. Osteogenic and adipogenic inductions of MSCs

The osteogenic induction medium was composed of high-glucose DMEM, 10% FBS, 50 μM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 100 nM dexamethasone (Sigma). Induction started after 24 h of the initial cell adhesion and

![Fig. 2. Fabrication procedure of RGD nanopatterns on PEG hydrogels. The top row demonstrates the formation of micelles of amphiphilic block copolymer PS-b-P2VP with gold acids enriched in the micellar cores. The middle row shows the process of preparation of a gold nanopattern on glass. The bottom row presents the process of transferring the Au nanopatterns from glass to a PEG hydrogel, which is formed via photopolymerization of the macromonomers of poly(ethylene glycol) diacrylate (PEG-DA), and then grafting RGD motifs onto the gold nanodots to eventually obtain an RGD nanopattern on the surface of a PEG hydrogel.](image)

Table 1

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Mn</th>
<th>Numbers of S &amp; VP units</th>
<th>c (mg ml⁻¹)</th>
<th>v (mm min⁻¹)</th>
<th>LA</th>
<th>Initial spacing (nm)</th>
<th>Eventual average spacing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4705-S2VP</td>
<td>42,500</td>
<td>288:119</td>
<td>7.0</td>
<td>12.5</td>
<td>0.60</td>
<td>34 ± 3.7</td>
<td>37</td>
</tr>
<tr>
<td>P4633-S2VP</td>
<td>115,500</td>
<td>759:247</td>
<td>4.0</td>
<td>25.0</td>
<td>0.40</td>
<td>48 ± 4.8</td>
<td>53</td>
</tr>
<tr>
<td>P5052-S2VP</td>
<td>217,000</td>
<td>1776:304</td>
<td>3.0</td>
<td>12.5</td>
<td>0.37</td>
<td>70 ± 4.0</td>
<td>77</td>
</tr>
<tr>
<td>P4556-S2VP</td>
<td>257,000</td>
<td>1728:732</td>
<td>3.0</td>
<td>16.7</td>
<td>0.35</td>
<td>79 ± 8.9</td>
<td>87</td>
</tr>
<tr>
<td>P3808-S2VP</td>
<td>362,000</td>
<td>2784:684</td>
<td>3.0</td>
<td>16.7</td>
<td>0.30</td>
<td>113 ± 3.5</td>
<td>124</td>
</tr>
</tbody>
</table>

a Concentration of the block copolymers PS-P2VP.
b Velocity in dipping.
c Loading amount of gold acid over copolymer.
d Inter-particle distance of gold nanodots on PEG surfaces before swelling in water.
e Average nanospacing on swelled PEG surfaces. The swelling ratio of the PEG hydrogel was 1.1.
lasted for 7 days. For adipogenic induction, cells were induced for 3 days in the induction medium composed of high-glucose DMEM, 10% FBS, 1× dmex- amethasone, 200 μm indomethacin, 10 mg/ml insulin and 0.5 μm methyl-
tobutyloxanthine (Sigma). Then, the cells were incubated in the maintenance
medium (high-glucose DMEM, 10% FBS, 10 mg/ml insulin) for another two days. At
last the maintenance medium was replaced by the induction medium, and the in-
duction lasted for 2 days more. In co-induction tests, cells were induced in a 1:1
mixture of osteogenic and adipogenic media. In order to diminish the interference
of cell proliferation, 0.5 μg/ml aphidicolin (Sigma) was added into the growth medium
from the forth to fifth day of induction to inhibit cell proliferation.

2.5. Osteogenesis and adipogenesis analysis

We employed alkaline phosphatase (ALP) as the marker of osteoblasts. Cells
positive for ALP would be stained in blue by Fast Blue RR/naphthol (Sigma). For
adipocytes, the fat vacuole was chosen as the marker, which would be stained in red
by Oil Red O (Sigma). In the case of sole inductions, osteogenic MSCs were rinsed in
PBS for 5 min, then fixed in 10% formalin for 15 min, again rinsed in Milli-Q water for
3 times (5 min each time), and then treated by the Fast Blue RR/naphthol solution in
water. For adipogenic MSCs, cells after fixation were rinsed in Milli-Q water and 60%
isopropanol in sequence, then stained with 30 mg/ml Oil Red O in 60% isopropanol,
and followed by another rinse in water. In the case of co-induction, fat vacuoles were
stained after ALP staining. The nuclei of sole induced and co-induced cells were both
stained by DAPI, which enabled counting of the cell numbers. The stained cells on
RGD nanopatterns of different nanospacings were photographed for statistical
analysis. Cells positive for ALP and fat vacuoles were considered as osteogenically
and adipogenically differentiated cells, respectively; those negative for both ALP
and fat vacuoles were considered undifferentiated.

2.6. Real-time polymerase chain reaction (PCR) analysis

After 7 days of sole induction or co-induction on the surfaces of 37-nm-spaced
and 87-nm-spaced nanopatterns following one day of cell adhesion, cells were
acted by the Magnesil mini-isolation System (Promega) to isolate total RNA of
treated cells. The concentration and purity of the isolated RNA were checked by Nanodrop
2000 (Thermo Scientific). 15 μg RNA isolated from different samples were reversely
transcribed into the first strand cDNA using PrimeScript RT reagent Kit with gDNA
Eraser (TaKaRa), and then real-time PCR was performed using Rotor-Gene SYBR
Green PCR Kit (Qiagen) via Rotor-Gene Q 2plex System (Qiagen).

ALP and Runx-related transcription factor 2 (Runx2) were employed as the target
genes of osteogenesis; lipoprotein lipase (LPL), peroxisome proliferator-activated
receptor γ (PPARγ) were chosen as the target genes of adipogenesis; and glyceral-
aldehyde-3-phosphate dehydrogenase (GAPDH) was taken as the housekeeping
gene. The sequences of the corresponding primers are summarized in
Supplementary Table S1. The samples were detected in a Rotor-Gene Q System
(Qiagen). The temperature protocol was set at 95°C for 5 min, then 40 cycles of 5 s at
95°C and 10 s at 60°C. Relative gene expression data were analyzed using the 2−DD
method.

3. Results

3.1. Fabrication of RGD nanopatterns on PEG hydrogels

We used block copolymers as the materials for the fabrication of RGD micelles. For
this system, longer chains usually lead to larger micelles and thus a larger period of the micelles after dip-coating, which
determines a larger spacing of the resultant gold nanodots [21,38]. The nanospacing was also in
fluence of the copolymer concentration and the pulling velocity in dip-coating. Those pertinent
fabrication parameters are listed in Table 1. The sizes of the Au nanodots were determined by the core sizes of the associated block
copolymer micelles and the loading amounts of HAUCl₄ in the micelles [15,38]. The resultant diameters of Au nanodots were about 10 nm.

After grafting RGD ligands to the gold nanodots via formation of S–Au bonds, the gold nanoarray was turned into an RGD nanoarray.
The term “nanospacing” here is defined as the period of the nanopattern, namely, the distance between nearest-neighbour
nanodots arranged hexagonally. Periods on glass were initially designed approximately 30, 50, 70, 90 and 110 nm, and the final
data would be known via microscopic imaging. The nanopatterns were successfully achieved and characterized, as shown in Fig. 3.
The PEG hydrogels would swell in the cell culture medium, which enlarged nanospacements to a certain extent. The eventual nanospacements were 37, 53, 77, 87 and 124 nm, as summarized in Table 1.

3.2. Cell adhesion on RGD nanopatterns of varied nanospacings

We cultured MSCs on those nanopatterns to examine the effect of nanospacing on cell adhesion in the cases of stem cells. The fluorescent micrographs of MSCs cultured for 24 h on nanopatterns are shown in the top and middle rows of Fig. 4. Corresponding statistical results about adhesion of MSCs are presented in the bottom row of this figure. The decrease of cell density and spreading area with the increase of nanospacing is consistent with previous reports about cell adhesion of other cell types (non-stem cells) [15,21,34].

The present study also made the calculation of cell shape parameters on nanopatterns including aspect ratio (AR) and cell
proximity. AR is defined as the ratio between the two principal axes, which were from the two eigenvalues of the corresponding cell
profile calculated by ImageJ software. Circularity is defined as area times 4π and divided by square of perimeter. Circularity 1.0 indicates a perfect circle, and the value close to 0 describes an extremely elongated polygon. Cell circularity was found to be increased with RGD nanopatterns. In the cases of small nanopatterns, cells exhibited more pseudopodia like filopodia and lamellipodia, which enlarged the cell perimeter and thus reduced the cell circularity. Neither cell areas nor those pseudopodia significantly contributed to the global cell shape, and thus AR was not very sensitive to nanospacing, as seen in Fig. 4.

3.3. Confirmation of persistent resistance of the PEG hydrogel to cell adhesion

To make sure this study focused on the nanospacing of RGD ligands, the pattern should have a high contrast of cell adhesion with
a strong and persistent non-fouling background even under a full serum level. Since the following differentiation studies would be
made for 1-day incubation and 7-day induction, MSCs were examined under 10% FBS for 8 days in order to verify the selective
adhesion of the RGD-rich region and the PEG hydrogel background. The hexagonal Au nanoarray was realized by dip-coating, and thus

Fig. 3. FE-SEM images of Au nanopatterns of varied nanospacings on the PEG surfaces.
Fig. 4. Cell adhesion on nanopatterned surfaces with nanospacings indicated in Table 1. The top and middle rows show, respectively, low-magnification and high-magnification fluorescent micrographs of MSCs cultured on nanopatterns for 24 h. Cells were stained to visualize F-actins (red), vinculins (green) and nuclei (blue). MSCs on surfaces of small RGD nanospacings got high densities, and exhibited more spreading morphology, more mature skeleton and stronger focal adhesion. The bottom graphs are statistical results of cell adhesion; the p values from Student t-tests are listed in Supplementary Tables S2–S5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
a dipline existed as the boundary between the coated and uncoated regions on glass initially and between the nanopatterned region and the PEG hydrogel background eventually, as schematically presented in Fig. 2.

Even after 8 days of culture with the full serum, our PEG hydrogel background resisted cell adhesion quite well, and a clear dipline was still observed in the fluorescence micrograph of MSCs. In Fig. 5, MSCs adhered only in the region with the RGD nanopattern, and the PEG hydrogel surface without the nanopattern hardly allowed cell adhesion. To the best of our knowledge, such a strong and persistent anti-adhesion background is very difficult to achieve, which convinces the present study of the effect of RGD nanospacing on differentiation of stem cells.

3.4. Effect of RGD nanospacing on sole induction of MSCs

We then examined induction of MSCs under osteogenic or adipogenic media. Osteogenically induced MSCs were stained by Fast Blue RR salt to show ALP, and adipogenically induced cells were stained by Oil Red O to show oil droplets. Some successful cases of osteogenesis or adipogenesis on RGD nanopatterns are demonstrated in the upper row of Fig. 6. The corresponding fluorescent micrographs of cell nuclei indicated clearly the position of each cell (Supplementary Fig. S1). The combination of the bright-field and fluorescent micrographs enables counting of the numbers of positively and negatively stained cells. Both osteogenic and adipogenic fractions increased with RGD nanospacing (Fig. 6), although such a trend became relatively insignificant when the nanospacing was over the critical adhesion value (>70 nm).

3.5. Effect of RGD nanospacing on co-induction of MSCs

Induction of MSCs could also be conducted in a 1:1 mixture of osteogenic and adipogenic media, as suggested by Chen group [31]. We employed this approach to co-induce osteogenic and adipogenic differentiations of MSCs on our nanopatterns. Both ALP and oil droplets were stained, and thus cells could be sorted into osteoblasts, adipocytes and undifferentiated cells. Now, the adipogenic fraction was decreased, and only the osteogenic fraction was enhanced with RGD nanospacing, as shown in Fig. 7. Cell density decreased (Fig. 4) and osteogenic differentiation fraction among adherent cells increased (Fig. 7) with the increase of nanospacing, and thus the absolute number density of osteogenically differentiated cells as a function of nanospacing was reasonably not monotonic but exhibited a peak at 53 nm, as presented in Supplementary Fig. S3.

Our experiments illustrate that a smaller RGD nanospacing does not necessarily mean a stronger cell response, if both cell adhesion and differentiation are taken into consideration.

3.6. Gene expression on nanopatterns of small and large nanospacings

We further quantified some genes of cells on nanopatterns with RGD nanospacings of 37 nm and 87 nm via quantitative PCR. Runx2 and ALP are two of the characteristic genes of osteoblasts; LPL and PPARγ are two of the characteristic genes of adipocytes. GAPDH was used as the housekeeping gene. The relative gene expression of cells on the 87-nm substrate was presented after normalized by that on the 37-nm substrate. A significant difference of gene expression was found between cells seeded on patterns of different RGD nanospacings. Our results of gene expression (Fig. 8) are basically consistent with those of differentiation fractions in either sole induction or co-induction (Figs. 6 and 7).

4. Discussion

In the present manuscript, we transferred a nanopattern from glass to a PEG hydrogel and examined the differentiation of MSCs. Such an ideal nanopattern appropriate for cell studies is expected to have a period much larger than the nanodots themselves, and thus cannot simply be obtained by close packing of gold nanodots. The block copolymer micelle nanolithography suggested by Spatz et al. [38] was thus employed to obtain the initial gold nanopattern. The resultant nanodots were as small as about 10 nm, while the nanospacings were as large as 37–124 nm. Considering that integrin has a size of about 12 nm [20], we believe that each nanodot eventually bonds to a single or none integrin after cell culture. So, the nanopattern of the RGD ligand eventually determined the spatial distribution of its receptor integrin, and the spacing of nanodots on the material surface controls the nanospacing of integrins in the cell membrane. It has been known that nanospacings of RGD ligands are quite important for cell adhesion and the critical interval is about 70 nm [15,21].

In this study, we successfully prepared RGD nanopatterns with five nanospacings crossing 70 nm (Fig. 3), and confirmed that the nanospacing effect on cell adhesion reported for fibroblasts and osteoblasts [15,21,37] also worked for stem cells. What is more, we quantitatively examined the effect of RGD nanospacing on cell shape parameters, and concluded that the cell circularity increased with RGD nanospacing while the average aspect ratio of cells did not change significantly (Fig. 4).

We further examined differentiations of MSCs on those nanopatterns. Since nanospacing influences adhesion of stem cells, it is predictive that nanopatterns of different nanospacings might influence the lineage commitments of stem cells. However, one
cannot predict whether or not the nanospacing influences stem cells beyond cell adhesion. Unexpectedly, the increase of RGD nanospacing is, although not beneficial for cell adhesion, beneficial for both osteogenic and adipogenic differentiations of MSCs in sole inductions (Figs. 6 and 8). Such a trend has been repeated by different members in our group.

The trend remained in co-induction for osteogenesis, yet reversed for adipogenesis (Figs. 7 and 8), which could be understood from competition between the two lineage commitments. So, the comparison between the results from the sole inductions and the co-induction illustrate that osteogenesis is more sensitive to RGD nanospacing than adipogenesis.

Then, how to explain the interesting phenomena of MSC differentiation on RGD nanopatterns? Previous research indicates that more spread MSCs exhibited better osteogenesis [31,41]. So, our results on nanopatterns for osteogenesis cannot be interpreted from the spreading effect, because a large nanospacing led to a less cell spreading (Fig. 4) but a higher osteogenesis in either sole osteogenic induction (Figs. 6 and 8) or adipogenic/osteogenic co-induction (Figs. 7 and 8). We have also examined the other candidate effects related to cell adhesion such as cell density and shape.

Previous results on micropatterned or non-patterned surfaces [31,41] indicate that a high cell density strongly favours adipogenesis of MSCs. In our present study, a small nanospacing led to a high density of adherent cells (Fig. 4) but a small fraction of adipogenesis in the adipogenic induction (Figs. 6 and 8); so our study on nanopatterned surfaces cannot be interpreted by cell density either. Aspect ratios of cells influence differentiation of MSCs [32]. No significant difference of aspect ratio of cells was found for MSCs on our nanopatterned surfaces (Fig. 4), and thus AR cannot serve as even an indirect cue underlying the nanospacing effect. An increased circularity disfavours osteogenesis of MSCs [32]. In this study, the increase of nanospacing enhanced cell circularity (Fig. 4) but was beneficial for osteogenic differentiation (Figs. 6–8). Therefore, the circularity can neither account for the nanospacing effect. Considering that the trends in Figs. 6–8 are not consistent with the existing knowledge about effects of cell adhesion on cell differentiation and sometimes were even altered, the present study illustrates that the inherent nanospacing effect on differentiation of stem cells is not weak.

Our unexpected finding triggers a very challenging fundamental question about the transduction route of the nanospacing signal as

![Optical micrographs and corresponding statistics of MSCs on nanopatterns upon 7 days of osteogenic or adipogenic induction following one day of cell adhesion.](image-url)
a cue to regulate stem cell differentiation. While it needs a long way to give a definite answer, the nanospacing effect could be tentatively anticipated to work via cytoskeleton, as schematically presented in Fig. 9. A small RGD nanospacing induces a strong focal adhesion and distinct cytoskeleton, and a large RGD nanospacing leads to a weak focal adhesion and indistinct cytoskeleton. We hypothesize that the bioconjugation between RGD and integrin is NOT the sufficient condition of a biological specific cell adhesion. The formation of a focal adhesion complex is dependent upon the lateral linking of F-actins by some other intracellular molecules such as z-actinin, as we conjecture. The critical nanospacing of about 70 nm might just measure the minimum necessary lateral

![Fig. 7. Co-induction of MSCs on nanopatterns for 7 days following one day of cell adhesion. The top images are bright-field micrographs of co-induced MSCs stained by both Fast blue RR salt and Oil Red O. The corresponding fluorescent micrographs of cell nuclei are shown in Supplementary Fig. S2. The bottom graph shows the fractions of differentiated and undifferentiated MSCs; the p values of t-tests are listed in Supplementary Tables S10–S12.](image)

![Fig. 8. Relative gene expression of sole and co-induced MSCs on indicated nanopatterns. The inductions lasted for 7 days following one-day adhesion of MSCs. * indicates a significant difference with p < 0.05. The p values of t-tests are listed in Supplementary Table S13.](image)
interval between two nearest-neighbour F-actins in the focal adhesion complex. The above interpretation of the critical nano-spacing essentially follows that in our previous cooperative study of specific adhesion of osteoblasts from the cell line MC3T3-E1 on RGD nanopatterns (the background was a self-assembly monolayer of PEG instead of a PEG hydrogel) [21]. In the present paper, we shed new insight that the RGD nanospacing can not only influence cell adhesion such as spreading areas and then indirectly adjust cell differentiation, but also directly tune cell differentiation on the molecular level. The lineage commitments of stem cells on RGD patterns of varied nanospacings might be determined by factors beyond cell adhesion. Yet to be further confirmed, it is probable that the nanospacing of active ligands might afford one of key regulators to transduce the outside-in signal of differentiation of stem cells under a material environment.

It seems worthy of emphasizing again that such an effect should be unambiguously revealed only based upon a persistently strong non-fouling background, which could resist cell adhesion during cell differentiation under a sufficient serum for a long time. Otherwise the study might not really be to the point of the nanospacing effect on cell differentiation. While the molecular mechanism remains open at the moment, the present study has unambiguously shaken an apparently self-evident mind that a better cell differentiation accompanies always with a better cell adhesion. Yet we
do not mean that cell adhesion and differentiation are completely independent with each other, for the nanospacing effect on differentiation of stem cells is more significant below the critical adhesion interval (<77 nm in Figs. 6 and 7). Both cell adhesion and cell differentiation should be taken into consideration in regenerative medicine.

5. Conclusions

RGD nanospacing might be an inherent regulator of differentiation of stem cells beyond cell spreading. Despite leading to less cell adhesion, the large nanospacing is beneficial for both adipogenesis and osteogenesis of MSCs in sole inductions, and osteogenesis is more sensitive to nanospacing based upon the experiments of co-induction. Cell adhesion and differentiation possibly result in different trends with nanospacing. The finding of the nanospacing effects is stimulating for new biomaterial design with appropriate spatial arrangement of ECM-mimetic ligands and might trigger pertinent comprehensive studies of mechanism of stem cell differentiation on the molecular level.

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Appendix A. Supplementary data

Supplementary data associated this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biomaterials.2013.01.021.

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