Effects of RGD nanospacing on chondrogenic differentiation of mesenchymal stem cells†

Zhenhua Li, Bin Cao, Xuan Wang, Kai Ye, Shiyu Li and Jiandong Ding*

Modification of material surfaces by an arginine-glycine-aspartate (RGD) peptide, a ligand of transmembrane integrin, can regulate cell adhesion and other events in tissue engineering and regenerative medicine. While both RGD effects and chondrogenesis are very important and have been much reportd, the studies on the influence of the spatial arrangement of RGD ligands on chondrogenic differentiation are rather limited. Herein, we examined the effects of RGD nanospacing on in vitro two-dimensional chondrogenic differentiation of mesenchymal stem cells (MSCs) for the first time. Using a unique nanolithography technology, hexagonal RGD nanopatterns were generated on nonfouling poly(ethylene glycol) (PEG) hydrogels. Two nanospacings (63 and 161 nm) were achieved, with one below and the other above the critical nanospacing. After one-day incubation and nine-day chondrogenic induction, expressions of collagen II proteins and chondrocyte-specific genes (SOX9, aggrecan and collagen II) were detected. The statistics illustrated that the large nanospacing led to a less spreading area and a higher chondrogenic differentiation extent. Further tests by the addition of an inhibitor SB203580 confirmed the positive regulation of the p38 phospho-relay cascade on the chondrogenic induction in this model system.

1. Introduction

In tissue engineering and regenerative medicine, mimicking of the extracellular matrix (ECM) is very important to develop new-generation biomaterials.1–8 It has been recognized that the peptide containing the arginine-glycine-aspartate (RGD) sequence is a crucial component of adhesive proteins in ECM.9–11 Therefore, RGD has been widely used in the modification of biomaterials,12–16 and RGD patterns have also been used for fundamental studies of cell–material interactions.17,18 Among the corresponding surface patterns for cell studies,19–21 an appropriate RGD nanopattern is very helpful and unique due to its high resolution at the molecular level. RGD can conjugate with its receptor integrin, a transmembrane protein with a diameter of approximately 8–12 nm.22 So, an RGD nanopattern with nanodots of less than 10 nm might eventually regulate the integrin distribution across the cell membrane.

To date, extensive efforts have been made in using RGD nanopatterns to investigate specific adhesion of various cells and the relevant proliferation,23 migration,24 and differentiation behaviors.25,26 A critical RGD nanospacing has been revealed, and cells presented obviously different adhesion behaviors on RGD nanopatterns with nanospacings below and above about 70 nm.27,28 A spacing less than 70 nm could significantly enhance cell adhesion with a clearer cytoskeleton and focal adhesion. This phenomenon has been repeated in several cell types such as a pre-osteoblast cell line MC3T3 and mesenchymal stem cells (MSCs) derived from bone marrow. Although the intracellular mechanism of the critical nanospacing on a substrate is not yet revealed, it is supposed to be related to some necessary lateral crosslinking between filamentous actins (F-actins) in a mature cytoskeleton network with respect to the formation of a focal adhesion complex.28

The present study is aimed to employ appropriate RGD patterns to examine the in vitro chondrogenic differentiation of MSCs. Due to the difficulty in self-healing of cartilage injuries, chondrogenesis has been a very challenging topic in tissue engineering and regenerative medicine.29–33 Besides plenty of reports on three-dimensional chondrogenic repair or regeneration,34–38 a well-designed two-dimensional surface could offer an alternative platform for the fundamental research of in vitro chondrogenic differentiation of stem cells.39,40 Nevertheless, no one has carried out the chondrogenic induction of MSCs on ordered RGD nanopatterns with well-controlled RGD nanospacings. It is thus an open question how the RGD nanospacing affects chondrogenic differentiation of MSCs. Besides, the design and preparation of the new-generation biomaterials for tissue engineering and regenerative medicine rely strongly on the understanding of cell–material interactions, which will benefit from the fundamental studies of cells on well-defined model material surfaces.
To clarify this question reliably, RGD nanoarrays should be generated on a nonfouling background, and the nonfouling property should be persistent for at least one week during the process of cell culture and induction in the presence of sufficient serum. Poly(ethylene glycol) (PEG) has been proved to be a perfect nonfouling molecule, and this molecule can be used in the forms of covalently grafting, component in copolymers or bulk PEG hydrogels. In cooperation with Spatz’s group, our laboratory has established a transfer lithography strategy to fabricate gold and then RGD arrays on PEG hydrogels. In this study, hexagonal RGD nanopatterns were fabricated on PEG hydrogels to explore the effects of RGD nanospacing on adhesion and chondrogenic differentiation of MSCs, as schematically presented in Fig. 1A. Two nanospacings, one below and the other above the critical nanospacing, were achieved; a ten-day chondrogenic culture including one-day incubation and nine-day chondrogenic induction were carried out, and the induction medium contained transforming growth factor-beta (TGF-β) and a serum; the expressions of collagen II proteins and characteristic genes of chondrocytes were detected and analyzed.

2. Materials and methods

2.1 Preparation of RGD nanopatterns on PEG hydrogels

Block copolymer micelle nanolithography and transfer nanolithography were combined to prepare the gold nanopatterns on PEG hydrogels using the procedure schematically presented...
in Fig. 1B, and the pivotal fabrication parameters are shown in Table 1.

Firstly, diblock copolymers polystyrene-block-poly(2-vinylpyridine) (PS-b-P2VP, Polymer Source) were chosen as the primary template polymers containing a nonpolar block (PS) and a polar block (P2VP). The reverse micelles were formed after the block copolymers were dissolved in anhydrous toluene (99.8%, Aldrich) with the polar blocks (P2VP) forming the cores and the nonpolar blocks (PS) forming the coronae. Subsequently, a metal precursor hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄·3H₂O, 99.99%, Alfa Aesar) was added. The added polar molecules were eventually loaded into the micelle cores mainly composed of poly(2-vinylpyridine) blocks following the principle of “like-dissolves-like”.

The loading amount (LA) was defined as the molar ratio of HAuCl₄·3H₂O to 2-vinylpyridine blocks. With the given copolymers and LA, we could calculate the mass of HAuCl₄·3H₂O for our experiments. Continuous stirring for at least 1 day was needed for dissolution of the block copolymers in anhydrous toluene and for homogeneous dispersion of HAuCl₄·3H₂O in micelle cores. The micelles of PS-b-P2VP copolymers loaded with HAuCl₄ were observed using a transmission electron microscope (TEM, Tecnai G2 20 TWIN, FEI) at 200 kV. The samples were prepared by self-assembly of micelles upon evaporation of toluene after the micelle suspensions were dropped on copper meshes.

Secondly, the copolymer–precursor micelles were dip-coated onto glass with pulling velocity shown in Table 1, and self-assembled as a monolayer after the evaporation of the toluene solvent. Afterwards, an oxygen plasma treatment (wattage of 100 W, G2 20 TWIN, FEI) at 200 kV. The samples were prepared by self-assembly of micelles upon evaporation of toluene after the micelle suspensions were dropped on copper meshes.

Thirdly, N,N'-bis(acryloyl) cystamine (Sigma) was grafted onto gold nanodots as a linker agent with a concentration of 1 mM in ethanol. Afterwards, poly(ethylene glycol) diacrylate (PEGDA, Mₙ = 700 g mol⁻¹, Sigma) was mixed with 0.05 wt% photo-initiator 2-hydroxy-2'-[2-hydroxyethoxy]-2-methylpropophenone (D2959, Sigma) and poured onto the glass surface with the gold nanooarray. Photo-polymerization was triggered by UV illumination at 365 nm with the sample protected by a nitrogen atmosphere. The gold nanooarrays were then transferred from the glass to the PEG hydrogels.

Finally, the patterned PEG hydrogels were incubated in 25 μM cyclo[Arg-Gly-Asp-D-Phe-Lys][PEG-COCH₂CH₂SH] [f: l-phenylalanine, K: l-lysine; abbreviated as: c-RGDfK]-thiol solution in water at 4 °C for 4 h. The c-RGDfK-thiol ligands (catalogue number PCI-3977-PI from Peptides International) were grafted onto gold nanodots by a chemical reaction between the thiol group and Au. The RGD nanopatterns on PEG hydrogels were ultimately realized. The prepared nanopatterns on glass and PEG hydrogels were characterized using an atomic force microscope (AFM, Multimode 8, Bruker) and a field-emission scanning electron microscope (FESEM, Ultra 55, Zeiss). In the AFM observation, we used an E scan head with a maximum scan range of 12 μm (length) × 12 μm (width) × 3 μm (height) and a probe called RTESP (material: 0.01–0.025 Ω cm⁻¹, antimony (n) doped Si, Bruker) in tapping mode. A data-processing software NanoScope Analysis (Bruker) was employed to deal with the testing results to acquire the distribution of the nanoarray and the height profile of the nanodots. In the FESEM observation, we selected the mode of secondary electron imaging with a working distance (WD) of 3–5 mm, an extra high tension (EHT) of 1.5–2.0 kV, and an aperture of 20 μm.

### 2.2 Isolation and culture of MSCs

MSCs were isolated from marrows of femur and tibia of 7 day-old neonatal Sprague Dawley (SD) rats, following our previous protocol. After being transferred to a culture flask, cells were cultured in a growth medium containing low-glucose Dulbecco’s modified Eagle medium (DMEM, Gibco), 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 100 U ml⁻¹ penicillin (Gibco) and 100 mg ml⁻¹ streptomycin (Gibco). Three days later, non-adherent cells were washed off gently with the renewal of the growth medium.

Passaging was carried out when cells reached 70–80% confluence. Firstly, cells were rinsed with warm low-glucose DMEM gently, and trypsinized by 0.05% trypsin–EDTA (Gibco) at 37 °C for 2–3 min to obtain a cell suspension. After termination of the trypsin–EDTA by 10% FBS in the growth medium, centrifugation was carried out at 800 rpm for 8 min to collect cells from the cell suspension. The cells were resuspended in the growth medium, and transferred to a culture flask. The second-passage MSCs were used in the subsequent chondrogenic induction of MSCs on RGD nanopatterns. All animal experiments were conducted in accordance with the “Principles of Laboratory Animal Care” (NIH publication #85–23, revised 1985) and approved by the ethics committee of Fudan University.

### 2.3 MSC seeding and chondrogenic induction on RGD nanopatterns

Prior to cell seeding, the nanopatterned samples were sterilized with 75% alcohol followed by extensive washing with a phosphate buffered saline solution.
buffered saline (PBS, Hyclone) solution, 6 times × 30 min, which has been confirmed to be sufficient for avoiding any significantly adverse effect of sterilization. Subsequently, the samples were transferred into 12-well plates (Corning). The MSC suspension with the growth medium as described above was added into 12-well plates at a density of 2 × 10^4 cells per well. Cells were cultured at 37 °C in 5% CO2 atmosphere. After 24 h, the non-adherent cells were washed off gently with the renewal of fresh chondrogenic induction medium. The induction medium contained high-glucose DMEM (Gibco), 7% FBS (Gibco), 10 ng ml⁻¹ transforming growth factor-beta 1 (TGF-β1, R&D), ITS + 1 liquid media supplement (100×) (Sigma), 100 mM dexamethasone (Sigma), 50 μg ml⁻¹ ascorbate-2-phosphate (Sigma), 2 mM L-glutamine (Gibco), 100 U ml⁻¹ penicillin (Gibco) and 100 mg ml⁻¹ streptomycin (Gibco). The chondrogenic medium was exchanged with a fresh one every 3 days, 3 times during the 9 day induction.

During the chondrogenic induction, 0.3 μg ml⁻¹ of aphidicolin (Sigma) was added at the first cycle for inhibiting cell proliferation. In some experimental groups, we added 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (20 μM, SB203580, Sigma). This pyridyl imidazole inhibitor blocks a mitogen-activated protein (MAP) kinase p38, 3-phosphoinositide-dependent protein kinase, and retinoblastoma hyperphosphorylation. In the case of chondrogenesis of MSCs, the p38 pathway was evoked by TGF-β1 under both two-dimensional monolayers and three-dimensional scaffolds. As a phospho-relay cascade, p38 pathway is a subtype of mitogen-activated protein kinase (MAPK) signaling. This protein and its signaling pathway are, in the present study, abbreviated as p38 MAP-kinase and p38 MAPK, respectively. While we do not know whether or not aphidicolin influences the activity of p38 MAK-kinase, it will not influence the central argument of this paper, namely, the RGD nanospacing effect on chondrogenic differentiation of MSCs, because the concentrations of aphidicolin among all corresponding experiment groups were the same in our study.

2.4 Fluorescence staining of cells on RGD nanopatterns

After cell culture for 24 h, F-actins and nuclei were stained for observations of cell adhesion. First of all, the cells on nanopatterns were rinsed gently with warm PBS. Then, we used 4% paraformaldehyde to fix the cells for 10 min, 0.1% Triton X-100 to improve the cell membrane permeability for 10 min, and PBS to rinse the cells again (3 times, 5 min each time). For labeling of F-actins, the preprocessed cells were incubated with 1 μg ml⁻¹ phalloidin–TRITC (phalloidin–tetramethylrhodamine B isothiocyanate, Sigma) at room temperature for 30 min. For labeling of nuclei, cells were treated with 2 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI, Sigma) at room temperature for 10 min.

After ten-day chondrogenic culture, collagen II was stained to evaluate the chondrogenic extent. The cells were first fixed and permeated with 4% paraformaldehyde and 0.1% Triton X-100. Then, 5% bovine serum albumin (BSA) was employed as the blocking buffer. Blocking was carried out at room temperature, and lasted for 30 min. A mouse monoclonal anti-collagen II IgG₂₀ (Santa Cruz Biotech) and a streptavidin–biotin–Cy3 complex immunofluorescence staining kit (SABC-Cy3) (SA1072, Boster) were chosen to stain collagen II following the manufacturers’ instructions. Positively stained cells emitted red fluorescence upon excitation. PBS served as a rinsing agent in the fluorescence staining process.

All fluorescently stained samples were observed under an inverted fluorescence microscope (Axiovert 200, Zeiss) equipped with a charge-coupled device (CCD, AxioCam HRc, Zeiss).

2.5 Analysis of fluorescently stained MSCs on RGD nanopatterns

The parameters for characterizing the cell morphology included the cell area, circularity and the aspect ratio (AR). They were obtained by outlining the micrographs of single cells using a free software Image J (freely available at website http://rsb.info.nih.gov/ij/). Circularity is defined as area times 4π divided by the square of the perimeter. It might be significantly influenced by the number of pseudopodia of a cell, because the latter increases the cell perimeter. AR is defined as the ratio of the major axis to the minor axis of a cell profile. So, in comparison with circularity, which reflects the local shape of a cell, the AR reflects the global shape of a cell fitted as an ellipse and might thus not be significantly influenced by pseudopodia.

The relative intensity of F-actins per cell on RGD nanopatterns was semi-quantified as follows: the red channel for the dye TRITC used for staining F-actins was split from the original micrograph and turned into an 8-bit greyscale image. The relative intensity of F-actins, i.e. RI (F-actin) of a cell was calculated by

\[ RI (F\text{-}actin) = S_{cell} (I - I_{0}) \]

Here \( I \) denotes the mean grey value of F-actins in a cell, \( I_{0} \) denotes the mean grey value of the hydrogel substrate background nearby the cell, and \( S_{cell} \) is the number of pixels in the cell project area outlined by Image J. For each independent sample, at least 200 cells were measured, and four independent samples were included in each experimental group.

Collagen II per cell stained by the dye Cy3 was also semi-quantified. The relative intensity of collagen II, i.e. RI (Col II) of a cell was calculated by

\[ RI (Col\ II) = S_{cell} (I - I_{0}) \]

Here \( I \) denotes the mean grey value of collagen II staining per cell, \( I_{0} \) denotes the mean grey value of the hydrogel background beside the cell, and \( S_{cell} \) is the cell area.

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

After ten-day chondrogenic culture on RGD nanopatterns, the total RNA of MSCs was extracted using the MagneSil Total RNA mini-Isolation System (Z3351, Promega). Afterwards, the reverse-transcription reaction was employed to synthesize cDNA from mRNA using a PrimeScript RT reagent kit (RR047A, Takara) including a genomic DNA elimination reaction. Then, the quantitative real-time polymerase chain reaction (qRT-PCR, Rotor Gene Q System, Qiagen) was performed using a SYBR Green
PCR kit (Qiagen) and predesigned primers (Invitrogen). The protocol temperature was 95 °C for 5 min, and then 40 circles of a combination of 95 °C for 5 s, 65 °C for 10 s, and 60 °C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as the housekeeping gene. The same mRNA expression level detection was carried out in the experimental groups with the signaling inhibitor of p38 MAPK. All operations were carried out according to the manufacturers’ instructions.

2.7  In vitro cytotoxicity

After one-day adhesion and nine-day chondrogenic induction, a live/dead viability assay was performed to evaluate the viability of MSCs on RGD nanopatterns in the chondrogenic induction medium. The solution of ethidium homodimer-1 (2 μM in PBS) and calcine AM (1 μM in PBS) was used to stain the cells for 30 min; and the samples were immediately observed under an inverted fluorescence microscope. Live cells emitted green fluorescence and dead cells emitted red fluorescence.

Furthermore, the quantitative viability of cells on nanopatterns was detected by cell counting kit-8 (CCK-8) assays on the fourth day (one day for adhesion, three days for chondrogenic induction). We exchanged the induction medium by 400 μl of fresh medium with 40 μl of CCK-8 solution in each well for incubation. After 3 h, a 300 μl solution in each well was transferred to a new 96-well plate to detect the absorbance at 450 nm in a microplate reader (Multiskan Mk 3).

The same viability/cytotoxicity assay was carried out in other experimental groups in the presence of the signaling inhibitor of p38 MAPK.

2.8  Data analysis

Independent experiments were averaged to obtain the cell-adhesion parameters, F-actin expression, collagen II expression and cell viability analysis. The \(2^{-\Delta\Delta CT}\) method was employed to analyze the relative gene expression. For each group, \(n = 4\) as usual. Part results were normalized by the results on nanopatterns with 63 nm RGD spacing. A difference was treated as significant when the \(p\) value in a Student’s \(t\)-test was less than 0.05 (marked with ‘**’).

3. Results

3.1  Fabrication of RGD nanopatterns with two nanospacings on PEG hydrogels

As described in Section 2.1, block copolymer micelle nanolithography and transfer nanolithography were combined to prepare the quasi-hexagonally ordered gold nanopatterns on PEG hydrogels. The TEM images in Fig. 2A indicate the micelles of the PS-b-P2VP copolymer loaded with the gold precursor. The dark part corresponds to the micelle cores mainly composed of P2VP blocks loaded with HAuCl4. The AFM images in Fig. 2B demonstrate the successful achievement of quasi-hexagonal arrays of gold nanodots on glass, and the dots meet the expected diameter, that is less than 10 nm.

The gold nanopatterns were then transferred from glass to the PEG hydrogels, as shown in Fig. 2C. The nanospacing was defined as the average distance of the centers between the nearest-neighbour nanodots and could be adjusted by the parameters listed in Table 1, in which the molecular weight of the
The copolymer had the greatest impact. The original inter-particle distances of gold nanodots on glass surfaces were 55 nm and 140 nm. The PEG hydrogels swelled in culture medium with a swelling ratio of 1.15, and thus the final nanospacings on swollen PEG hydrogel surfaces were 63 nm and 161 nm, respectively, with one below and the other above the critical nanospacing of about 70 nm.

### 3.2 Cell adhesion on RGD nanopatterns

Cell adhesion is a key event when cells are cultured on a material. Fluorescence micrographs and the corresponding quantitative adhesion parameters are shown in Fig. 3. The cell density and spreading area decreased with the increase of the RGD nanospacing, consistent with previous reports on other cell types. The aspect ratio (AR) and cell circularity were calculated from the cell profile acquired by using the Image J software. Here, the AR is defined as the ratio of the major axis to the minor axis of a cell profile, and a perfect circle has an AR of 1:1. Circularity is defined as area times \( \frac{4\pi}{	ext{perimeter}^2} \), with 1.0 indicating a perfect circle.

The AR was not significantly different between two RGD nanopatterns, which is not hard to understand since no factors specially influence the major or minor axis of a cell profile alone. Nevertheless, because the cells on patterns with the small nanospacing presented more pseudopodia that promoted the cell perimeter, the circularity of cells increased with the nanospacing.

Using the method demonstrated in Fig. 4A, the relative intensity of F-actins per cell on RGD nanopatterns of different nanospacings were semi-quantitatively analyzed, with the results
shown in Fig. 4B and C. The fluorescence intensity of F-actins of cells on patterns with the small nanospacing was significantly higher than that of the large nanospacing, in accordance with the peak values in the distribution graphs of integrated grey value per cell. So, more F-actins were assembled in cells on patterns of the small nanospacing.

3.3 Persistent anti-adhesive ability of PEG hydrogels in stem cell induction

Only when the background of the RGD pattern possesses powerful and persistent anti-adhesion throughout the whole experimental period, the effects of the RGD nanospacing on chondrogenic differentiation of MSCs can be conclusive without the interference from non-specific adhesion. The dip-line in block copolymer micelle nanolithography provided a way to check the persistent anti-adhesive ability of the PEG hydrogels. After ten-day chondrogenic culture of MSCs including the first-day incubation and the ninth-day chondrogenic induction, the dip-line was still well distinguished (Fig. S1, ESI†), which convinced the subsequent cell experiments.

3.4 Chondrogenic induction of MSCs on RGD nanopatterns

After ten days of the chondrogenic culture, collagen II was detected as the marker of chondrogenesis. The proteins were immunofluorescently stained, as schematically presented in Fig. 5B. Some typical images are presented in Fig. 5A. For further semi-quantitative estimation of the collagen II expression, the relative intensity of collagen II per cell was calculated using the method described in Section 2.5, with the statistical results shown in Fig. 5C. The averaged collagen II expression of cells on RGD patterns with the large nanospacing was relatively higher than that of the small nanospacing.

Meanwhile, the gene expressions of SOX9, aggrecan, and collagen II after ten-day chondrogenic culture were analyzed using qRT-PCR, with the predesigned primers shown in Fig. 6. The characteristic genes of chondrocytes were basically up-regulated with an increase of the RGD nanospacing. Expressions of both collagen II proteins and characteristic genes of chondrocytes illustrated that the large RGD nanospacing was beneficial for the chondrogenic differentiation of MSCs.

3.5 Chondrogenic differentiation of MSCs on RGD nanopatterns treated with the inhibitor SB203580

Mitogen-activated protein (MAP) kinases including p38, extracellular signal-regulated kinase-1/2 (ERK-1/2), and c-Jun N-terminal kinase (JNK) are activated by TGF-β while promoting the cartilage-specific gene expression. The Smad signaling is also closely associated with the TGF-β-induced chondrogenesis. Among these, the p38 phospho-relay cascade, a subtype of MAPK signaling, has been known as a positive regulator of TGF-β-induced chondrogenesis of mesenchymes in many cell types on different materials. TGF-β was included in our induction medium. SB203580, a kinase inhibitor, was added to this system of the chondrogenic induction of MSCs to explore whether or not this p38 MAPK pathway played a role in the chondrogenic differentiation of MSCs on RGD nanopatterns. According to our pre-experiments, the appropriate concentration of SB203580 was about 20 μM, at which the inhibitor influenced the cell morphology and viability a little, as demonstrated in Fig. 7A and B. After ten-day chondrogenic culture, the majority of cells on the
Fig. 5  Collagen II expression of MSCs after ten-day chondrogenic culture. (A) Representative fluorescent micrographs of collagen II staining of MSCs on RGD nanopatterns with indicated nanospacings. (B) A schematic presentation of immunofluorescence staining of collagen II using the SABC-Cy3 method described in Section 2.4. (C) The statistical results of the relative fluorescent intensity of collagen II on indicated RGD nanopatterns ($p = 0.08$).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer (5’-3’)</th>
<th>RGD nanospacing (nm)</th>
<th>Relative grey value</th>
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<td>SOX9</td>
<td>Fw: CTGAAAGAGAGAGAGAGAG</td>
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<td>1.00</td>
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<td></td>
<td>Rv: TTTCTCTCCCTCCCTCC</td>
<td>161</td>
<td>1.50</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Fw: TATGAGATGATGCTCCAG</td>
<td>63</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Rv: AAGCTTCCTACCTCATCTC</td>
<td>161</td>
<td>1.50</td>
</tr>
<tr>
<td>Collagen II</td>
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<td></td>
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<td></td>
<td>Rv: GTGTACACAGGCTGTCTCTG</td>
<td>161</td>
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Fig. 6  Relative gene expressions of SOX9, aggrecan and collagen II after ten-day chondrogenic culture of MSCs on the indicated RGD nanopatterns. The table in the top left shows the predesigned primers.
nanopatterns still emitted green fluorescence in the live/dead viability assay. Besides, the cytotoxicity of SB203580 at this concentration was examined by CCK8, as shown in Fig. 7C. There was no significant difference between the groups with and without SB203580 treatment on RGD nanopatterns. These data ensured the reliability of the subsequent experimental results.

Gene expressions of SOX9, aggrecan, and collagen II with and without 20 μM SB203580 were further detected. As indicated in Fig. 7D, the characteristic genes of chondrocytes were basically down-regulated with the inhibition of the p38 phospho-relay cascade. These results confirmed the positive regulation of the p38 phospho-relay cascade in this system, namely TGF-β-induced chondrogenic differentiation of MSCs on RGD nanopatterns. On the other hand, the remaining significant difference in the expression of aggrecan between two nanospacings in the presence of SB203580 illustrated that p38 MAPK was not the exclusive pathway.

4. Discussion

In stem cell-based cartilage tissue engineering, the effect of scaffold materials on chondrogenic differentiation of stem cells is a fundamental topic. Grafting of functional groups, especially an RGD peptide has received extensive attention. An RGD peptide can be recognized by an integrin, a protein across the cell membrane. Bioconjugation is achieved between a transmembrane receptor integrin and an RGD ligand in ECM, which triggers specific cell adhesion. Furthermore, the integrin distribution across the plasma membrane can be regulated by the RGD ligand distribution pre-designed on material surfaces,
and may eventually affect cell behaviors including adhesion, proliferation, migration and differentiation. In the current manuscript, based on an innovative two-dimensional nanomaterial in our laboratory, we tried to explore the effects of RGD spacing on the chondrogenic differentiation of MSCs at the nanoscale.

RGD nanopatterns with different nanospacings were prepared using a combination of block copolymer micelle nanolithography, transfer nanolithography and grafting of RGD ligands. Two RGD nanospacings with one below and the other above the critical nanospacing were designed, and the final average nanospacings were 63 and 161 nm, respectively. Non-specific cell adhesion was thoroughly prevented by the PEG hydrogels. Therefore, the results of the relationship between the RGD nanospacing and cell behaviors could be unambiguous, free of interference of non-specific adhesion.

MSCs seeded on RGD nanopatterns with the large nanospacing exhibited a smaller spreading area and a larger circularity close to a circle. The F-actin networks were less formed on nanopatterns with the large nanospacing. With the detection of the expressions of collagen II proteins by immunofluorescence staining and three characteristic genes of chondrocytes (SOX9, aggrecan, and collagen II) by qRT-PCR, we further demonstrated that the large RGD nanospacing contributed to the chondrogenic differentiation of MSCs.

According to the reported dedifferentiation process of chondrocytes, normal chondrocytes presented a phenotype more close to a round phenotype with smaller spreading areas compared to dedifferentiated chondrocytes.\(^\text{56}\) Besides, chondrocytes cultured with a round phenotype could keep the differentiated phenotype and did not exhibit obvious actin stress fibers, while a spread phenotype with clear F-actins preferred dedifferentiation.\(^\text{57}\) Furthermore, despite no completely consistent conclusions, most of the studies indicate that a relative weak cytoskeleton is beneficial for the chondrogenic differentiation of stem cells.\(^\text{56,59}\) In our present study, MSCs on RGD nanopatterns with the large nanospacing exhibited a phenotype more analogous to the natural chondrocytes than that with the small nanospacing, which was strengthened by cell adhesion (qualitative cell morphology, quantitative adhesion parameters and semi-quantitative fluorescence intensity of F-actins) to cell differentiation (expressions of chondrocyte-specific genes). So, the large RGD nanospacing contributed to a better chondrogenic differentiation of MSCs on this two-dimensional nanomaterial in spite of worse cell adhesion.

Our results of the effects of the RGD nanospacing on adhesion and chondrogenic differentiation of MSCs are schematically summarized in Fig. 8. Specific cell adhesion requires not only the bioconjugation between RGD ligands and transmembrane receptor integrins, but also the integrin clustering and the

Fig. 8 Schematic presentation of the effects of the RGD nanospacing on the chondrogenic differentiation of MSCs. When an RGD nanospacing on a material surface is larger than the critical nanospacing of about 70 nm, focal adhesion complexes and intracellular cytoskeletons could not be effectively formed, contributing to a less cell spreading and a higher chondrogenic extent. In contrast, focal adhesion complexes were well formed in MSCs on the pattern of the small nanospacing of less than 70 nm, resulting in a more cell spreading but a lower chondrogenic extent due to unknown mechanisms.
formation of complexes composed of integrins and adapter proteins in cytoskeletons, in which talins and α-actinins might be two of the key adapter proteins. The formation of a focal adhesion complex might depend on the lateral crosslinking of F-actins by adapter proteins, among which the length of talins plus α-actinins is around 60 nm.60,61 Further taking the molecular size of F-actins themselves into consideration, the 70 nm critical spacing may refer to a necessary interval of adjacent F-actins. In this study, the RGD nanospacing was pre-determined by the underlying gold nanopattern. When the nanospacing was larger than the critical value, MSCs exhibited a “constricted” form with unclear focal adhesions and cytoskeletons, less cell spreading, but a higher chondrogenic extent due to unknown mechanisms worthy of further investigation.

An integrin, the receptor of an RGD, is composed of α and β subunits, and each of the two subunits have a series of subtypes. It has been suggested that αvβ3 receptors play distinct roles in adhesion and spreading of MSCs on RGD-modified gels and α5 is also important for chondrogenic differentiation. Thus, the RGD nanospacing might affect the recognition of the RGD motif by different integrin receptors, which further influences the subsequent chondrogenic differentiation of MSCs. Yet this speculation as one of the candidate reasons of the RGD nanospacing effect needs to be checked in the following studies.

It is well known that the p38 phospho-relay cascade, a subtype of MAPK signaling, positively regulates TGF-β-induced chondrogenesis of mesenchymes in different systems. With the addition of the signaling inhibitor SB203580, we confirmed that p38 MAP-kinase acted as a positive regulator in the chondrogenic differentiation of MSCs on RGD nanopatterns, as schematically shown in Fig. 9. On the RGD nanopattern with the large nanospacing, the expressions of three characteristic genes of chondrocytes (SOX9, aggrecan, and collagen II) were all down-regulated.

The remaining significant difference in the expression of aggrecan illustrated that p38 MAPK was not the exclusive pathway. The difference arises from the fact that the pyridinyl imidazole inhibitor SB203580 might block some other kinases such as protein kinase B phosphorylation and retinoblastoma hyperphosphorylation, independent of p38 MAP-kinase. The other kinases such as the Smad family have typical characteristics for TGF-β signaling of chondrogenic differentiation; and the influence of mitogen-activated kinases ERK1/2 and JNK can also not be ruled out according to the present data. Anyway, other unknown pathways might exist in the differentiation process, as emphasized by the dashed line in Fig. 9.

5. Conclusions

The present study has examined in vitro the MSC behaviors due to adhesion to chondrogenic differentiation while interacting with the unique RGD nanopatterns on persistently nonfouling PEG hydrogels. We found that the large nanospacing was, despite its adverseness for cell adhesion, favorable for the chondrogenic induction of MSCs in the presence of TGF-β. The p38 MAPK acted as a positive but not the only pathway in the chondrogenic differentiation of MSCs on RGD nanopatterns. So, we have revealed that an appropriate nanoscale spatial arrangement of ECM ligands is vital for chondrogenic induction. Further understanding of the underlying outside-in signal transduction and the intracellular signaling pathway of interactions between cells and materials are called for.

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