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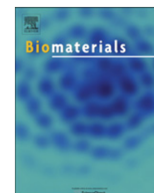

Effect of RGD nanospacing on differentiation of stem cells

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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 focussing 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

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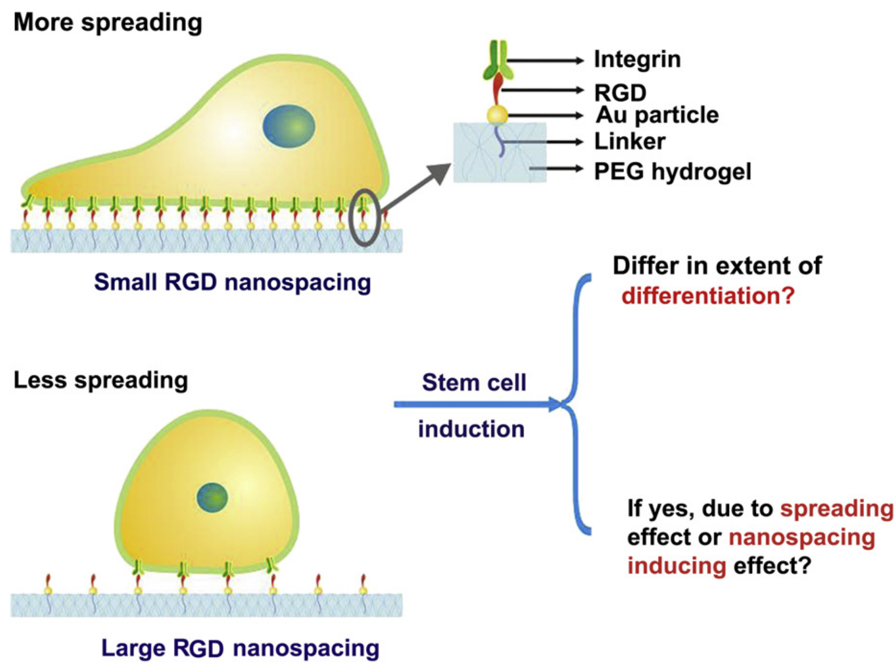

Fig. 1. Schematic presentation of stem cells on nanopatterns of cell-adhesive RGD ligands on a PEG hydrogel, a strong and persistent anti-fouling background. While it is known that cells exhibit different adhesion behaviours on RGD nanopatterns of varied nanospacings, the present study is aimed to further examine the possible effect of nanospacing on differentiation of stem cells.

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 $HAuCl_4$ 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 macromonomer PEG-DA (M_n 700, Sigma) and initiator 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (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 thiol group and gold, RGD peptides were grafted onto gold nanodots. An RGD nanopattern on the PEG hydrogel was eventually generated.

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