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Expression of Oct4 in human embryonic stem cells is dependent on nanotopographical configuration

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ABSTRACT

The fate of adult stem cells can be influenced by physical cues, including nanotopography. However, the response of human embryonic stem cells (hESCs) to dimensionally well-defined nanotopography is unknown. Using imprint lithography, we prepared well-defined nanotopography of hexagonal (HEX) and honeycomb (HNY) configurations with various spacings between the nanostructures. In serum-free hESC culture medium, basic fibroblast growth factor (bFGF) is required to maintain expression of Oct4, a pluripotent gene. Unexpectedly, hESCs cultured on nanotopography could maintain Oct4 expression without bFGF supplementation. With bFGF supplementation, the HEX nanotopography maintained Oct4 expression whereas the HNY configuration caused down-regulation of Oct4 expression. Thus, we observed that the lattice configurations of the nanotopography cause hESCs to respond to bFGF in different ways. This differential response to a biochemical cue by nanotopography was unforeseen, but its discovery could lead to novel differentiation pathways. Consistent with studies of other cells, we observed that nanotopography affects focal adhesion formation in hESCs. We posit that this can in turn affect cell–matrix tension, focal adhesion kinase signaling and integrin–growth factor receptor crosstalk, which eventually modulates Oct4 expression in hESCs.

1. Introduction

The success of regenerative medicine depends in large part on the ability to direct the cell fate of stem cell populations in vivo to get organs to heal themselves, or the ability to direct stem cells ex vivo to the desired cell lineages prior to transplantation. In both cases, it is necessary to elucidate the signals that direct stem cell fate. These signals can be soluble factors, such as cytokines, or insoluble factors, such as the extracellular microenvironment. The use of cytokines, growth factors or small molecules in cell culture has been the predominant method for directing the cell fate of stem cells. In contrast, strategies utilizing the extracellular microenvironment to direct cell fate of stem cells have only recently gained momentum. In recent years, research has identified some of the different aspects of the microenvironment that dictate stem cell fate and has shown that both biochemical and biophysical signals of the microenvironment are important [1–3]. The biophysical signals that have been identified include cell–cell contacts [4,5], mechanical forces [6,7], matrix elasticity [8–11], topographical patterns [12,13], integrin clustering [14] and the effects of extracellular matrix (ECM) tethering [15]. Of these, matrix elasticity and topography are appealing and important signals to be cognizant of when designing strategies to direct stem cell fate because they can be engineered as stable and reproducible biophysical signals both in vitro and in vivo.

Interactions between nanotopography and cells result in significant outcomes because the cell biomolecular machinery is in the nanometer-size regime. Thus, cell responses modulated by nanotopography are well documented and include adhesion, alignment, proliferation, motility and survival [16–21]. Importantly, nanotopography has been shown to influence the cell fate of human mesenchymal stem cells (hMSCs) in vitro. hMSCs, when stretched and aligned on long nanogratings, will up-regulate the expression of neuronal genes [22]. Other nanostructures, such as 70–100 nm hexagonal closed-packed titanium oxide nanotubes, differentiate hMSCs into osteoblastic cells without using osteogenic medium [23]. However, supplementation with osteogenic medium appears to switch the hMSCs to differentiate into osteoblastic cells only on smaller 15 nm titanium oxide nanotubes and not on the larger diameter nanotubes [24]. When analyzed together, the latter two studies suggest that the response of hMSCs to soluble cues (the

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osteogenic supplement) is dependent on either nanostructure spacing or configuration. Switching of the hMSC response was also demonstrated with polymeric nanopits; a square array of nanopits supported self-renewal of hMSCs, whereas a similar array of nanopits with a slight (50 nm) offset became osteogenic [25]. These studies with hMSCs clearly demonstrate that nanotopography is able to influence human adult stem cell fate, and additionally that the configuration of the nanotopography plays an important role in determining that fate.

Human embryonic stem cells (hESCs) are very different from hMSCs and adult stem cells in their biology and cell lineage potential. Adult stem cells can typically only differentiate into several types of specialized cells, whereas pluripotent embryonic stem cells are able to differentiate into every cell type in the body. Therefore, there is a motivation to devise strategies to control hESC fate such that very specific and functional cell lineages are derived from them for cell-based regenerative therapies. Because hESCs and hMSCs are so different biologically, it is interesting to investigate whether nanotopography will influence hESC fate in similar ways. Early studies suggest that hESCs can be aligned by nanogratings [26] which consequently induced the expression of neuronal genes [27]. Recently, Chen et al. [28] reported that the expression of Oct4 in hESCs was reduced when cultured on nanoroughened glass. These studies have demonstrated that nanotopography does influence hESC gene expression and potentially cell fate. However, what has yet to be demonstrated is whether different nanotopographical configurations switch the response of hESCs to soluble cues, as observed in MSCs. If, indeed, the biochemical responses of hESCs can be modulated by nanotopographical configurations, the implication of this finding will be significant. If true, in order to achieve the therapeutic potential of hESCs, the nanoenvironment of the cells must be well defined so that robust, repeatable and reliable differentiation of hESCs to a particular lineage is ensured. Additionally, research that focuses on the interaction between hESCs and the nanoenvironment to maintain pluripotency is still in its infancy. Instead, most of the research work has been focused on identifying soluble cues, such as small molecules, cytokines and growth factors, that maintain the pluripotency of hES cells [29–32]. However, an early study demonstrated that mouse ES cells cultured on nanotopography (electrospun polyamide nanofibers) maintained self-renewal [33], thus providing the motivation to also study the effects of nanotopography on hESCs self-renewal.

On the basis of the foregoing, we hypothesized that hESC fate can be directed by differences in nanotopography configuration. This can be a response due directly to the different biophysical cues or to modulation of the cell biochemical response by the biophysical cues, or a combination of both. To address this hypothesis, we developed a nanofabrication process to produce polystyrene nanopillars in hexagonal (HEX) and honeycomb (HNY) lattice configurations (see Section 2.1 for details; see also Fig. 1a–h). This nanofabrication process for producing the nanotopographical cues (NTCs) is more complex than the processes used to produce nanopitted topography [25] or nanoroughened surfaces [28], but is able to produce dimensionally well-defined nanotopography in a reliable and reproducible fashion, thus enabling more quantitative results. Currently, the design and fabrication of the nanopillars described in this paper results in nanopillars having a width to height ratio of 1–1.5, with each individual nanopillar measuring 30–40 nm in width. Also, within each HEX and HNY lattice configuration, we varied the spacing between the nanopillars (see Fig. 1i–p). The spacing was a parameter that had shown an effect on hMSC cell response, and we surmised that it might also play a role in modulating hESC cell response.

The hESC response to the HEX or HNY nanopillar topography was characterized by several methods: immunofluorescent staining of a pluripotent marker, cell proliferation, the number of focal adhesions and cell migration. The number of focal adhesions and cell migration response reflected on the direct influence nanotopography has on focal adhesion dynamics. Modulation of the focal adhesions then affected downstream signals that eventually led to changes in cell proliferation and pluripotency. To probe the biochemical response, the cell culture medium was supplemented with basic fibroblast growth factor (bFGF). This is used to maintain the pluripotency of hESCs, so a switch in cell response to bFGF will result in a loss of pluripotent marker expression. Based on these experiments, we have identified nanopillar lattice configurations that help maintain the expression of a pluripotent marker, Oct4, in hESCs without bFGF supplementation in serum-free medium. We also demonstrate that nanoscale lattice configurations can switch the response of hESC to bFGF, underscoring the importance of the needs to fully define the nanoenvironment and to fully characterize the response of hESCs to growth factors when cultured on different nanotopographies. Thus, knowledge of the precise role of the nanoenvironment in maintaining or directing hESC fate is necessary to unlock the full potential of these cells.

2. Materials and methods

2.1. Fabrication of NTCs

To make NTCs, a silicon "mother" mold was first fabricated using the following micro- and nanomachining techniques. First, a polystyrene (PS) nanosphere (NS) mask was deposited onto a 22×22 mm silicon wafer using a homemade Langmuir trough. The silicon wafer was immersed in the subphase (water) of the trough and the nanospheres were gently dispersed onto the air–subphase interface as per Weekes et al. [34] (Fig. 1a). The NSs were compressed slowly with the movable trough barrier (2 mm min^{-1}) until a close-packed hexagonal monolayer of NSs was formed (Fig. 1b). The subphase was then gradually drained by a vacuum aspirator to deposit the NS monolayer onto the silicon wafer [35] (Fig. 1c and d). In our study, NSs with initial diameters of 50, 100, 200, 300 and 400 nm (Thermo Scientific 3000 series) were used. The NS-coated silicon wafer was then removed from the trough and left to dry overnight.

PS NTCs may be cast from silicon "mother" molds but, since the "mother" molds are rigid, shearing of the PS nanopillars occurs frequently during separation of the nanopatterned substrates from the mold. To circumvent this problem, a compliant "daughter" mold was made from polymerizing heptadecafluorodecyl methacrylate (HFDMA; Sigma–Aldrich) over the silicon "mother" mold. After cleaning the silicon "mother" mold, an anti-stiction coating, perfluorodecyltrichlorosilane (Gelest, Inc.), was applied onto the "mother" mold using the MVD100E system (Applied Microstructures, Inc.). HFDMA:divinylbenzene (DVB):2,2-dimethoxy-2-phenylacetophenone, mixed in a weight ratio of 250:7:1, was sandwiched between a clean 22×22 mm glass coverslip and the Si "mother" mold. The HFDMA pre-polymer mixture was polymerized by ultraviolet radiation (365 nm, 2 mW cm^{-2}) for 5 h and the glass coverslip peeled from the "mother" mold, thus forming the HFDMA "daughter" mold (Fig. 1g). The PS NTCs were made from thermally polymerizing styrene monomer (Sigma–Aldrich):DVB:benzoyl peroxide (Sigma–Aldrich) in a weight ratio 31:4:1. The styrene pre-polymer mixture was sandwiched between a 22×22 mm glass coverslip and the HFDMA "daughter" mold, then thermally polymerized at 98 °C for 25 min. The glass coverslip was peeled from the HFDMA "daughter" mold, thus forming the PS NTCs (Fig. 1h). The NTCs in the HEX and HNY configurations fabricated and used in this study are shown in Fig. 1i–p.