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Control of proliferation and osteogenic differentiation of human dental-pulp-derived stem cells by distinct surface structures

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ABSTRACT

The ability to control the behavior of stem cells provides crucial benefits, for example, in tissue engineering and toxicity/drug screening, which utilize the stem cell's capacity to engineer new tissues for regenerative purposes and the testing of new drugs in vitro. Recently, surface topography has been shown to influence stem cell differentiation; however, general trends are often difficult to establish due to differences in length scales, surface chemistries and detailed surface topographies. Here we apply a highly versatile screening approach to analyze the interplay of surface topographical parameters on cell attachment, morphology, proliferation and osteogenic differentiation of human mesenchymal dental-pulp-derived stem cells (DPSCs) cultured with and without osteogenic differentiation factors in the medium (ODM). Increasing the inter-pillar gap size from 1 to 6 μm for surfaces with small pillar sizes of 1 and 2 μm resulted in decreased proliferation and in more elongated cells with long pseudopodial protrusions. The same alterations of pillar topography, up to an inter-pillar gap size of 4 μm, also resulted in enhanced mineralization of DPSCs cultured without ODM, while no significant trend was observed for DPSCs cultured with ODM. Generally, cells cultured without ODM had a larger deposition of osteogenic markers on structured surfaces relative to the unstructured surfaces than what was found when culturing with ODM. We conclude that the topographical design of biomaterials can be optimized for the regulation of DPSC differentiation and speculate that the inclusion of ODM alters the ability of the cells to sense surface topographical cues. These results are essential in order to transfer the use of this highly proliferative, easily accessible stem cell into the clinic for use in cell therapy and regenerative medicine.

1. Introduction

Stem cells have unique characteristics regarding proliferation, differentiation and plasticity that make them a very promising cell source for tissue engineering and regenerative medicine. However, with the ethical issues concerning the use of embryonic stem cells and with the limited access to the location of most adult stem cells, the introduction of new, accessible adult human cell niches for tissue engineering is of great interest. Human mesenchymal dental-pulp-derived stem cells (DPSCs) have recently been identified within the dental pulp, and studies have confirmed them to be multipotent capable of differentiating along the odontogenic/osteogenic, chondrogenic, adipogenic, endothelial and neural lineages [1–4], as well as having the ability to self-renew [5]. Furthermore, these cells have been found to be bone-cell-like mechano-responsive in vitro and to form bone-like tissue in vivo, suggesting them to be very useful for tissue engineering applications of bone [6,7]. A detailed understanding of how to control the expansion and differentiation of these easily accessible stem cells will provide a strong basis for the use of the cells for e.g. tissue engineering and regenerative applications. Also, it may result in a more rational approach for the design of next-generation orthopedic implants to guide stem cell response on surfaces and improve osseointegration.

It is well known that biomechanical cues presented in the form of micro- and nanoscale surface topographies are of significant importance in regulating cell behavior [8–13]. More specifically, it has been shown that topographies ranging from the nano- to microscale influence cellular attachment [14,15], orientation [16] and proliferation [17–21]. Recently, several studies have also investigated the effect of surface topography on stem cell differentiation [22–27]. Dalby et al. found that the specific

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nanotopographical pattern influences the ability of mesenchymal stem cells (MSCs) to differentiate along the osteoblastic lineage [22]. They cultured cells without osteogenic differentiation factors in the medium (ODM) and found that highly ordered topographies (nanopits) produce weak cellular adhesion and osteoblastic differentiation, while cells cultured on nanopits with the same size and density and a controlled disorder of ± 50 nm from their true center significantly increased osteospecific differentiation to levels comparable to the results from MSCs cultured on planar surfaces with ODM. A different approach was taken by Oh et al., who cultured MSCs on substrates covered with nanotubes with a narrow range of diameters of 30, 50, 70 or 100 nm, respectively [23]. The nanotubes with 100 nm diameter regulated the differentiation of MSCs toward an osteoblastic lineage in the absence of ODM, while MSCs on the other nanotube surfaces did not differentiate under identical culture conditions. Also, the introduction of micropatterning of cell adhesive regions has been shown to have an influence upon cell differentiation. In one study it was possible to control the early differentiation of keratinocytes, an in vivo process essential to achieve and maintain epithelial tissue integrity (tissue homeostasis), through the topographical characteristics of fibronectin coated pillars [24]. The study revealed that, by decreasing the distance between pillars from 14 to 8 μm, the period in which the early differentiation marker keratin 1 was expressed was extended, indicating that differentiation to mature keratinocytes was delayed [24]. Other research suggests that stem cells are influenced by topography in a contextual manner, with their response also dependent on external factors. In one study human MSCs were cultured on nanogrooves under low oxygen conditions (2%), and this dual effect promoted the retention of stem cell markers OCT4 and SOX2 [28]. The effect was observed neither on planar surfaces nor under normal oxygen conditions (5%) alone.

The mechanism by which the topography exerts its effect on cell fate is by altering focal adhesion assembly and cytoskeletal stress, resulting in adaptive gene- and protein-level changes. This process of transferring such external mechanical stimuli into the nucleus is by mechanotransduction. Mechanotransduction can be both indirect, involving changes in signaling cascades induced at focal adhesions with a downstream effect on gene expression [29–35], or direct, whereby a physical pulling of the cytoskeleton on the nucleus may alter gene transcription by changing the organization of nuclear components [36,37]. Despite this knowledge, details regarding the link between topographical cues and changes in gene expression are not understood. This makes it impossible at present to elucidate general trends from published studies and, accordingly, we still do not have an understanding of the cell–surface interaction that allows us to tailor a biomaterial with specific cellular activity at the cell–biomaterial interface. A more systematic approach to test for the effect of topography on cells would clarify whether there are some general trends connecting surface topography with differentiation of stem cells. These trends may lead to a more fundamental understanding of the underlying mechanisms controlling the fate of stem cells.

The aim of this study was to determine topographical parameters at the micrometer scale governing osteogenic differentiation and proliferation of DPSCs. To perform this study in an efficient and methodical manner, we measured their response on a combinatorial library consisting of 13 × 13 (169) distinct topographies using the Bio Surface Structure Array (BSSA) platform [17,38,39]. The BSSA libraries consist of one unstructured control at the center of the wafer, 10 series (A–J) each with 16 unique combinations of pillar size, *X*, inter-pillar gap size, *Y*, and series K (referred to as "sharkskin like" [40]) with eight unique combinations of rectangular pillar sizes, *X*, and an inter-pillar gap size, *Y* = 1 μm. By performing this type of systematic screening it is possible to unravel the type of surface topographies that potentially could influence, and ultimately control, the proliferation and differentiation of DPSCs. The location of the focal adhesions and the arrangement of the cell cytoskeletal protein actin were determined in order to visualize the topographical effect on cell attachment and morphology known to have a large effect on later stage processes such as proliferation and differentiation [22,41,42]. Proliferation was measured by counting the number of DNA positive cells, and the osteogenic differentiation by identifying the total deposition of the osteogenic markers osteocalcin (OCN), osteopontin (OPN) and calcium. Effects of the topographies were measured with or without ODM. We hypothesize that using this rational approach for the identification of a surface topography with optimal effect on proliferation and osteogenic differentiation may lead to more defined parameters to be used for the design of biomaterials.

2. Materials and methods

2.1. Fabrication of the BSSA combinatorial libraries

The procedure for the fabrication of the 13 × 13 arrays has previously been described in Ref. [17]. Briefly, the arrays were etched in boron-doped *p*-type Si wafers using a standard photolithographic process. A 1.2 μm resist without hard bake was etched with Cl_2, HBr and NF_3 to produce side wall angles of 85° with etch rate non-uniformity amounting to 2–3% (max–min). After the removal of the resist and cleaning, the surface was sputter-coated with 100 nm of tantalum at a rate of 50 nm min^{-1} in a palette-based batch process. Tantalum is known to be an excellent in vitro and in vivo biocompatible material as it forms the stable and biocompatible Ta_2O_5 oxide layer when coming into contact with oxygen from the air [43,44]. The quality of the microtopographies was verified using scanning electron microscopy (SEM) (data not shown).

Eleven different series (A–K) of distinct pillar patterns and shapes were present in the array, with series A–J consisting of 16 unique combinations of pillar size, *X*, with *X* = 1, 2, 4 and 6 μm and inter-pillar gap size, *Y*, with *Y* = 1, 2, 4 and 6 μm. The last series K consisted of eight different sharkskin-like structures of rectangles with different lengths placed in rows, all with a gap size of 1 μm (Fig. 1). All structures of the array were 2.4 μm high. This

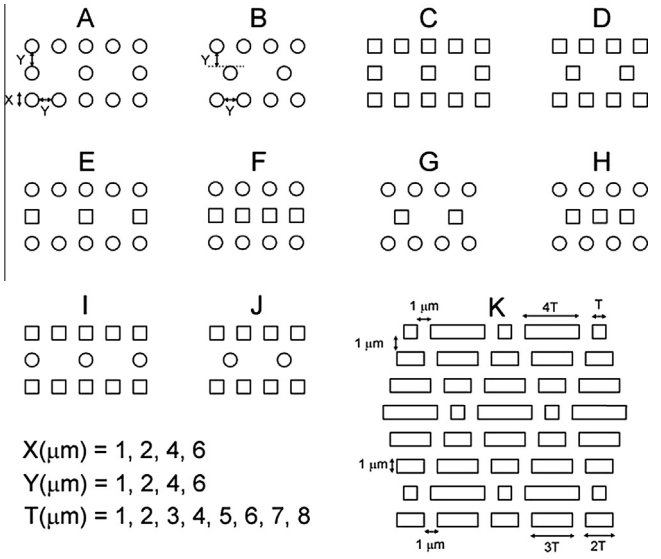

Fig. 1. The wafer design consisting of 13 × 13 distinct surfaces, each 3 × 3 mm^2 in size. The 169 surfaces consisted of geometry series A–K, with A–J consisting of pillars with *X* = 1, 2, 4 and 6 μm and inter-pillar gap sizes of *Y* = 1, 2, 4 and 6 μm, and with geometry *K* consisting of 8 unique combinations of rectangles with different lengths (*T*, 2*T*, 3*T* and 4*T*, where *T* = 1, 2, 3, 4, 5, 6, 7, 8 μm). Fig. taken from Ref. [38].