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The effect of substrate surface nanotopography on the behavior of multipotnent mesenchymal stromal cells and osteoblasts

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ABSTRACT

Hexagonally arranged Gold nanoparticles with controllable diameters and inter-particle distances were deposited on thick SiO_2 layers on top of Si wafers and used as masks during subsequent reactive ion etching. In this way, arrays of nanopillars are obtained with well-defined diameters (10/30 nm), inter-pillar distances (50–120 nm) and heights (20–35 nm), all on the nanoscale. Such nanotopographies served as substrate for multipotent mesenchymal stromal cells (MSC) and human osteoblasts (OB) allowing to study cellular responses to purely topographically patterned interfaces. Focus was put on adhesion, proliferation and differentiation of the cells. It turned out experimentally that *adhesion* is comparable for both cell types practically independent of topographical details at the substrate surface. Topography induced *proliferation* enhancement, however, is again independent of geometrical details in case of MSC, but significantly sensitive to pillar height in case of OB with a clear preference towards short nanopillars (20 nm). A high sensitivity to topographic details is also observed for osteogenic *differentiation* of MSC, in that case with a preference towards higher nanopillars (50 nm). The present experimental data also allow the important conclusion that cell proliferation and differentiation can be optimized simultaneously by fine-tuning nanoscaled topographical parameters.

1. Introduction

The road towards tissue engineering with its goal to provide synthetic biomimetic materials substituting damaged tissue or, at least, to support self-healing by integration of biomaterials in tissues, is paved with numerous obstacles one has to contend with. For instance, in vivo, the functionality of a biological tissue strongly depends on its three-dimensional (3-d) histoarchitecture. Thus, any attempt approaching this functionality in vitro should try to mimic such 3-d local architectures rather than to rely on ideal flat supporting surfaces [1]. Indeed, it turned out that the macro structure of surfaces, in a range up to micrometers, enhances successfully the integration of biomaterials [2]. Consequently, the cellular response to surface features in the micrometer range, such as grooves, ridges and wells, has been intensively investigated over the last years [3–6]. Meanwhile, cell sensitivity even to nanoscaled topographic patterns has been recognized [7], probably reflecting the importance of geometric cues from the extracellular matrix (ECM) for triggering cell responses. ECM, in turn, contains a variety of nanoscale components, such as hydroxyapatite crystals, collagen fibrils, or proteoglycans [7–9]. Thus, a nanoscaled surface topography at the cell interface serves as an attempt to mimic the in vivo cell environment leading to an interaction with tissue specific cells and modulation of their functions, including maintenance of tissue homoeostasis and cell differentiation or avoiding inflammation processes [10,11].

In skeletal tissues, the nanostructure dynamically influences the secretion of growth and differentiation factors and therefore the behavior of several different cell types like osteoblasts, osteoclasts, or their progenitors like mesenchymal or hematopoietic stem cells [9,12]. In addition, both, the chemical characteristics and the nanotopography of surfaces are essential for initial protein adsorption and therefore the cellular adhesion via integrin receptors [5,13].

Currently, several different techniques are used to fabricate nanostructured surfaces like imprinting approaches, methods based on self-assembled colloids or e-beam lithography [14–16]. To obtain surface topographies with more pronounced three-dimensional features, reactive ion etching has been successfully

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applied by several groups [17–20]. However, due to the diversity of reported results, mainly due to combining various topographic length scales often in a quite uncontrolled way or topographic and chemical patterns like adding protein coatings [16,18], elucidation of dominating mechanisms appear difficult. Also the use of different cell types or immortalized cell strains adds further problems for an immediate comparison of experimental data.

For that reason, for the present work exclusively well-controlled nanoscaled topographic surface structures were fabricated and the cellular response to these structures was studied with emphasis on cell adhesion, proliferation and differentiation. To this end, hexagonally arranged arrays of nanopillars in SiO_2 were prepared by applying a combination of block copolymer micellar lithography (BCML) and reactive ion etching (RIE) allowing independent control of inter-pillar distances as well as their height and diameter. Due to this preparational control together with the abandonment of any additional chemical surface functionalization, especially the role of pillar height could be addressed. The missing non-adhesive chemical coating of the area between nanopillars furthermore allows detecting possible preferential anchoring sites of cellular adhesion points on top or in between nanopillars. Finally, the present study aims at providing convincing experimental data in order to decide whether optimizing topographic parameters can simultaneously enhance proliferation and differentiation of osteogenic cells.

2. Materials and methods

2.1. Generation of nanotopography

Controlled fabrication of surface nanotopography was accomplished in a class 100 clean room facility. Approximately 300 samples from 3″ n-doped Si wafers with 1 μm thermally grown SiO_2 surface layer (CrysTec, Berlin, Germany) were prepared in order to analyze and characterize the cell–surface interaction. In a first step, wafers were spin-coated with PMMA resist and were cut into pieces of 5 mm × 10 mm. The resist was removed and substrates were cleaned by ultrasonic acetone and isopropanol baths, respectively, for 3 min each and then blow-dried with nitrogen. To pattern ordered arrays of nanopillars into the SiO_2 surfaces, Au nanoparticles were produced by block copolymer micellar lithography (BCML) and deposited on top of the SiO_2 surfaces by dip coating [21]. For this step, Polystyrene-b-poly(2-vinyl-pyridine) diblock (PS-b-PVP) copolymers with monomer lengths of 1850 and 900, respectively (Polymer Source Inc., Dorval, Canada), were stirred for one week in VLSI grade toluene as a solvent (J. T. Baker, Griesheim, Germany) leading to the formation of inverse micelles. Subsequently, Gold(III) chloride hydrate ($HAuCl_4 \cdot H_2O$) (Sigma–Aldrich, Munich, Germany) is added allowing to selectively load the core of the micelles with Gold by stirring for another week. The resulting micellar solution is then dip coated onto the substrates resulting in a single layer of Au-loaded hexagonally arranged micelles. By exposing such surfaces to a H_2-plasma for 90 min at 160 W (TePla 100-E Plasma Processor, Munich, Germany) the organic species forming the micelles are completely removed and the Au-precursor is reduced into metallic Au nanoparticles keeping their position as pre-defined by the hexagonal micellar array [22]. The essential advantage of this approach is the complete control over the final Au nanoparticle size as well as over their mutual distance with both geometrical parameters exhibiting narrow distributions. While particle diameters are determined by the total Au precursor load (in the present work, based on a micellar load factor of 0.5, the diameter of the primary Au particles is fixed to 10 nm), the interparticle distance can be influenced by both, the total length of the diblock-copolymer as well as by the pulling-out velocity applied during the dip coating process [23].

Once the Au nanoparticles are deposited on top of substrate, they serve as nanomasks for the subsequent reactive ion etching (RIE) process. To improve the etching resistance of these masks, the above described BCML technique was combined with an additional seed-mediated particle growth [24]. For that purpose, a 5 mM solution of $HAuCl_4$ in a mixture of phthalatesters (product number: 1160, Cargille Labs, New Jersey, USA) is prepared under ambient conditions and a drop is pipetted onto the Au nanoparticles. Exposure to UV-irradiation (Osram Hg lamp, Munich, Germany) provided by a mask aligner (Karl SUSS MJB 3 Mask UV 400, Garching, Germany) results in a selective photochemical deposition of Au on top of the pre-fabricated nanoparticles. In this way their starting diameters were enlarged from 10 nm up to 50 nm in a controlled manner. After exposure, samples were cleaned in acetone and isopropanol baths for 6 min each and blow-dried with nitrogen. Additional annealing at 720 °C for 4 h finally produces uniformly shaped Au nanomasks.

For the subsequent RIE process, a CHF_3/CF_4 plasma was applied and etching carried out with a constant DC bias of 96 V at ambient temperature to enhance anisotropy (Oxford PlasmaLab 80 Plus, Yatton, UK) [25,26]. The height of the resulting nanopillars was varied by controlling the etching time. After the RIE step, residual Au nanomasks were removed by a Lugol's solution followed by cleaning substrates with Millipore water and blow-drying with nitrogen. Finally, the nanostructured substrates were sterilized by heat inactivation for 20 min at 121 °C in humidified air at 1 bar pressure.

2.2. Cell culture

The human material for cell isolation was collected with informed consent by following the guidelines from the ethic committee of Ulm University. Human multipotent mesenchymal stromal cells (MSC) and human osteoblasts (OB) were isolated under standard procedures as described earlier [27,28]. All cells were cultivated in 75 cm^2/cell culture flasks (BD Falcon, Heidelberg, Germany) at 37 °C, 95% humidity, and 5% CO_2 using DMEM medium, supplemented with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin and 10% L-glutamine (all by Biochrom AG, Berlin, Germany). The medium was changed every three days.

For seeding on the substrates, cells were harvested by 1% trypsin/EDTA, centrifuged at 900 g for 10 min, subsequently suspended in cell culture medium adjusted to 20.000 cells/mL. Sterile substrates were placed within cell culture polystyrene well with a 3.8 cm^2 growth area (BD Falcon, Heidelberg, Germany). The substrates were covered with 1 mL cell containing medium and incubated over night as described above. Next day, the substrates were transferred into new polystyrene well in order to remove the cells that did not adhere on the surface. Cells were cultivated for 1 day or 7 days under standard conditions as described above. At least five different donors of primary cells were used per experiment. For differentiation experiments, MSCs from one donor were cultivated in standard medium on the different nanotopographic surfaces.

2.3. Scanning electron microscopy

The starting arrangements of Au nanoparticles on SiO_2 substrates as well as the final patterned nanopillar arrays were analyzed by high resolution scanning electron microscopy (Hitachi S5200 HRSEM, Krefeld, Germany) with an acceleration voltage of 30 kV. From the corresponding SEM images the diameter of the Au nanomasks and the resulting interpillar distance could be determined by applying ImageJ 1.40 g software. In order to characterize the pillar shape and dimensions, the sample holder was either tilted by an angle of 30° or a special holder was used to acquire cross-section images.

For the characterization of MSCs and OBs on the nanostructures, samples were cultivated as described above. Before fixation, the samples were washed twice with phosphate buffered saline (PBS) solution and subsequently incubated with 2.5% Glutaraldehyde with 1 M Saccharose (all Sigma–Aldrich, Munich, Germany) in PBS for 1 h at RT. After an additional washing step – three times with PBS - samples were incubated in 2% Osmium tetroxide for 2 h at RT. After additional washing in PBS for several times, the samples were put into ethanol solutions of increasing concentration from 30% to 100% and then dried in supercritical CO_2.

The dried probes were mounted on holders for electron microscopy and sputter coated with 5 nm Platinum or 5 nm Gold/Palladium. For HRSEM analyses of the cells, lower acceleration voltage (10 kV) was applied to prevent damaging and tilt angles of 0°, 30° and 85° were chosen in order to make the interaction between lamellipodia–filopodia and nanopillars clearly visible.

2.4. Immunofluorescent staining

For analyzing overall cell morphology, the actin cytoskeleton and the distribution of focal adhesions a fluorescent staining with anti Vinculin and Phalloidin-FITC (all Sigma–Aldrich, Munich, Germany) was employed. Proliferation was analyzed by DAPI (Sigma–Aldrich, Munich, Germany) staining of cell nuclei. Osteogenic differentiation was proved by anti-Osteocalcin staining (Abcam, Cambridge, UK). In order to visualize the staining on the nanotopographies, non-fluorescent antibodies were marked with a secondary antibody, labeled with Alexafluor 568 (Life Technologies, Darmstadt, Germany).

For all stainings, the medium from the cultivated substrates was removed and the substrates were washed twice with PBS (Sigma–Aldrich, Munich, Germany) before fixation with 3.7% Formaldehyde/PBS over 30 min. After three additional washing steps with PBS, the cells were incubated for 10 min in 0.2% Triton X-100 (Merck KGaA, Darmstadt, Germany) at RT. After a final washing with PBS, the substrates become covered with DAKO Protein Block reagent (DAKO, Hamburg, Germany) for 30 min.

After removal, the substrates were incubated with the antibodies for anti-β-Actin (1:200), anti-Vinculin (1:400) at 4 °C over 16 h. After a washing step with PBS, the samples were incubated with the secondary antibody, modified with Alexafluor 568 (1:1000) for 1 h in the dark. The samples were washed three times, followed by a Phalloidin-FITC (1:200) staining. In terms of testing osteogenic differentiation, cells were stained exclusively with anti-Osteocalcin (1:100) before using the Alexafluor 568 labeled secondary antibody under same conditions. Finally, in all cases the samples were stained by DAPI (1:10) for 30 min in the dark in order to count the total cell number.