Biomaterials 61 (2015) 316–326

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Surface topography enhances differentiation of mesenchymal stem cells towards osteogenic and adipogenic lineages

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ARTICLE INFO

Article history:
Received 24 April 2015
Accepted 16 May 2015
Available online 18 May 2015

Keywords:
Mesenchymal stem cells
Microstructure
Nanotopography
Laser ablation
Gene expression
Osteogenesis

ABSTRACT

Surface topography impacts on cell growth and differentiation, but it is not trivial to generate defined surface structures and to assess the relevance of specific topographic parameters. In this study, we have systematically compared *in vitro* differentiation of mesenchymal stem cells (MSCs) on a variety of groove/ridge structures. Micro- and nano-patterns were generated in polyimide using reactive ion etching or multi beam laser interference, respectively. These structures affected cell spreading and orientation of human MSCs, which was also reflected in focal adhesions morphology and size. Time-lapse demonstrated directed migration parallel to the nano-patterns. Overall, surface patterns clearly enhanced differentiation of MSCs towards specific lineages: 15 μm ridges increased adipogenic differentiation whereas 2 μm ridges enhanced osteogenic differentiation. Notably, nano-patterns with a periodicity of 650 nm increased differentiation towards both osteogenic and adipogenic lineages. However, in absence of differentiation media surface structures did neither induce differentiation, nor lineage-specific gene expression changes. Furthermore, nanostructures did not affect the YAP/TAZ complex, which is activated by substrate stiffness. Our results provide further insight into how structuring of tailored biomaterials and implant interfaces – e.g. by multi beam laser interference in sub-micrometer scale – do not induce differentiation of MSCs *per se*, but support their directed differentiation.

1. Introduction

Self-renewal and differentiation of stem cells is influenced by chemical and physical features [1–3]. In their physiological microenvironment, the so called “stem cell niche”, mechanical stimuli are provided by the extra cellular matrix (ECM) as well as cell–cell interactions and contribute to cell fate decisions [4]. Cells can “sense” substrate elasticity [5,6] and surface patterns ranging from 10 nm to 100 μm [7,8]. This recognition is particularly mediated by integrin receptors [9–11], which in combination with several other proteins like vinculin or zyxin form focal adhesions [12,13]. Tailored biomaterials with specific structures – such as grooves, ridges, pits, or pillars – can mimic the topographic landscape of the niche. However, these structures are notoriously complex with multiple combinations of parameters and symmetry layout. For a better understanding of relevant topographic features it is therefore important to systematically vary one specific parameter at the time in order to determine its impact on cellular functions. Grooved patterns are ideally suited in this context since 1) the width of either grooves or ridges can be systematically modified, 2) orientation of cells along these structures can readily be observed [14], and 3) such structures may reflect the fibrillar organization of ECM, e.g. of collagen fibrils [1].

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http://dx.doi.org/10.1016/j.biomaterials.2015.05.030
0142-9612/

The method of choice for surface structuring depends on the biomaterial and on the pattern. Frequently used techniques include photolithography [15] or soft lithography [16]. High resolution structures can also be generated by reactive ion etching. In this method, the impact of plasma ions removes material from the surface and such synergism between chemical and physical bombardment facilitates fast etching rates of the features to be patterned, down to micrometer range [17]. Alternatively, direct multi-beam interference employs the modulated energy distribution by the interference pattern of laser beams to ablate the surface of a substrate. It allows the direct nanostructuring of most types of materials without the need of additional steps, thereby decreasing costs of periodic surface functionalization [18,19]. In contrast to common laser techniques, laser-interference is not limited by light diffraction and hence facilitates generation of subwavelength structures [20]. Well-defined and consistent patterns ranging from 20 μm down to sub-micrometer can be produced in a large area within very short period of time – however, so far laser interference technology was rarely used to structure biomaterials [21].

Due to their relevance in regenerative medicine, mesenchymal stem cells (MSCs) are among the most studied cell types in the biomaterial field. They can be isolated from various tissues, such as bone marrow and adipose tissue, and comprise a subpopulation that is capable of differentiation towards multiple cell types of the mesodermal lineage including osteocytes, adipocytes, and chondrocytes [22]. It has been suggested that specific surface patterns can either maintain MSCs in an undifferentiated state [23] or drive them towards osteogenic [24], adipogenic [25], or chondrogenic lineages [26]. Cell shape, cytoskeletal tension and RhoA signaling are thought to direct this commitment [27]. Several studies focused on the impact of micro- and nano-grooved substrates in osteogenic differentiation [28–32] whereas only few studies reported effects on adipogenesis [33,34]. Osteogenesis and adipogenesis are triggered by different biochemical stimuli and appear to be rather mutually exclusive [35,36]. Thus, it is essential to better understand which precise specifications of surface patterns can direct differentiation towards one lineage or another.

In this study we designed a microgrooved polyimide chip featuring a combination of 25 different structures with systematic variation of the width of grooves and ridges. In addition, we generated groove and ridge structures by multi beam laser interference. The rational to combine these two different approaches was to bridge a wider spectrum of topographies ranging from 15 μm structures down to sub-micrometer scale. The impact of these structures on morphology, proliferation, focal adhesions and *in vitro* differentiation of MSCs was subsequently analyzed.

2. Material and methods

2.1. Production of microgrooved polyimide substrates

Ten mm in diameter glass wafers were cleaned, treated with oxygen plasma and coated with an organosilane (VM-651, HD Microsystems). Polyimide (PI) PI-2611 (HD Microsystems, Neu-Isenburg, Germany), supplied as polyamic acid dissolved in n-methyl-2-pyrrolidone, was spin-coated on the glass surface at 500 rpm. The PI layer was subsequently cured for three hours at 400 °C. The final thickness was 5 μm as determined with a profilometer (Tencor P-10, KLA Tencor, Milpitas, USA). Afterwards, a 100 nm thick chromium layer was evaporated on the PI and structured by standard optical lithography to serve as an etching mask. Reactive ion etching of the grooves was then performed using oxygen plasma with 150 W power for 33 min in a TEGAL 901 PLASMA ETCH RIE system (Petaluma, CA, USA).

2.2. Production and characterization of nano-grooved polyimide foils

The linear polarized beam of a diode pumped Nd:YAG laser (Q301-HD, JDS Uniphase Corporation, Milpitas, USA) with a coherence length of 5.4 mm, a pulse duration of 38 ns and a wavelength of 355 nm was refined through a spatial filter and then split into two beams. Through the deflection by a set of mirrors the two beams were guided to intersect on the surface of a 50 μm thick PI foil (Upilex, Ube, Japan) and ablated its surface. The periodicity of the resulting patterns was determined by the incidence angle α of the intercepting beams and by the laser wavelength. Pattern geometry and its periodicity were measured with an atomic force microscope (AFM; Rados N8, Bruker, Herzogenrath, Germany). The surface area of the PI foil that was not exposed to the laser beams was used as flat, non-structured control.

2.3. Isolation and characterization of MSCs

MSCs were isolated from lipoaspirates after patient's written consent using guidelines approved by the Ethic Committee of the University of Aachen (Permit number: EK163/07). Cell isolation and culture was performed as previously described [37]. Culture medium consisted of Dulbecco's Modified Eagles Medium-Low Glucose (DMEM-Low Glucose; PAA, Pasching, Austria) with 2 mM L-glutamine (Gibco/Invitrogen, Eugene, OR, USA), 100 U/mL penicillin/streptomycin and 10% human platelet lysate (hPL) which was pooled from five platelet units of healthy donors [38–40]. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 with medium changes twice per week. MSC preparations were always used at similar passage number (between 2 and 4) and their immunophenotype and *in vitro* differentiation potential was validated as described before [41].

2.4. Fluorescence microscopy

For the visualization of cells on the microgrooved PI chip, MSCs were stained with the PKH26 dye according to manufacturer's instruction (Sigma Aldrich, Hamburg, Germany) and then cultured for 48 h on the chip. Cells were fixed with 4% PFA (Carl Roth GmbH, Karlsruhe, Germany) and stained with DAPI (4′,6-Diamidin-2-phenylindol; Molecular Probes, Eugene, Oregon, USA). Pictures were taken using an EVOS fl fluorescence microscope (Life Technologies, Darmstadt, Germany). Due to reactive ion etching the grooves revealed higher autofluorescence (Suppl. Fig.1).

For actin filament and focal adhesion (FA) analysis cells were grown for 3 days on the PI substrates and then fixed in 3.7% formaldehyde in cytoskeleton buffer (150 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM glucose, 1 mg/ml streptomycin, 10 mM MES, pH 6.2). Subsequent steps were performed as described before [42]. Vinculin was stained with the antibody clone hVin1 (Sigma Aldrich, Hamburg, Germany) and actin was stained with Alexa Fluor488 phalloidin (Life Technologies, Darmstadt, Germany). Samples were covered with 20 μl GelMount mounting medium (Sigma Aldrich, Hamburg, Germany), covered with a 170 μm thick cover slip. For the detection of Yes-associated protein (YAP), MSCs were grown on the different substrates for 24 h, fixed with 4% PFA in PBS, permeabilized with Triton X-100 0.1%, and then stained with primary anti-YAP mouse monoclonal antibody (sc101199, Santa Cruz Biotechnology, Dallas, USA 1:200 dilution in BSA 1%) and secondary goat-anti-mouse Alexa 488 antibody (1:300 dilution in BSA 1%). Microscopy was performed on an inverse confocal laser scanning microscope (LSM710, Carl Zeiss, Jena, Germany) using a 63× Plan-Apochromat oil immersion objective (Ph3, NA 1.4) and appropriate filter sets. Images were recorded with a cooled, back-illuminated