Biomaterials 35 (2014) 2743–2751

Contents lists available at ScienceDirect

Biomaterials

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

Tuning the material-cytoskeleton crosstalk via nanoconfinement of focal adhesions

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ARTICLE INFO

Article history:
Received 11 October 2013
Accepted 13 December 2013
Available online 3 January 2014

Keywords:
Cell adhesion
Cell morphology
Progenitor cell
Adhesive nanopatterns
Mechanotransduction
Polydimethylsiloxane

ABSTRACT

Material features proved to exert a potent influence on cell behaviour in terms of adhesion, migration and differentiation. In particular, biophysical and biochemical signals on material surfaces are able to affect focal adhesion distribution and cytoskeletal assemblies, which are known to regulate signalling pathways that ultimately influence cell fate and functions. However, a general, unifying model that correlates cytoskeletal-generated forces with genetic events has yet to be developed. Therefore, it is crucial to gain a better insight into the material-cytoskeleton crosstalk in order to design and fabricate biomaterials able to govern cell fate more accurately. In this work, we demonstrate that confining focal adhesion distribution and growth dramatically alters the cytoskeleton's structures and dynamics, which in turn dictate cellular and nuclear shape and polarization. MC3T3 preosteoblasts were cultivated on nanograted polydimethylsiloxane substrates and a thorough quantification – in static and dynamic modes – of the morphological and structural features of focal adhesions and cytoskeleton was performed. Nanoengineered surfaces provided well-defined zones for focal adhesions to form and grow. Unique cytoskeletal structures spontaneously assembled when focal adhesions were confined and, in fact, they proved to be very effective in deforming the nuclei. The results here presented provide elements to engineer surfaces apt to guide and control cell behaviour through the material-cytoskeleton-nucleus axis.

1. Introduction

Material features, in the form of chemical-physical characteristics, proved to be very effective in controlling different aspects of cell behaviour such as adhesion and migration up to cell differentiation. In a landmark study, Chen et al. demonstrated that local geometric control of cell shape, by means of cell confinement on adhesive islands, was sufficient to regulate cell growth or apoptosis [1]. More recently, Engler et al. have shown that the mechanical behaviour of the culturing substrate was a powerful cue to specify MSC lineage towards neurons, myoblasts and osteoblasts. In particular, stiff matrices lead to enhanced cytoskeletal tension and osteogenesis, while compliant matrices directed MSCs towards neurogenesis and myogenesis [2]. Surface topographies also have dramatic effects on cell fate. For example, Dalby et al. demonstrated that topographic signals alone, in the form of a slightly irregular pattern of nanopits, were sufficient to induce osteogenic differentiation of MSC [3]. More recently, the same group has reported that ordered arrays of nanopits allowed MSC to retain a stem cell phenotype in vitro, up to eight weeks [4].

Although the underlying biomolecular mechanisms are not thoroughly understood, there is growing evidence that cell contractility and cytoskeletal-generated forces play a crucial role in triggering signalling pathways responsible for cell differentiation [5]. Additionally, some evidence demonstrated that contractility was sufficient to deform the nucleus and that such a deformation had direct consequences on gene expression [6]. Altogether, these observations suggest that material features altering cell contractility have a profound impact on regulating cell fate through the cytoskeleton–nuclear axis. Along these lines, recent literature postulated that the material-cytoskeleton crosstalk and therefore cell-generated forces might be tuned via focal adhesion (FA) assembly [7]. Indeed, FAs play an important role in this process being the mechanical link between the cytoskeleton and the extracellular environment. Adhesions are multiprotein structures that involve the recruitment, clustering and turnover of several molecular components. They change in shape and dimensions according to the forces

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.023

acting on them. Similarly, the cytoskeleton changes its configuration according to the spatial arrangement and size of adhesions [8]. Nascent adhesions are initially formed when few integrin heterodimers (transmembrane receptors) bind to extracellular ligands. The cytoplasmic part of these clusters can be stabilized by reinforcing proteins like talin, vinculin, paxillin, α-actinin and focal adhesion kinase [9]. Nascent adhesion may either disassemble or mature into larger focal complexes. The latter event requires the action of myosin II, and presumably contractility, along with the clustering of additional integrin dimers and cytoplasmic proteins, which increases the dimension of the complex with enhanced stability [10]. Increasing stresses generated by contractile actin, causes focal complexes to turn into focal adhesions, which can reach several micrometres in length. Here, several hundreds of proteins are assembled in a multilayered fashion and stabilize the mechanical link with the cytoskeleton by means of high levels of vinculin and zyxin [11]. Adhesion maturation only occurs if specific environmental and dynamic requirements are fulfilled. Firstly, material surfaces have to present a continuous distribution of ligands. Furthermore, ligands with a lateral spacing above 73 nm proved to impair integrin clustering and therefore adhesion maturation [12]. Integrins extend about 20 nm in the extracellular space, therefore, this is likely the maximum distance in which they can bind to ligands, for example when these are in recesses or nanocavities [13]. Furthermore, FAs have often been presented as dynamic entities, owing not only to the fact that they increase their size, but also because they have the ability to 'slide' in a treadmill-like fashion under the effect of contractile forces [14]. Having all these geometrical characteristics in mind, it is possible to pattern material surfaces with adhesive patches that are conducive for FA establishment and growth, juxtaposed to others that are not, thus obtaining a strong control over FA distribution and therefore cytoskeletal assemblies.

One of the possibilities to achieve the above consists in embossing topographic patterns on material surfaces. Surface features having specific dimensions and aspect ratios prevent cell membrane curvature and conformal contact with the features themselves [15]. In this case, topographic features create patterns of accessible and impervious zones for integrins, which affect adhesion formation and maturation. Several technologies have been proposed to gain a tight control on the geometric characteristics of the surface features, such as width, pitch and depth. Among these, soft lithography, electron or ion beam lithography and nano imprinting lithography proved to be very effective in patterning large material surfaces with high spatial resolution [16]. Furthermore, these technologies can be implemented on a large variety of materials: metals, elastomers, polymers and hydrogels. Therefore, the use of topographic patterns represents a robust and reliable method to control FA growth and cytoskeletal assemblies. Yet, many aspects of the influence of the material features on the FAs/cytoskeleton/nuclear shape axis still remain unclear. Mastering this crosstalk is essential in order to conceive functionalization strategies that are effective in controlling cell behaviour.

This study is based on the hypothesis that FA confinement is responsible for specific cytoskeletal assemblies and therefore intracellular stress states, which ultimately affect not only cell, but also nuclear shape. To this aim, we correlated quantitative analyses performed with a high resolution microscopy with time dependent observations, which provided a comprehensive vision of the effects of FA confinement at different levels: from adhesion plaques to nuclear shape.

2. Materials and methods

2.1. Preparation of nanopatterned substrates

Patterned substrates were obtained by replica moulding of polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) on polycarbonate master. The pattern consisted of an area of 1 cm^2 containing parallel and straight channels with a groove and ridge width of 700 nm and depth of 250 nm. PDMS was prepared by mixing elastomer base and curing agent at 10:1 weight ratio. The solution was degassed, poured onto the master and then cured at 37 °C for 24 h. Control (uniform) PDMS substrates were produced by pouring the base and curing mix on a 35 mm polystyrene petri dish (Corning) and curing at 37 °C for 24 h. To improve cell adhesion, PDMS substrates were treated with oxygen plasma. Briefly, the treatment was performed with a Plasma Femto (Diener) equipped with 13.56 MHz 100W generator for the plasma excitation. Plasma exposure was 1 min and then substrates were sterilized by UV exposure for 15 min and then incubated with serum supplemented culture medium overnight prior to cell culturing experiments.

2.2. Cell culture

MC3T3-E1 preosteoblasts (ATCC) were cultured in αMEM with deoxyribonucleosides, ribonucleosides and 2 mM L-glutamine, supplemented with 10% foetal bovine serum, penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) (GIBCO). The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every two days. After 3 days of culture, cells were detached with trypsin/EDTA (0.25% w/v trypsin/0.02 mM EDTA) (GIBCO) and seeded on nanopatterned or uniform substrates at a density of $5{\cdot}10^3$ cells cm^{-2}.

2.3. Immunofluorescence

Stress fibres and FAs were examined by immunofluorescence confocal microscopy. Cells cultured on nanopatterned and flat substrates were fixed at 4 and 12 h after seeding. Cell fixation was performed with 4% paraformaldehyde for 20 min and then cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS 1×. Samples were blocked in PBS/BSA 1% solution (Sigma) for 30 min, to avoid non-specific binding. Focal adhesions were recognized by incubating samples with anti-vinculin monoclonal antibody (dilution 1:200; Chemicon) for 2 h at 20 °C. After incubation, substrates were washed 3 times with PBS (3 min per wash) and incubated with Alexa Fluor 488 conjugated goat anti-mouse antibody (dilution 1:1000; Molecular Probes) for 30 min at 20 °C. Actin filaments were stained by incubating samples with rhodamine conjugated phalloidin (dilution 1:250; Sigma) for 30 min at 20 °C. Nuclei were stained by incubating samples with DAPI (dilution 1:1000; Sigma) for 15 min at 37 °C.

Fluorescent images of focal adhesion and actin bundles were collected with a Leica TCS SP5 confocal microscope (Leica Microsystems). Samples were excited with 488 nm (vinculin) and 543 nm (actin) laser lines, and the emissions were collected in the 500–530 nm and 560–650 nm ranges respectively. DAPI stained nuclei were visualized in two-photon microscopy mode. Excitation was set at 700 nm and the emission was collected in the 400–450 nm range using a band pass filter.

2.4. Drug treatment

Blebbistatin (Sigma) was used to inhibit myosin II contractility. Cells grown on patterned or on flat control substrates were treated for 40 min with a 15 μM solution of blebbistatin in DMSO (Sigma). The treatment started either after 3 h or 11 h post seeding and lasted for 40 min. Afterwards, the samples were fixed and stained for the cytoskeleton and vinculin as described above.

2.5. Live-cell confocal microscopy

MC3T3-E1 cells were seeded in 35 mm petri dish at 80% confluence and let overnight in incubator at 37 °C and 5% CO_2. Cells were then transfected with LifeAct-GFP (Ibidi) and mKate2-Paxillin (Evrogen). The transfection complex was prepared in Opti-MEM I reduced serum medium (GIBCO) and Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. The amount of DNA/Lipofectamine was determined by following the supplier's instruction. Briefly, after 4 h incubation with 1.5 μg of pDNA in lipoplexes, cells were incubated with a medium containing 10% FBS 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Then, the transfected cells were re-plated on nanopatterned and flat substrates. Normal growth medium supplemented with 60 mM Hepes (Sigma) was used during the acquisition.

Time-lapse videos were acquired with a Leica TCS SP5 equipped with heated sample holder environment (37 °C) and the images were collected with a 63× oil objective. Preliminary experiments were carried out to optimize confocal settings in order to minimize phototoxicity and photobleaching.

Time-lapse experiments started 30 min after cell seeding on the substrates and frames were taken every 2 min for 12 h.

2.6. Image analysis

Cell polarization was assessed from TRITC-phalloidin stained cells that were analyzed with the MomentMacroJ v1.3 script (hopkinsmedicine.org/fae/mmacro.htm) run in Fiji. Briefly, the macro calculates the second moment of grey scale images. For our purposes, we evaluated the principal moments of inertia (i.e. maximum and minimum) and the cell polarization was defined as the ratio of the principal moments (max/min). The angle of polarization was defined as the angle that the principal axes of inertia form with the reference axes. To determine actin bundle orientation, selected regions of confocal images of TRITC-phalloidin stained cells