Bioactive Nanoimprint Lithography: A Study of Human Mesenchymal Stem Cell Behavior and Fate

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Abstract—**Biomaterials aim to mimic *in vivo* extracellular matrices where cell interactions occur on the nanoscale. Thus, incorporation of nanosized components is interesting in the preparation of bioactive surfaces. We present a technique using nanoimprint lithography to create chemical nanopatterns on silicon surfaces functionalized with bioactive motifs. Due to high throughput and versatility, a wide range of geometries and dimensions can be efficiently patterned. In our study, we prepared and characterized two types of bioactive nanodots (150 nm diameter with 350 nm spacing, and 80 nm diameter with 110 nm spacing) functionalized with cell adhesion-promoting RGD peptides. We examined mesenchymal stem cell adhesion and commitment on these modified material surfaces with respect to homogeneous RGD and non-functionalized surfaces. We report that bioactive nanostructures induce fibrillar adhesions on human mesenchymal stem cells with an impact on their behavior and dynamics specifically in terms of cell spreading, cell-material contact, and cell differentiation.**

Keywords—**nanoimprint lithography, mesenchymal stem cell, surface functionalization focal adhesion, differentiation.**

I. INTRODUCTION

In biomaterials design, an effective product combines the right type of cells, an appropriate scaffold, and a smart choice of signaling molecules to be incorporated in the system [1]. Human mesenchymal stem cells (hMSCs) are widely used in regenerative medicine as their multi-potent capabilities show promise in repairing damaged tissues and organs [2]. In addition, nanopatterned scaffolds have gained interest in tissue engineering due to the unique potential of mimicking an *in vivo* extracellular matrix (ECM) [3]. It is important to understand the mechanisms by which stem cells differentiate in the nanoscale environment, particularly in relations to cell dynamics such as responses in cell morphology and focal adhesions (FAs) to bioactive nanostructures. We develop a method of preparing and characterizing nanostructured RGD motifs using nanoimprint lithography (NIL) and surface functionalization [4,5]. We demonstrate that bioactive surface nanostructures have an impact on hMSC behavior by inducing fibrillar FA formation, increasing cell-material contact, and affecting hMSC commitment.

II. MATERIALS AND METHODS

A. Materials

Silicon wafers were purchased from Active Business Company GmbH, Germany. Poly(methyl methacrylate) (PMMA) was purchased from Agilent Technologies, Belgium. 3-aminopropyldimethylethoxysilane (APDMS) and 2-[Methoxypoly(ethyleneoxy)propyl]trimethoxysilane (PEO silane) were purchased from ABCR GmbH, Germany. Dry dimethylformamide (DMF) and 3-Succinimidyl-3-MaleimidoPropionate (SMP) were purchased from Sigma-Aldrich, France. Dry toluene was purchased from Fisher Scientific, Belgium. Customized GRGDSPC peptides were synthesized by Genecust, Luxembourg.

B. Nanoimprint Lithography

PMMA was spin-coated onto Si to create a polymer mask. A Si mold was pressed onto the polymer mask using an Obducat nanoimprinter. The sample was heated at 170 °C for 3 minutes, then the pressure was increased to 60 bars and left for 3 minutes to perform the imprint. The system was then cooled down to 70 °C and the mold was detached from the sample. Samples were subjected to a descum process in O_2 plasma to remove residual PMMA from the patterned regions.

C. Surface Functionalization and Characterization

Imprinted samples were placed in a Schlenk reactor injected with APDMS. The reaction was run overnight at 80°C. Samples were then washed in acetone using a Soxhlet apparatus, and immersed overnight in a solution of non-adhesive PEO silane in dry toluene. Passivated samples were immersed in a SMP solution in dry DMF for 2 hours at room temperature. Samples were then rinsed with Milli-Q water and immersed in an RGD peptide solution. The reaction was run for 4 hours at room temperature under gentle agitation. After surface preparation, atomic force microscopy (AFM) was performed in contact mode on functionalized nanopatterned samples to characterize surface topography and roughness.

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D. Cell Culture and Immunofluorescence

Human mesenchymal stem cells (hMSCs) were seeded at a density of 10^4 cells/cm^2. After 24 hours or 4 weeks in culture (for adhesion and differentiation studies, respectively), hMSCs were fixed with paraformaldehyde. Fixed cells were permeabilized and blocked with 1% bovine serum albumin. Cells were then incubated with primary antibody and secondary antibody successively, then stained for F-actin using Alexa Fluor® 488 phalloidin. Cell nuclei were counterstained with DAPI. After DAPI staining, samples were mounted with coverslips on microscope slides.

E. Image Acquisition and Analysis

A Leica DM5500B epifluorescence microscope was used to image hMSCs. Cells with morphologies representative of each condition were imaged. Fluorescent images of at least 50 cells at each surface condition were taken for quantitative analysis, both for adhesion and differentiation studies. Quantification of FA count, FA area, projected cell area, and STRO-1 expression was carried out using ImageJ software.

III. RESULTS

A. Nanopattern Characterization

Two types of ordered nanodots ($D_{150}S_{350}$ and $D_{80}S_{110}$, with D and S denoting the nanodot diameter and inter-dot spacing in nanometer, respectively) were prepared. AFM was performed in contact mode on both types of surfaces to show the chemical contrast between the PEO background and the nanopatterned regions (Figures 1A and 1B for $D_{150}S_{350}$ and $D_{80}S_{110}$ respectively). Data was acquired in height mode to obtain chemical topography, with 3D rendering shown in Figures 1C and 1D.

B. Stress Fiber Organization and Focal Adhesion Formation

To evaluate cell behavior, hMSCs were cultured on RGD-grafted $D_{150}S_{350}$ and $D_{80}S_{110}$ surfaces as well as bare, polished silicon surfaces (Si poli) and homogeneous peptide-grafted silicon surfaces (RGD H) as controls. Cell morphology was observed at each condition (Figure 2). Adherent hMSCs on bare Si samples are smaller and lacked defined cytoskeletal organization, whereas on RGD H samples, cells are larger with a more organized cytoskeletal structure as shown by the arrangement of the F-actin stress fibers. $D_{150}S_{350}$ and $D_{80}S_{110}$ show a mixture of cell shapes and sizes, but cytoskeletal arrangement remains organized with defined stress fibers.

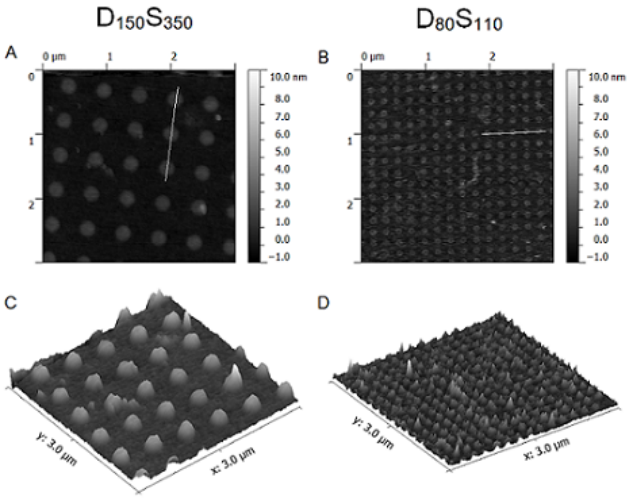

Fig. 1 Contact mode AFM is performed on (A) $D_{150}S_{350}$ and (B) $D_{80}S_{110}$ surfaces after the grafting of SMP (hetero-bifunctional cross-linker) in the nanopatterns. The difference in intensity between the background and the nanodots is indicative of topographical and chemical contrast between the SMP and the PEO silane. 3D rendering of the (C) $D_{150}S_{350}$ and (D) $D_{80}S_{110}$ surfaces was performed to illustrate chemical topography.

To observe the formation of FAs, we stained for vinculin, an important protein at the site of integrin-mediated FAs. Figure 2 highlights the typical appearance of FAs found on each type of surface with magnified views of selected regions shown in Figures 2A to 2D. FAs on Si poli were scarce, while RGD H induced thin, sparse clusters of vinculin both around the periphery and around the nucleus of the cell. In contrast, FAs were concentrated exclusively around the cell periphery on $D_{150}S_{350}$ and $D_{80}S_{110}$, with thicker and more pronounced fibrillar contacts, representing locally concentrated integrin clustering. Notably, FAs on RGD H are arranged in a random fashion, whereas on $D_{150}S_{350}$ and $D_{80}S_{110}$, the elongated FAs are aligned along the orientation of the stress fibers.

C. Focal Adhesion Area

To implicate the role of FA clustering, we quantified the area of each FA. For each type of material, the individual FAs are classified based on their area: > 25 um^2, 10 – 25 um^2, 5 – 10 um^2, and < 5 um^2. Figure 3A is a hMSC on a $D_{150}S_{350}$ surface with FAs in the first three classes. Magnified views of FAs are shown in Figures 3B, 3C, and 3D for FA areas of 25 um^2, 10 um^2, and 5 um^2 respectively. Figure 3E expresses the number of FAs in each class as a percentage of the total number of FAs for each material. FAs on both $D_{150}S_{350}$ and $D_{80}S_{110}$ were more abundant in each class compared with homogeneous surfaces, whether bare Si or RGD-grafted.

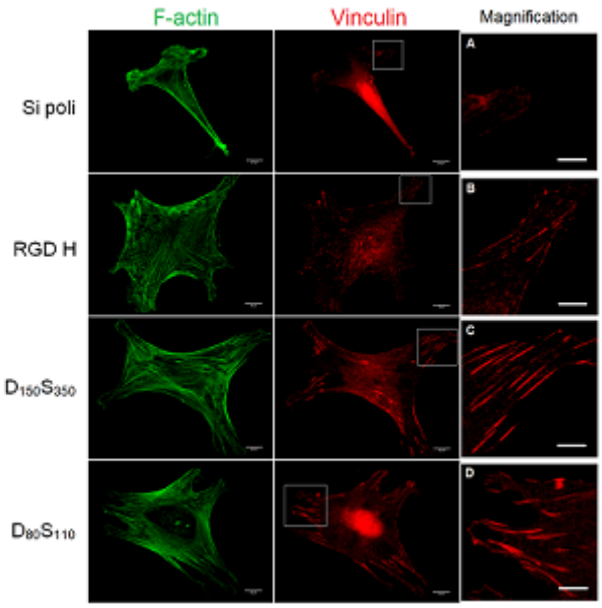

Fig. 2 Immunofluorescent staining showing typical morphologies of hMSCs grown on various substrates, with F-actin in green and vinculin in red, scale bar = 20 µm. Cells are larger on RGD-grafted substrates, with a more organized cytoskeletal structure and more distinct points of focal adhesion on nanopatterns as represented by vinculin clustering. Magnifications of focal adhesions are shown in (A) – (D), scale bar = 10 µm.

D. STRO-1 Expression

To investigate whether FA fibrillar shape formation induced by nanopatterns changes hMSC commitment, we cultured hMSCs for 4 weeks and stained for STRO-1, a mesenchymal stem cell-specific marker (Figures 4A to 4D). The STRO-1 fluorescence signal was quantified by measuring the mean signal density of each cell (Figure 4E). We noted a significant decrease in STRO-1 expression on RGD H, and again a significant decrease on nanopatterned RGD-grafted surfaces. A lower STRO-1 activity implies that stem cell population has decreased over 4 weeks on nanopatterns relative to homogeneous controls. While a part of the population is still STRO-1 positive (hence retaining its "stemness"), the decrease indicates that more cells have differentiated on nanopatterns than homogeneous controls.

IV. Discussion

Stem cell differentiation is affected by molecular composition and physical forces present in their ECM environment [6]. Nanoscale topographies have a noticeable effect on stem cell behavior and fate [7]. The study of hMSCs on bioactive nanopatterned surfaces offers interesting insights. We prepared materials with chemical

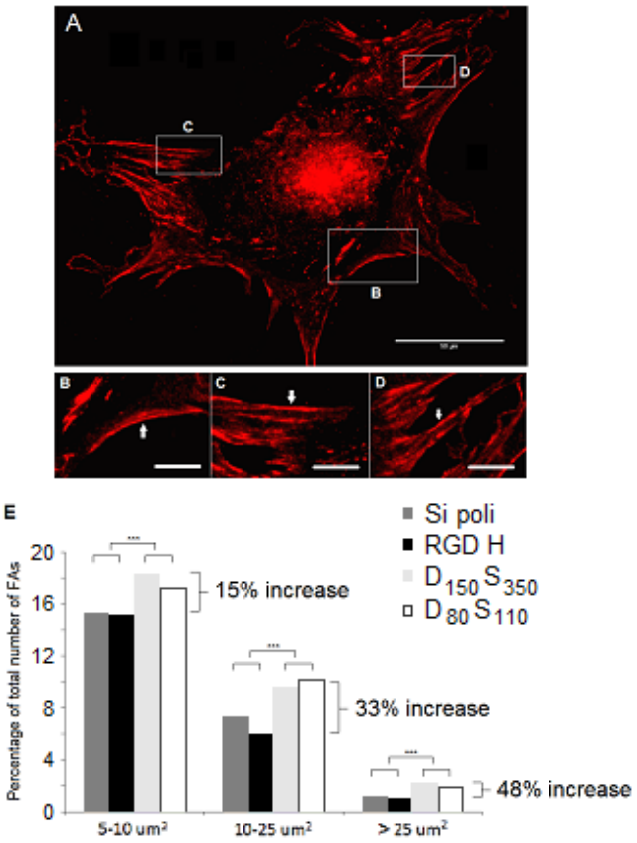

Fig. 3 A hMSC expressing vinculin is shown in (A), scale bar = 50 µm. FAs with sizes (B) 25 μm^2, (C) 10 μm^2, and (D) 5 μm^2 are magnified, scale bar = 10 µm. FAs are sorted into three classes. The number of FAs (vinculin clusters) in each class is expressed as a percentage of the total number of FAs (E). Large FAs are more abundant in nanopatterns compared with control surfaces, with a 48% increase for FA areas > 25 um^2, 33% increase for FA areas between 10 and 25 um^2, and a 15% increase for FA areas between 5 and 10 um^2. Two sample t-test was used to compare significant difference between the percentages. *** represents a *p-value* of less than 0.05.

nanopatterns, providing a bioactive stage for specific cell response. The onset of efficient cell adhesion requires the clustering of integrins and their receptors which, when mature, establish FAs and enhance adhesive strength, in turn initiating a host of related signal transductions [8]. We note the formation of fibrillar FAs on nanopatterned surfaces (Figure 2), which are absent on bare Si and RGD H surfaces. Moreover, these fibrillar FAs are aligned in the direction of stress fibre elongation, serving as anchors for that maintain fiber tension. On the other hand, FAs on RGD H orient in a random and disordered way, without the fibrillar shape and cytoskeletal alignment observed on nanopatterned surfaces. Since cytoskeletal contractility is implicated in cellular signal transduction, the alignment of fibrillar FAs with stress fibers

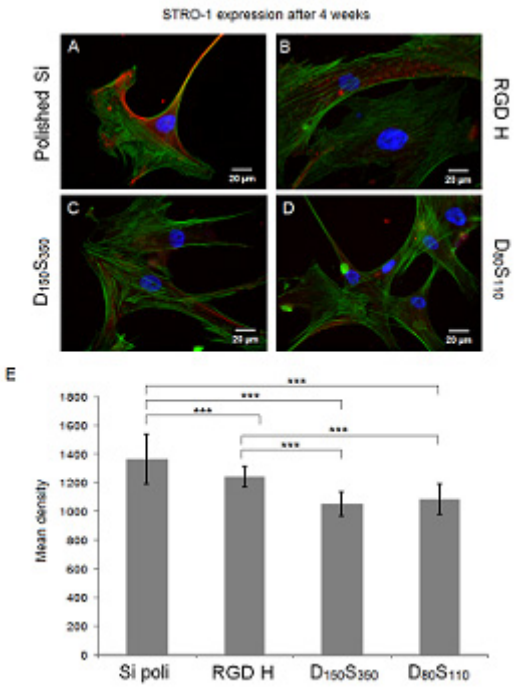

Fig. 4 Commitment studies of hMSCs after 4 weeks. (A) – (D) STRO-1, a hMSC marker, is immunofluorescently stained and shown in red, with F-actin stained in green and cell nucleus in blue, scale bar = 20 µm. (E) The amount of STRO-1 present in the cell is expressed as mean fluorescent density. Compared with bare Si controls, STRO-1 activity is lower on RGD H surfaces and still lower on patterned RGD surfaces, both $D_{150}S_{350}$ and $D_{80}S_{110}$, indicating that the cells have lost some "stemness" and differentiated into mature lineages. *** represents a p-value of less than 0.05.

may upregulate this contractility through tension caused by the pulling action of FAs, inducing stem cell behaviors such as differentiation. The amount of mature fibrillar FAs is also significantly greater on surfaces with nanopatterned bioactivity (Figure 3), confirming the impact of nanopatterns on FA configuration.

As FAs have a direct effect on cell mechanotransduction and signaling pathways, we attempted to establish a direct link between FA activity and hMSC commitment and performed differentiation-specific immunofluorescence staining on hMSCs cultured on various surfaces for 4 weeks. STRO-1 is expressed when cell stemness is present (Figure 4). The decrease in STRO-1 expression on $D_{150}S_{350}$ and $D_{80}S_{110}$ relative to RGD H is a sign that cells are less "stem" on nanopatterns than RGD H surfaces after 4 weeks in culture, indicating that they have differentiated into a mature cell lineage. We can attribute this change in commitment behavior to the way the FAs form stable contacts between the cells and the substrate, causing changes in the cytoskeletal contractility and altering the cell mechanism and chemical signal pathways. hMSC multi-potency allows them to differentiate into any of a variety of mature lineages, and it is currently unknown whether our nanopatterns favor a particular route for hMSC differentiation. Future comprehensive differentiation studies will address this interest.

V. Conclusions

The patterning technique we have developed in this study allows the deposition of biomolecules on nanopatterned surfaces for the study of stem cells on spatially organized bioactivity. NIL is a comprehensive technique of surface fabrication as it is versatile in terms of the geometries that can be patterned and the types of biomolecules that can be grafted. As we have demonstrated in our study that nanopatterned RGD peptides can induce a noticeable effect on the specific fibrillar adhesion and differentiation behavior of hMSCs, the same approach can be applied on other types of biomolecules to examine the lineage commitment and differentiation of hMSCs in future studies.

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References

1. Liao S, Chan CK, Ramakrishna S. (2008) Stem cells and biomimetic materials strategies for tissue engineering. Mater Sci Eng C 28:1189-1202
2. Caplan AI. (2007) Adult Mesenchymal Stem Cells for Tissue Engineering Versus Regenerative Medicine. J Cell Physio 213:341-347
3. Anselme K, Davidson P, Popa AM et al. (2010) The interaction of cells and bacteria with surfaces structured at the nanometre scale. Acta Biomater 6:3824-3846
4. Pallandre A, Glinel K, Jonas AM et al. Binary Nanopatterned Surfaces Prepared from Silane Monolayers. Nano Lett 4:365-371
5. Porté-Durrieu MC, Guillemot F, Pallu S et al. (2004) Cyclo-(DfKRG) peptide grafting onto Ti–6Al–4V: physical characterization and interest towards human osteoprogenitor cells adhesion. Biomaterials 25:4837-4846
6. Discher DE, Mooney DJ, Zandstra PW. (2009) Growth Factors, Matrices, and Forces Combine and Control Stem Cells. Science 324:1673-1677
7. Dalby MJ, Gadegaard N, Tare R et al. (2007) The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. Nat Mater 6:997-1003
8. Adams JC. (2001) Cell-matrix contact structures. Cell Mol Life Sci 58 :371-392

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