Mesenchymal Stem Cell Differentiation

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Contributor: Marie-Christine DURRIEU

Human mesenchymal stem cells (hMSCs) respond to the characteristics of their surrounding microenvironment, i.e., their extracellular matrix (ECM). The possibility of mimicking the ECM offers the opportunity to elicit specific cell behaviors. The control of surface properties of a biomaterial at the scale level of the components of the ECM has the potential to effectively modulate cell response. Ordered nanoscale silicon pillar arrays were fabricated using reverse micelles of block copolymers on full wafers, with standard deviations lower than 15%. Bioactive synthetic peptides were covalently grafted on nanoarrays to evaluate possible synergies between chemistry and topography on osteogenic differentiation of hMSCs. Functionalization with RGD (Arg-Gly-Asp) and BMP-2 (bone morphogenetic protein-2) mimetic peptides lead to an enhancement of osteogenic differentiation. Bare nanopillar arrays of reduced pitch were found to promote faster hMSC differentiation. These findings highlight the relevance of investigating possibilities of engineering in vitro systems which can be fine-tuned according to the envisaged cell response.

nanotopography surface functionalization mimetic peptide mesenchymal stem cell osteogenic differentiation

1. Introduction

Biomaterials can be engineered to improve and actively guide cell response in a controlled way [1]. To achieve that, material surfaces should be able to mimic the in vivo microenvironment to which a cell is normally in contact with, i.e., to mimic its extracellular matrix (ECM) [2]. Since most cell-ECM interactions occur at nanoscale (e.g., growth factor-receptor interaction), control of biomaterial surface properties at this scale level is of utmost importance. Most reported studies rely on the creation of micro-/nanoscale topographies, the fine-tuning of surface chemistry of a material or the tuning of the stiffness of materials for the cell type under investigation to perform such control [3][4][5][6][7]. Mesenchymal stem cells (hMSCs) have been one of the main cell types used in studies of modulation of cell fate through the control of materials design [4]. hMSC are easy to culture and manipulate in ex vivo culture and these cells are a very promising option for bone tissue engineering applications, due to their osteogenic differentiation potential (among the potential to differentiate into other lineages, namely adipogenic or chondrogenic) [8].

Nanofabrication methods commonly used in electronic applications grant powerful tools to produce nanoscale features which can be translated into platforms for cell-substrate interaction studies. Though these fabrication methods can potentially be applied to a multitude of materials, state-of-art approaches are normally developed for

silicon. Several variations of nanotopographies, namely pillars, rods, pits, and their organization on the surface (i.e., ordered/disordered) have been used in the investigation of hMSC differentiation towards osteoblastic lineage [3][7][9][10][11][12][13]. The pillar arrays are further, more stable configurations than rods attached to the surface via their long axis. There is no real consensus on which geometry is the most efficient on the promotion of osteogenesis [14]. Even studies investigating identical nanotopographies can report contradictory results [5][15]. Material topography is a very powerful parameter for the control of cell behavior, but it is necessary to keep in mind that any slight change of chemistry, at the level of material surface or of culture media composition, besides cell origin (e.g., adipose-/bone marrow-derived hMSCs, donor age) can have an impact on cell response of the same amplitude as topography [4][16][17][18]. We reported that osteogenic differentiation of hMSCs on silicon nanopillar arrays is enhanced when cells are cultured on nanopillars of larger diameter (100 nm) and height (80 nm) [18]. It was observed that depending on the age of the donor, osteogenic differentiation was further enhanced for smaller or larger spacing between features (140–200 nm) for younger and older donors. Our earlier work had focused on the impact of nanotopographies without chemical factors immobilized onto the silicon surfaces. Our efforts in the current work are however directed at the impact of surface chemistry due to introduction of BMP and RGD (Arg-Gly-Asp) peptides on the nanotopographies that can independently control the response of the hMSCs.

Molecules of varied sizes, ranging from full-length ECM proteins to short linear peptides have been investigated as possible ways of assigning bioactivity to a material surface [4][19][20][21][22][23][24][25]. Although the use of full-length ECM proteins has proven to be a successful way of controlling cell behavior on bioactive materials, their use is hindered due to intrinsic limitations (e.g., poor stability, safety concerns) [19]. To overcome these shortcomings, synthetic peptides encompassing only the amino acids (aa) necessary to support a particular biological activity have been investigated [20][26]. Mimetic peptides can be synthesized with high purity, lower costs, and specific active sites can be engineered in a controlled way [19]. Moreover, contrary to proteins, conformation and density of short molecules can be controlled when bound to a material [19]. The most representative motif used for the improvement of cell adhesion is the sequence of aa arginine-glycine-aspartic (RGD), which in vivo mediates the binding of ECM proteins (e.g., fibronectin) to transmembrane integrin receptors [27][28][29]. The growth factors most commonly used for the enhancement of osteogenic differentiation of hMSCs are bone morphogenetic proteins (BMPs), particularly BMP-2 [30][31]. Most studies take advantage of only the sequence responsible for the osteogenic activity of this molecule to functionalize biomaterials for bone tissue engineering applications [19][32][33]. The combination of a peptide promoting cell adhesion with one promoting cell differentiation for the co-functionalization of a biomaterial has been reported to further enhance differentiation when compared with the grafting of only one peptide sequence, such as a BMP-2 mimetic peptide [34][35][36][37][38]. Several studies can be found reporting also synergistic effects of combining nanotopographies with chemical cues on osteogenic differentiation of hMSCs or osteoblast progenitors [39][40][41].

2. Current Insights

Reverse micelles of initial block copolymer (BCPs) granted the possibility of creating ordered polymeric arrays with uniformity over large areas (full wafers) which could be used as masks for the patterning of the underlying

substrate with high processing reproducibility. The ability of creating organized nanostructure arrays with high throughput is essential for the subsequent utilization of these samples for cell culture. Such characteristics are essential for the use of silicon nanopillars on studies of hMSC differentiation. A more detailed discussion on this point has been previously reported [18]. XPS characterization showed that surface modification process was successful on the topographies tested. Moreover, since identical atomic % were observed on all surfaces after peptide grafting, it could be concluded that the surface chemistry on all patterns was similar. It was essential to confirm that surface chemistries were homogeneous across a sample area and identical between topographical conditions due to the high sensitivity of hMSCs to small alterations of surface chemistry of a material [34][42].

A uniform distribution of the molecules on the surface was expected on both nanopillar arrays. The theoretical size of unfolded BMP-2 mimetic peptide bound to the surface was ~6 nm, which is less than a fifth of the lowest separation for the array with reduced periodicity (A, 36 nm separation between features). Hence, separation between nanopillars should not result in a preferential distribution of molecules on the nanopatterns. Previous studies demonstrated that cells adhere only to the top part of the pillars when cultured on nanopillar arrays, thus, making molecular distribution on pillar tops to be much more important compared to the inter-feature concavities [43][44]. The top parts of the pillars are even more accessible for surface-functionalization as compared to a flat surface due to their three-dimensional profiles. Additionally, curvature on the pillar tops is not prominent, with typical rounding radii of 45 nm, which is approximately 10 times larger than the thickness of the molecular layer thickness. Such curvature should not adversely impact the molecular binding to the surface.

hMSCs were cultured for two weeks in basal medium independently of the assay (immunofluorescence or RT-qPCR). Flat and nanopatterned samples were tested right after fabrication or functionalized with RGD or/and BMP-2 mimetic peptide to investigate which could be the best surface for the promotion of osteogenic differentiation of hMSCs.

The nanostructure arrays were selected according to results we previously reported [18]. In that study, modulation of osteogenic differentiation of hMSCs by silicon nanopillar arrays was investigated in vitro. A set of six nanopillar arrays of different dimensions was used as substrates for hMSC culture in basal medium. It was observed that osteogenic differentiation was enhanced for cells cultured on nanopillars of large diameter (100 nm) and height (80 nm). Moreover, it was verified that spacing between features needed to be tuned according to the age of the cell donor to further increase the rate of osteodifferentiation. In agreement with our previous study, after two weeks, non-modified Nanoarray A appeared to be the best surface for the control of hMSC commitment and differentiation towards the osteoblastic lineage, as shown by immunofluorescence and RT-qPCR results [18]. When comparing the expression of the different markers from cells cultured on this pattern with the remaining samples (flat or B), significantly higher levels were observed on Nanotopography A.

Such agreement between immunofluorescence and RT-qPCR results was not verified for biofunctionalized samples. Nonetheless, precise correlations between proteomic and genomic analysis are normally impossible to establish [45][46]. It is necessary to consider protein stability issues, variations in the efficiency of RNA translation, along with possible experimental errors and background noise related to each assay [45][46]. Although cells cultured

on non-modified silicon express higher levels of osteogenic markers, and, particularly, markers of late differentiation as OPN, it is necessary to keep into consideration that this is the cell response at two weeks [47]. The observation of RT-qPCR results in more detail indicates that, though osteogenic differentiation may occur slower during the two weeks in culture, it is expected that biofunctionalized surfaces have a significantly higher contribution for the osteoblastic differentiation. The exceptionally high expression of Runx2 on biofunctionalized surfaces (10-fold of the expression observed on bare surfaces), particularly for cells cultured on samples where RGD and BMP-2 peptides were co-immobilized, indicates that a much larger number of cells is starting to differentiate towards the osteoblastic lineage. These trends in marker expression are in agreement with previously reported works [47][48][49][50].

Taken together, it is possible to conclude that the impact of surface topography appears to be more effective on the quick modulation of hMSC differentiation than the surface functionalization tested. Considering that Runx2 and COL1A1 are markers of early osteoblast differentiation, whereas OPN and OCN are late markers of differentiation, it can be assumed that Nanopillar Array A (diameter 105 ± 14 nm, periodicity 141 ± 12 nm, height 75 ± 6) is the best nanotopography for the promotion of osteogenesis [47][51]. Though leading to a slower differentiation, co-functionalization of the surfaces, independent of the topography, contributes to a significantly higher expression of the markers studied, hence, to the osteogenic differentiation of a larger fraction of the population. Investigation of the expression of the markers selected at later time points could be of interest towards better understanding of these phenomena.

These results can be of great interest for different in vitro applications. If a large number of differentiated cells is required, then silicon functionalized with RGD and BMP-2 mimetic peptide should be the most suitable option (though undergoing a slower differentiation, a larger fraction of the hMSCs population appears to differentiate when cultured on these bioactive surfaces). Contrarily, if a longer time of storage of the samples before cell culture is necessary, than non-modified Nanotopography A (small inter-spacing) should be the condition chosen. Non-functionalized samples can potentially be stored indefinitely, which is not the case of samples onto which bioactive molecules were grafted as such molecules have a shorter shelf-life [35].

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