

Preparation of nano-patterned substrates via Dip-pen Nanolithography for stem cell applications

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ABSTRACT

Micro- and nano-scale patterns are known to have a high impact on cell adhesion and are very interesting for stem cell research since they represent highly defined and reproducible systems which might be beneficial for obtaining a homogeneous stem cell culture. Homogeneity is a basic requirement for subsequent therapeutic application in the field of regenerative medicine. In this work, we are using nano-patterned substrates created with Dip-pen Nanolithography (DPN) together with the immobilization of BMP-2 in order to achieve this highly controlled environment for stem cell differentiation experiments.

Keywords: Dip-pen Nanolithography, stem cell differentiation, nano-patterning, BMP-2

1 INTRODUCTION

In the past two decades, the emerging nanotechnologies gave access to the field of nanomedicine, which amongst other include approaches to biosensing [1], basic cell biology, tissue engineering and regenerative medicine in order to treat various diseases [2]. The role of micro- and nano-technology in these areas is evident since already existing fabrication technologies could not provide access to nanoscale structures. The reason for this is in the body's cells: cell adhesion is a crucial process for anchorage dependent cells. In vitro experiments have proved that, in order to be viable, spread and proliferate, these cells need to attach to adhesive surfaces [3]. The cell receptors that play a role in attachment and signal transduction are at the micro- and nano-length scale themselves [4], as well as the intracellular proteins that enable the cells to exert forces onto the substrate [5]. In literature, there are many examples of cells being influenced by chemical or topographical cues as micro- or nano-features, wherein the cell alignment, polarization, migration, proliferation and gene expression is altered [6]. Recent studies have shown that the geometrical order of the micro- or nano-pattern is important [7] and controls cell proliferation and even differentiation.

Given these novel tools, it is evident to not only apply them in somatic cell culture but moreover take advantage of them in stem cell research, focussing on their potential ability to generate or maintain a population of cells with homogeneous phenotype, which is the ultimate challenge when working in the area of stem cell differentiation, in order to fully take advantage of their therapeutic potential.

Stem cells have been a hot topic in the last few years and there were many publications and press releases about their promising application in regenerative medicine [8]. They are undeveloped cells and possess the ability of self renewal, differentiation and tissue regeneration. There are two different types of human stem cells – embryonic stem cells (ESC) and adult stem cells. Latter ones can be obtained from different adult cell sources like bone marrow, umbilical cord blood, adipose and neural tissue. They are called multipotent and are not able to differentiate into any type of cell, but only into several phenotypes. A well known cell line are the mesenchymal stem cells (MSC) which upon exposing to appropriate stimuli can be differentiated into either osteoblasts, myoblasts or neurons [9]. The advantage of this cell type (non-ESC) is that they can be obtained from adult tissue and could be used in stem cell therapy (re-implantation of stem cells in an individual after in-vitro differentiation). ESCs are obtained from the embryo and are called pluripotent since they can differentiate into every cell type found in the body [10]. Although ESCs are the ideal model for regenerative medicine, they are very controversial considering the ethical issue of extraction of cells from embryos.

Opposing the theory of potential benefits of stem cells, in practice there are still many unsolved problems concerning effective stem cell culture. Like every other cell type, stem cells are sensitive to many different signalling cues coming from their microenvironment [11, 12]. Therefore, a high amount of control over these cues is necessary in order to achieve homogeneous cell populations. Homogeneity can be influenced, among other things, by the administration of so called soluble exogenous stimuli (biochemical factors, often presented as media supplements). The interactions of cells with these soluble factors, as well as with extra-cellular matrix (ECM) proteins, are usually studied by multiwell plate assays. This

method, when parallelised, is time consuming and expensive due to the high quantities of growth factors required. Moreover, standard culture protocols lead to inhomogeneous cell populations composed of differentiated and undifferentiated cells. Promising results were obtained when using chemically modified substrates [13].

Our approach tries to avoid uncontrolled exogenous stimuli as far as possible and rather uses surfaces with immobilized factors presented to the cell in a controlled way. A clear difference in differentiation behaviour could be shown when comparing factors in solution to factors immobilized on the surface [14]. Figure 1 [adapted from 15] shows a possible explanation of these differences. While soluble factors can bind to the receptor from every side, the immobilized factors can lead to effects as integrin clustering that trigger alternative pathways.

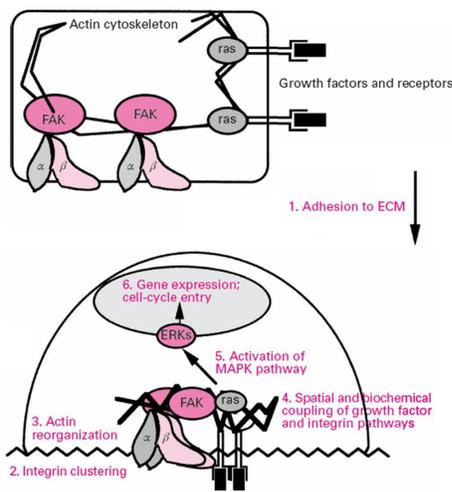


Figure 1: Immobilized factors create integrin clustering

Our work hypothesis is that, being able to construct a highly controlled surface at the nano-scale, it should be possible to induce a homogeneous cell response to surface factors and, therefore, a more homogeneous cell population as compared to conventional cell experiments [16].

Present strategies of immobilizing molecules for cell studies include proteins of the ECM like collagen, laminin, vitronectin and fibronectin [6] or motifs from these proteins that are known to have an influence on cell adhesion by interacting with the transmembrane proteins called integrins, namely the amino acid sequence arginine-glycine-aspartate (RGD) [17]. Apart from that, also transforming growth factors (TGFs) are popular candidates for immobilization [18], amongst them BMP-2 [19].

Among the various methods to create micro and nano-patterned surfaces, we chose a quite novel technique called Dip-pen Nanolithography (DPN), which was introduced in 1999 by Chad Mirkin [20]. It very fast proved to be a powerful tool for the chemical patterning of surfaces with nano size resolution. There are already many different applications [21], however, only few results are published where DPN was used for cell or stem cell applications. Up

to now, the drawbacks of this method were the missing of an automation feature and the limited area size. But in order to create surfaces for cell culture, certain claims have to be met such as large surface area of pattern (for embryonic stem cells in culture at least 500µm in length and width [22]) and biological compatibility of substrates and ink. To this end, the new NLP2000 (Nanoink) is used to create large areas of homogeneous nano-pattern in order to enable the subsequent use of these substrates in stem cell culture to test their differentiation potential. This device enables up-scaling of the present DPN because it works without laser feedback and has no restriction in the maximum scanner range. On the other hand it still uses common AFM cantilevers in order to deposit the ink on the substrate, what means the patterning can be conducted with more than one tip at the same time.

An important pre-condition for successful factor immobilization is the complete control over orientation and density of the factor on the surface. If the factor immobilization on the substrate yields denatured proteins or the cell simply is not able to recognise the binding site due to very dense surface packing or wrong orientation, this leads to wrong results. There are several possibilities how a protein can be immobilized on a substrate, but not all of them leave the protein active for cell recognition, as was shown by Kashiwagi et al. [23]. Instead of absorption, direct chemical linking or peptide linking, we take advantage of the well-known biorecognition mechanism of the biotin-streptavidin pair in order to achieve a reliable and reproducible factor immobilization. It could already be shown in our group that the attachment of biotinylated BMP to a surface induces stem cell differentiation in C2C12 cells towards osteoblasts. Figure 2 shows the differentiation pathway of C2C12 cells into osteoblasts when stimulated with BMP-2 [24].

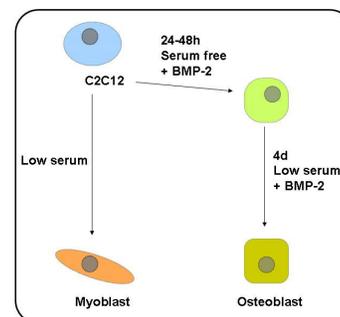


Figure 2: C2C12 differentiation pathway

Before fabricating the nano-patterned substrates, the experiments were performed at the micrometer scale in order to test the feasibility of the whole experimental set-up. Furthermore, detection with conventional fluorescence microscopy is easier at the micro scale and does not have the risk of pattern features being too small for the resolution limit of the microscope (around 500nm diameter). Experiments for the micrometer scale include the sandwiching of biotin and streptavidin in order to establish

the BMP-2 pattern on the substrate in a stable manner. Subsequently, the biotinylation of BMP-2 was conducted and the reaction outcome determined via Western Blot. Furthermore, the presence of BMP-2 on the substrates was verified by detection with a fluorescent antibody. Finally, C2C12 cells were seeded onto the substrates and also on reference samples and monitored for differentiation.

The nano-pattern experiments include the optimization of the DPN technique in order to create large-area patterns with biotin-SH in order to culture cells.

2 MATERIALS AND METHODS

2.1 Materials

Gold substrates were bought from NTB (Buchs, Switzerland), biotin-PEG-thiol was a gift from Prof. Fernando Albericio (IRB, Barcelona, Spain [25]), Silicon oxide master (5 μ m holes) was provided from Amo GmbH (Aachen, Germany), Perfluorooctyl-trichlorosilane was from Fluka (Barcelona, Spain), PDMS Sylgard 184 was from Dow Corning (Midland, USA), Carrier free recombinant human BMP-2 was purchased from Vitro S.A. (Madrid, Spain), bovine albumin serum (BSA) from Sigma (Barcelona, Spain), (+)-biotin N-hydroxysuccinimide ester was from Aldrich Quimica S.A. (Madrid, Spain), ZebaTM Desalt Spin columns and ImmunoPure[®] Streptavidin Texas Red[®] Conjugated were obtained from Cultiex S.L. (Madrid, Spain), ethanol, methanol and acetonitrile was from Panreac (Barcelona, Spain), PBS was obtained from Invitrogen S.A. (Barcelona, Spain). DPN tips and inkwells were bought from Nanoink Ink. (Skokie, USA).

2.2 Micro contact printing

The silicon oxide master was cleaned with and subsequently silanized with Perfluorooctyl-trichlorosilane following standard protocols. After silanization, PDMS was mixed at a ratio of 10:1 (elastomere base : curing agent), poured into a heat resistant petri dish over the master and cured for 1h at 80°C in the oven. Finally, the replica (furthermore called stamp) could be lifted off from the master and cut out of the excess PDMS. The quality of the master was controlled by AFM, the quality of the PDMS stamp by interferometry.

In order to deposit the biotin-PEG-thiol (further called biotin-SH) on a gold substrate, the stamp was cleaned with ethanol and activated with a UV-cleaner (Bioforce Nanosciences, Ames, IA, USA) before incubation with 10mM biotin-SH in ethanol for 3min. After drying with nitrogen, the stamp was placed with the active side facing down on a previously cleaned gold surface and kept in contact for 10min. Biotin-SH was left for reaction overnight, the sample subsequently rinsed with MilliQ, and passivated against non-specific protein adsorption for 1h at room temperature with a 2% bovine albumin serum (BSA) solution in MilliQ water. After rinsing with MilliQ, the

sample was incubated with 0.1mg/mL Streptavidin Texas Red (SA-TR) in MilliQ for 1h at room temperature in the absence of light. Finally, the substrate was rinsed with PBS and MilliQ water and carefully dried with nitrogen.

Fluorescence images were taken with an Eclipse E1000 upright microscope (Nikon, Netherlands) using a green excitation G-2A long pass emission filter and a charge-coupled-device (CCD) camera.

2.3 Dip-pen Nanolithography

An NScriptor system (Nanoink, Skokie IL, USA) was used to deposit the biotin-SH ink on gold. Gold surfaces were cleaned, marked with a diamond scribe and placed on the provided sample holder of the NScriptor system. An AFM tip was inked in a 5mM solution of biotin-SH in ethanol and dried with nitrogen. DPN experiments were conducted at 22°C and a relative humidity between 30 and 50%. Directly after patterning, the deposition was controlled with the same tip at an elevated scanning velocity (between 3 and 5Hz).

2.4 Biotinylation of BMP-2

Carrier free (CF) recombinant human BMP-2 (further more called BMP-2) was reconstituted following the protocol provided by the distributor to a working concentration of 50 μ g/mL. The biotinylation took place at the protein's amino residues; therefore 5 μ g of BMP-2 were incubated with 2.5nmol of biotin-NHS in PBS for 1.5h at room temperature. Separation of biotinylated BMP-2 from remaining free biotin-NHS was done by centrifugation using a Zeba Desalt Spin Column of 0.5mL volume.

2.5 Cells

C2C12 mouse cells were a kind gift from Prof. Francesc Ventura (Bellvitge Hospital, L'Hospitalet de Llobregat, Spain). They were expanded using Dulbecco's modified Eagle's medium (DMEM, Gibco, Spain) which contained 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, 1% L-Glutamine and 1% sodium pyruvate.

3 PRELIMINARY RESULTS

3.1 Micro contact printing

In Figure 3, the resulting pattern can be seen after printing of biotin-SH on gold, passivation with BSA and incubation with SA-TR. The fluorescence can only be seen in the circular areas, almost no contamination is found outside. On top of that, the biotin-SA bond seems stable enough to endure washing steps.



Figure 3: Fluorescence images of biotin-SH with SA-TR

3.2 Dip-pen Nanolithography

DPN was performed with the NScriptor system provided by the Nanotechnology platform (PCB, Barcelona, Spain).

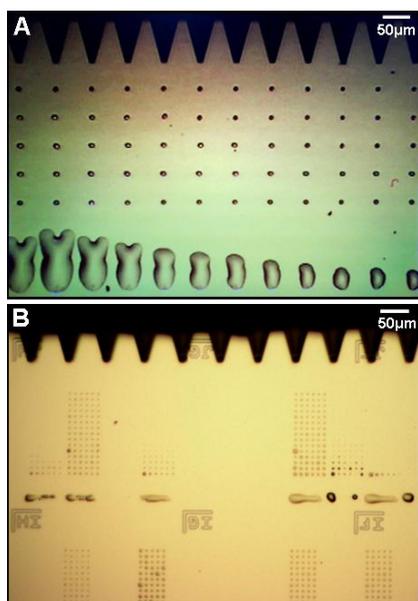


Figure 4: Patterning with Nscriptor (A) and NLP2000 (B)

Preliminary experiments were performed to characterize the biotin-SH ink using different solvents like acetonitrile, ethanol and water. Due to the poor solubility of the biotin-SH, further experiments have to be conducted, including the use of surfactants in order to increase the deposition rate of the ink. Experiments with the NLP2000 system (Nanoink Inc., Skokie, USA), revealed its potential for cell culture applications. The ability to create large areas of homogeneous nano-structures along with the automation capability makes the instrument of choice for further experiments. In figure 4, the dots seen in A (NScriptor) were deposited moving the tips manually 50µm upwards. The pattern in B (NLP2000) was deposited by entering the desired pattern sequence in the software and starting lithography – a highly automated process.

3.3 Biotinylation of BMP-2

Previous results from our group indicated that biotinylated BMP-2 is still active and substrates which are prepared using the sandwich structure (see figure 5) can induce differentiation in C2C12 cells towards osteoblasts (results not shown). On the other hand, when BMP-2 was directly printed on the substrate without inducing some sort of orientation of the BMP-2, the cells would not differentiate. This leads us to the conclusion that the induced directionality of BMP-2 sticking out from the surface is important for cell differentiation and that on the other hand, the biotinylation step does not alter the protein activity of BMP-2.

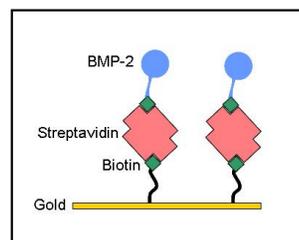


Figure 5: Orientation of BMP-2 via sandwich structure

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