Patterned growth and differentiation of human cord blood-derived neural stem cells on bio-functionalized surfaces

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Bio-functionalized surfaces were prepared to study the adherence and differentiation capacity of neural stem cells derived from human umbilical cord blood (HUCB-NSC). Cell growth platforms containing arranged arrays of adhesive molecules were created by microcontact printing on a biologically inert surface. Biomolecules used to prepare microarray platforms included the extracellular matrix protein fibronectin and the polyaminoacid poly-L-lysine. HUCB-NSC plated on microplatforms at various serum conditions showed serum and molecule type dependent capacity for adhesion and differentiation. Poly-L-lysine allowed the maintenance of stem-like non differentiated cells attached to the surface, whereas fibronectin promoted spreading and neural commitment. Serum deprivation did not influence the attachment of HUCB-NSC to fibronectin, but significantly enhanced the attachment to poly-L-lysine and promoted dBcAMP induced neuronal differentiation. A bio-pattern of squares with interconnecting lines was used to guide neuronal differentiation by directing cell protrusion outgrowth. Tailoring the geometry of the bio-pattern enabled directing and monitoring of the neural stem cells' development in the large scale multiparameter biotests.

Key words: human umbilical cord blood neural stem cells, micropatterning, bio-functionalized surfaces

INTRODUCTION

The promise held by neural stem cells (NSC) for cell therapy of neurodegenerative disorders is now widely accepted and stem cell research is mainly focused on such NSC application (Pluchino et al. 2005, Buhnemann et al. 2006). In parallel, the NSC culture can be considered as a model system for *in vitro* screening of putative toxic or transforming factors on their proliferation, viability and differentiation potential into neuronal cells. While great progress has been made in exploration of both: medical (*in vivo*) (Bliss et al. 2007, Habisch et al. 2007, Harris et al. 2007, Kozłowska et al. 2007, Stroemer et al. 2008) and toxicological diagnos-

tic (*in vitro*) (Coecke et al. 2007, Conti et al. 2008) potential of NSC, much still need to be understood before the complex biological system of stem cells can be effectively controlled and utilized. Spatiotemporal signals are important regulators of cellular behavior. They include extracellular matrix (ECM) proteins, neighboring cells, soluble factors (hormones, drugs or toxicants) as well as geometric components and physical forces (Doetsch et al. 1997, Lin et al. 2002, Raghavan and Chen 2004, Trosko 2006, Durbec et al. 2008, Jurga et al 2009). *In vivo* NSC of different developmental stages coexist within the same complex niche and respond to these signals by switching "on" or "off" intracellular molecular pathways which direct cellular mutual interactions and behavior (Trosko 2003, 2006). Spatiotemporal signals further govern the orchestration of such developmental events as cell proliferation, migration and differentiation that eventually

give rise to distinctive, complex and functional tissues and organs. Monitoring these developmental processes requires combining proper *in vitro* cellular models with advanced cell growth platforms. Through careful control of their physico-chemical properties and the biological competence of their active zones, such platforms can provide information on how surface modifications can affect cell interactions and cell developmental processes (Chen et al. 1997, Tourovskaia et al. 2005, Falconnet et al. 2006, Peterbauer et al. 2006). Stem cell technology combined with "intelligent" alignment of biomolecules on microarray platforms provides control over temporal and spatial presentation of microenvironmental stimuli to interacting cells (Raghavan and Chen 2004, Soen et al 2006). If applied to neurotoxicity testing, it could serve as a new tool for better understanding the mechanisms involved in compound-induced adverse reactions of the organism during development of the nervous system (Bhogal et al. 2005, Coecke et al. 2007).

The Human Umbilical Cord Blood Neural Stem Cell (HUCB-NSC) line is an example of a model system where key neurodevelopmental processes were investigated by conventional and emerging techniques (Buzanska et al. 2005, Ruiz et al. 2007, 2008a,b). The advantage of the HUCB-NSC line is that cells are of human origin, non-transformed and can be cultured/harvested at different developmental stages (Buzanska et al. 2006a,b).

In this report micropatterned surfaces were created as a spatial arrangement of functional domains to study the adherence and differentiation capacity of HUCB-NSC. This included creating bio-functional surfaces by microcontact printing of poly-L-lysine or fibronectine on antifouling surfaces in a customized geometry. HUCB-NSC plated on microplatforms at various serum conditions, showed serum and molecule type dependent capacity for adhesion and differentiation. Poly-L-lysine allowed maintenance of stem-like non differentiated cells attached to the surface, in contrast with fibronectin which promoted spreading and neural commitment. Serum deprivation did not influence the attachment of HUCB-NSC to fibronectin, but significantly enhanced attachment to poly-L-lysine and promoted dBcAMP induced neuronal differentiation. A bio-pattern of squares with interconnecting lines was used to guide neuronal differentiation by directing cell protrusion outgrowth. Tailoring the geometry of the bio-pattern enabled monitoring and directing of the neural stem cells' development.

METHODS

Surface functionalization

Moulds for the microcontact printing process were fabricated by casting polydimethylsiloxane (PDMS) silicone elastomer against a silicon master. The PDMS (Sylgard 184) was used in a 10:1 mixture of base elastomer-curing agent and hardened at 65°C for 4 h. The microstructured PDMS were used as stamps for the direct printing of poly-l-lysine and fibronectin on Petri dishes coated with a plasma polymerized polyethylene oxide-like, PEO-like, film. The PEO-like films were deposited by plasma-enhanced chemical vapor deposition in a capacitively coupled reactor, using a glow discharge in diethylene glycol dimethyl ether vapor (Sigma Aldrich) (Brétagnol et al. 2006),

Poly-L-Lysine FITC-labeled (PLL), from Sigma Aldrich, was diluted at 25 µg/ml at pH 8.4 in carbonate buffer. Fibronectin (FN), from Sigma Aldrich, was used diluted in printing buffer at 42 µg/ml. These solutions were used as inking solutions of the PDMS stamps. Before inking, the PDMS stamps were ultrasonicated in ethanol for 5 min and cleaned in mild O_2 plasma (200W, 1.2 torr) for 30 seconds. The PDMS stamps were then inked at room temperature with the PLL and the fibronectin solutions for 15 min and 45 min, respectively. After inking, the excess solution was removed and the stamps were dried in a N_2 stream. The inked stamps were placed in contact with the substrates for 5 min. Before seeding the cells, the patterned surfaces were sterilized by UV for 15 min.

Surface characterization

Ellipsometry allows maps of the thickness of the different layers of a multilayer based on the difference of their refractive indexes to be obtained. Ellipsometric data were acquired with a variable angle imaging ellipsometer (model EP3 by Nanofilm Surface Analysis GmbH, Germany). All imaging measurements were performed in air at room temperature at an angle of incidence of 42°, using a monochromatized high power Xe lamp at a wavelength of $\lambda = 554.3$ nm. A polarizer-compensator-sample-analyser (PCSA) null-ellipsometric procedure was used to obtain maps of the Δ and Ψ angles for the selected area. Thickness maps were calculated from the Δ and Ψ maps by point-by-point modeling with the ellipsometer software EP3View

using a two-layer model comprising the PEO-like and stamped PLL layers. The thickness and the refractive index (n_{PEO}=1.51~1.52) of plasma-deposited PEO-like were independently determined by an angle-resolved measurement. The thickness of the underneath PEO-like layer was between 11 and 12 nm in all the samples. A refractive index of 1.46 was used both for the poly-peptide layer (Richert et al. 2004) and for the protein layer (Guemouri et al. 2000). From the results of the thickness of the protein layer, the mass density can be determined by the method of Cuypers and others (1983). Optical imaging of the cells was carried out by a Zeiss inverted fluorescent microscope Axiovert 200 equipped with a CO_2 incubator.

Cell culture and differentiation conditions

A neural stem cell line obtained from a non-hematopoietic fraction of human umbilical cord blood (HUCB-NSC) (Buzanska et al. 2006a) was kept in culture either as a non-differentiated floating popula-tion of cells in serum free condition: F12/DMEM + B27 (1:50) and EGF (20 ng/ml) or a mixed population of committed adherent progenitors and floating undif-ferentiated cells in F12/DMEM containing 2% fetal bovine serum (FBS, Gibco), insulin-transferrin-seleni-um (ITS,1:100, Gibco), antibiotic antimycotic solution AAS (1:100, Gibco), at 37°C, 5% CO_2 and 95% humid-ity. HUCB-NSC cultures were propagated every two weeks by trypsinization (Trypsin/EDTA 0.025%, Gibco) in 1:4 ratio. For cell array generation, the cells were plated at 2.5×10^4 cells/cm^2 density on the printed slides. After cell seeding, the samples were placed into a cell culture incubator (37°C and 5% CO_2) overnight, followed by the change of the medium in order to remove the excess of non adherent cells. During the course of the experiments (up to 1 week) the medium (maintenance with 2% of serum or serum-free) was changed every two days.

Cell array inspection and immunostaining

Cell growth platforms prepared by a microcontact printing technique contained separate or line intercon-nected patches (squares) of adhesive biomolecules with identical size for each biomolecule (squares of 120 µm side, separated 100 µm, both isolated and intercon-nected *via* 15 µm wide lines). HUCB-NSC culture arrays were inspected each second day and contrast

phase photos were taken with AxioCam MRC5 camera combined to Axiovert 200 fluorescent microscope (Zeiss).

For immunostaining cells were fixed in 4% para-formaldehyde for 20 minutes, rinsed extensively in PBS and permeabilized for 20 minutes in 0.1% Triton X-100. Primary antibodies: monoclonal neuronal class III β-Tubulin Antibody (TUJ1, 1:500, Covance) and polyclonal rabbit antibodies against S-100β (1:1000, Swant) or glial fibrillary acidic protein (GFAP) were applied overnight. AlexaFluo 488 and AlexaFluo 546 (1:2000, Jackson) were used to visualize β-Tubulin III and S-100β, respectively. Cell nuclei were contra-stained with Hoechst 33342 (1:1000, Molecular Probes) for 20 min. The slides were either closed with mount-ing solution (Fluoromount-G from Southern Biotech). Data were calculated and statistically evaluated by the one way ANOVA (Graph Pad Prism 4.1, GraphPad Software, San Diego, CA) from three independent experiments.

RESULTS

Characterization of the poly-L-lysine and fibronectin microarrays

The PEO-like coating with protein spots and the micro-stamped surfaces were analyzed by ellipsome-try. With this technique the thickness of the different biomolecules deposited on PEO when silicon is used as substrate for analysis can be evaluated.

Characterization of the micro-stamped surfaces shows that microcontact printing allows the patterning of highly packed protein layers. The surface protein
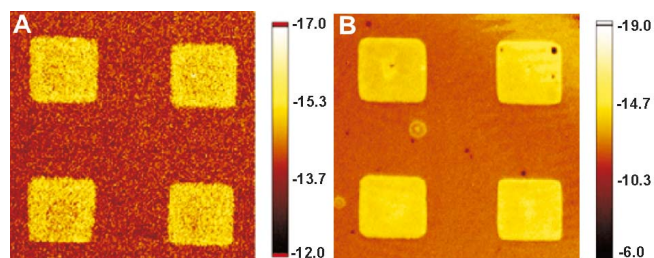

Fig. 1. 2D thickness maps of Poly-L-Lysine FITC-labeled (PLL) (A) and fibronectin (B) stamped by microcontact printing on plasma-deposited polyethylene oxide (PEO), calculated from ellipsometric Δ- and Ψ-maps. The thickness is given in nm with respect to the Si surface. Size of the maps: 400 µm × 400 µm.

coverage does not decrease after rinsing the samples. Figure 1 shows the thickness maps of the micro-stamped PLL and fibronectin patterns. For the PLL, the thickness of the peptide layer depends mainly on the conformal contact applied, since similar thick patterns were obtained using different printing solutions (a detailed study has been reported in Ruiz et al. 2008a). In the samples used in this study, the effective medium height of the PLL patterns was 1.2 ± 0.1 nm, which corresponds to a PLL mass density of 159 ng/cm^2. The fabrication of the fibronectin patterns using 42 µg/ml solutions resulted in a layer thickness of 3.2 nm, as measured by ellipsometry, which corresponds to a mass density of 356 ng/cm^2.

Geometry and biomolecule composition of the microarrays

Microarray layouts of different geometries and biomolcules were prepared to control stem cell adhesion and differentiation (Fig. 2A,B). The cell-repellent property of PEO-like film, deposited on the surface of the slides (Fig. 2, arrows) determined the confinement of the cells on the printed patterns (Fig. 2C,D). For this
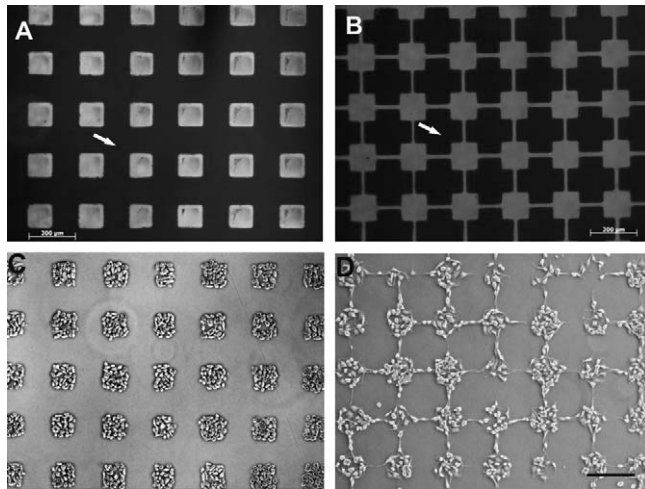
study printed poly-L-lysine and fibronectin patterns with patches of equal size 120 µm separated by 100 µm distance were used to study neural stem cell attachment properties. Arrays with patches of 120 µm interconnected by lines of 15 µm thickness (Fig. 2B,D) and separated by 100 µm distance were designed to guide neuronal cell protrusion outgrowth and were used in this study to estimate neural stem cell differentiation potential. Both, separated and interconnected by lines patterns were composed either with poly-L-lysine (Fig. 2C) or fibronectin (Fig. 2D) printed on the biologically inert surface.

Adhesion of the HUCB-NSC to the bio-functionalized surfaces

Molecule type dependent adhesion

The adhesive properties of HUCB-NSC to the patterns of poly-L-lysine and fibronectine are presented on Fig. 3A,C and Fig. 3B,D, respectively. HUCB-NSC were plated on poly-L-lysine and fibronectin at a density of 2.5×10^4 cells/cm^2. The excess of the floating, non-attached cells were removed after overnight incubation and phenotypes of the cells immobilized on the microprinted patterns were investigated under the contrast-phase microscope. Non attached cells which were present in the medium for 16 hours did not influence

Fig. 2. Layout configurations of PLL patterned by microcontact printing at 25 µg/ml concentration (A, B) and neural stem cells (HUCB-NSC) array on activated surface (C, D). Note the clear contrast between PEG (cell repellent substrate, arrows) and areas printed with adhesive PLL. (A), (C) PLL is deposited on PEO as an array of 120 µm per 120 µm patches separated by 100 µm distance; (B) the same array of PLL squares as in (A), but with additional interconnecting 15 µm in diameter PLL lines. The scale bar (200 µm) is the same for (A) and (B), (C) and (D).
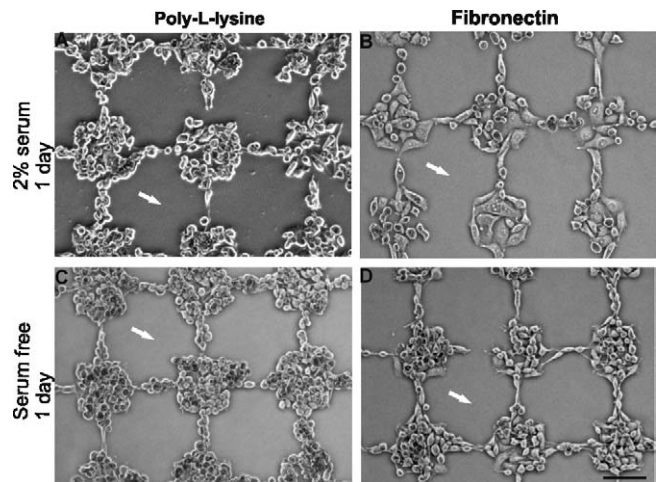

Fig. 3. Contrast phase images of HUCB-NSC attached to PLL (A, C) and fibronectin (B, D) microarrays. Cells were plated at a density of 2.5×10^4 cells/cm^2 and incubated for 1 day in medium containing 2% serum (A, B) or in serum free conditions (C, D). Arrows show PEG – cell repellent substrate. The scale bar is 100 µm.

the non-adhesive, cell-repellent properties of PEO-like film, thus strong contrast between active areas printed with biomolecules and non adhesive zones covered by the PEO-like film was maintained (Fig. 2 and Fig. 3, arrows). The confinement of the cells on the printed patterns is well defined and the effect of the type of the molecule on the mode of cells immobilization to the surface is visible. HUCB-NSCs cover the entire area of fibronectin domains and are attached well, revealing a flattened morphology (Fig. 3B,D). This confirms the specific receptor mediated interaction between the extracellular matrix protein and the cell membrane. In contrast, array on poly-L-lysine are less confined. The adherent cells are smaller than on fibronectin, rounded and attached to the surface without visible spreading (Fig. 3A,C). This observation reveals non-specific, electrostatic attachment of the cells to the underlying polyaminoacid. Such non-receptor mediated attachment allows immobilization and maintenance on the patterned surface of non-differentiated stem cells. For both fibronectin and poly-L-lysine cells attached to the patterns in a reproducible manner.

Serum condition dependent adhesion

The shape and number of the adherent cells depend upon the type of adhesive substrate and serum conditions. Quantitative data are presented on Fig. 4. The fibronectin induces strong adhesion and this is not dependent on the presence of serum. The spread phenotype of the cells interacting with fibronectin limits the number of cells per pattern/unit. A similar amount of cells is attached to the fibronectin pattern in serum (19 ± 4.6 cells per 120/120 μm square) and serum-free condition (23 ± 5.6 in serum-free medium). The number of attached cells to PLL squares in 2% serum (32 ± 8.9) is significantly higher as compared to fibronectin ($P<0.01$), because cells interacting with poly-L-lysine do not spread and the same area of adhesive molecule allows for the recruitment of additional cells. Serum deprivation significantly enhanced the attachment to PLL pattern ($P<0.01$). The number of cells immobilized on the active unit of PLL surface in serum free conditions reached the value 51 ± 6.5 cells per unit square.

Differentiation of HUCB-NSC on the microcontact printed arrays

Maintenance of the HUCB-NSC cells on the PLL and fibronectin patterns

For neuronal differentiation HUCB-NSC were incubated for 7 days in culture medium (2% serum or serum-free) supplemented with 100 μM dBcAMP. Control cultures were run parallel to dBcAMP treated cultures. The number of cells per unit of PLL and fibronectin active surfaces (120/120 μm square) in the control and dBcAMP treated cultures in different serum conditions is shown on Fig. 5. For control cultures the number of cells attached to the surface after 7 days of incubation was similar or higher (for PLL) to the adhesion values after 1 day of culture. In serum free control cultures this value was 35 ± 7.3 for FN and
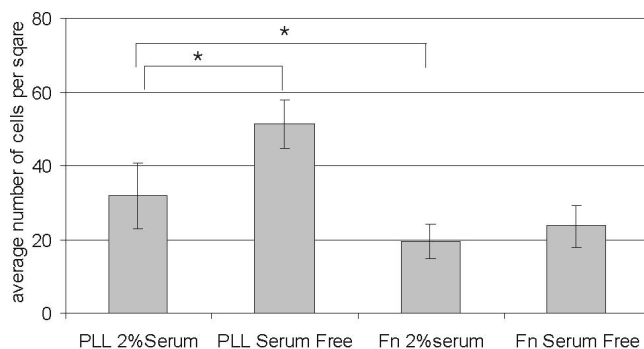

Fig. 4. Histogram of the number of HUCB-NSC attached to PLL and fibronectin square (120 μm per 120 μm) after 1 day culture in different serum conditions. Asterisks show statistically significant differences between indicated pairs ($P<0.01$).
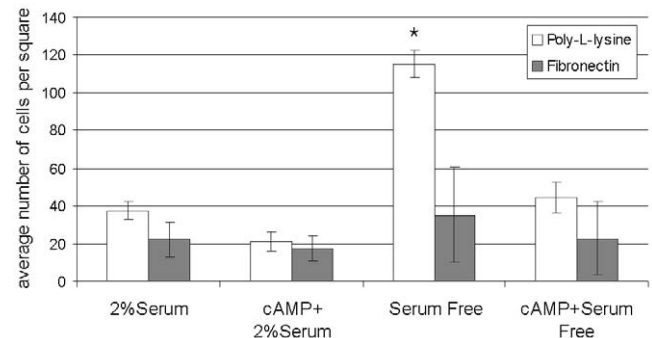

Fig. 5. Histogram of the number of HUCB-NSC attached to PLL and fibronectin square (120 μm per 120 μm) in 7 DIV culture growing in different serum concentrations either in control or differentiating (+100 μM dBcAMP) media. PLL serum free sample shows statistically significant difference (asterisk) as compared with other samples ($P<0.01$).

125 ± 29.3 for PLL. The number of cells on PLL in serum free condition is significantly higher than on any other PLL or fibronectin sample (Fig. 5, asterisk). dBcAMP significantly lowered the number of cells remaining on the pattern ($P<0.01$) only on PLL in serum free conditions.

Phenotypes of differentiating HUCB-NSC attached to PLL and fibronectin patterns

The geometry of the patterns was modified by adding lines of 15 μm thick interconnecting the printed squares in order to better visualize differentiation of HUCB-NSC by monitoring of the cells axonal/dendrite protrusion outgrowth.

After 7 days of culture control cells on the PLL patterns in 2% serum condition remained rounded, loosely attached, mostly non-differentiated and migrating out of the pattern. The pattern was no longer stable and confined (Fig. 6A). Incubation with dBcAMP allowed only single, attached cells to differentiate and to grow axon-like projection in the guided direction (Fig. 6B, arrow). In serum free conditions the control PLL pattern of HUCB-NSC was more stable and allowed for the maintenance of many rounded, non-differentiated cells (Fig. 6C). In the presence of dBcAMP cells remained attached to the PLL pattern but the majority were rounded and do not differentiated (Fig. 6D). Fibronectin control patterns in serum

(Fig. 6E) and serum-free (Fig. 6G) conditions were similar. Cells remained strongly attached, some of them were flattened and some were rounded, revealing a morphology of dividing cells. After differentiation in the presence of dBcAMP only well attached cells remained on the pattern and differentiate showing extensive growth of neural projections (Fig. 6F,H, arrows). More cells differentiating into a neuronal phenotype are observed on fibronectin patterns in serum free conditions.

Neuronal and astrocytic differentiation of HUCB-NSC attached to PLL and fibronectin patterns

Immunofluorescent analysis of the expression of neural cell type specific markers was applied to estimate the extent of neuronal and astrocytic differentiation of HUCB-NSC attached to PLL and fibronectin patterns. Figure 7 shows the panel of immunoimages of control and dBcAMP differentiated cells, grown on PLL and fibronectin patterns in serum and serum free conditions. Cells committed to neuronal lineage express β-tubulin III (green color), while cells which attain astrocytic characteristics express GFAP and S100β (red color). Some cells, though neuraly committed are still before final fate determination and express both: β-tubulin III and GFAP. We call them "astroneurons" and the overlapping green and red color of neuronal and astrocytic markers gives orange staining
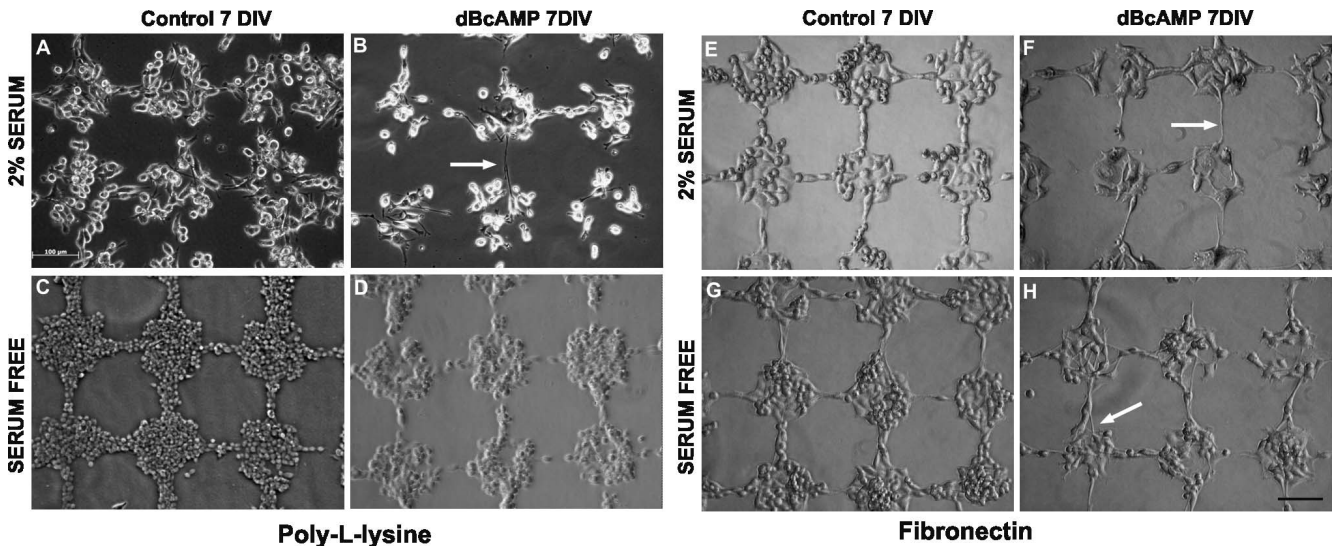

Fig. 6. Photomicrographs (Phase contrast) of 7 DIV HUCB-NSC attached to PLL (A–D) or fibronectin (E–H) microarrays. Cells were grown in control or differentiating (+100 μM dBcAMP) media or in presence or absence of 2% serum. Note that patterning interconnections guide neuronal protrusion outgrowth (arrows).
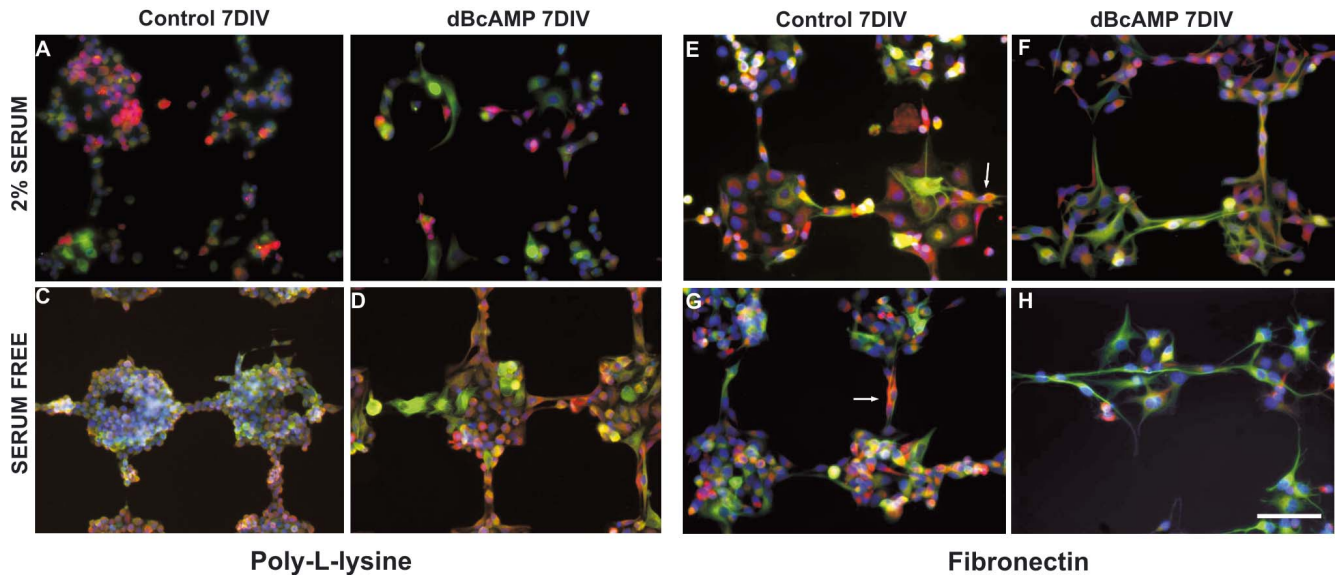
Fig. 7. Immuno-images of HUCB-NSC grown on PLL (A–D) and fibronectin (E–H) microarrays for 7 days in serum (A, B, E, F) and serum free conditions (C, D, G, H) differentiating in the presence of dBcAMP (B, F, D, H) or growing as a control cultures (A, C, E, G). Neuronal marker β-tubulin III is shown green for all images. Astrocytic markers are stained red for S100β (A–D) and GFAP (E–H). Cells co-expressing GFAP and β-tubulin III reveal an orange color (E, G, arrows). Cell nuclei are contra-stained with Hoechst (blue). The scale bar is 100 μm for all images.

(Fig. 7E,G, arrows). Cell nuclei are contra-stained with Hoechst (blue color). Figure 8 summarizes the distribution of: undifferentiated, astro-neuronal, neuronal and astrocytic cells in control and differentiated HUCB-NSC cultures grown on PLL and fibronectin patterns in different serum conditions. Table I shows the extent of cell type specific differentiation on PLL and fibronectin micropatterns, given as the percentage of cells expressing β-tubulin III (neuron-like), GFAP or S100β (astrocyte-like) and the population of cells co-expressing β-tubulin III and GFAP (astroneuron-like). On PLL control micropatterns (spontaneous differentiation) neuronal commitment was attained in higher number of cells than on fibronectin patterns, especially in 2% serum condition (Table I). This difference is significant for 2% serum condition ($P<0.05$), but is not significant ($P<0.05$) for serum deprived control cultures. Intermediate stage of differentiation resulted in appearance of "astroneuron-like" cells expressing GFAP and β-tubulin III and was promoted by the presence of serum, regardless of attachment conditions (either PLL or fibronectin, Table I).

Among the dBcAMP differentiated cells growing in 2% serum conditions, PLL attached cells expressed β-tubulin III more frequently than cells on fibronectin ($45.9 \pm 4.9\%$ as compared to $24.5 \pm 5.9\%$). However,

the morphology of these cells indicates that cells patterned on fibronectin, although in minority, are much more advanced in neuronal differentiation (note small green cells on Fig. 7B lacking protrusions compared to green, elongated neuronal cells with projections growing out on Fig. 7F). In serum free conditions dBcAMP induced differentiation was the most potent for FN patterned cells in terms of the number of differentiated cells and also their morphology ($58.2 \pm 4.7\%$ *versus* $28.4 \pm 7.7\%$, $P<0.05$, Fig. 7H *versus* 7D). Neuron like cells express β-tubulin III and extend axon-like protrusions, following geometry of the fibronectin pattern (Fig. 7H).

DISCUSSION

In this report we have tested the influence of the micropatterned and bio-functionalized two-dimensional surfaces on neural stem-cell adhesion and differentiation. We have shown that the patterning of adhesion molecules in arranged arrays confine the number of cells in spots that are easily tracked on the fabricated surfaces. Different techniques can be used for patterning biomolecules on surfaces. As compared with other methods such as non-contact spotting, the particularity of microcontact printing is that it allows

Table I

The extent of cell type specific differentiation expressing β-tubulin III (neuron-like), GFAP or S100β (astrocyte-like), population of cells co-expressing β-tubulin III and GFAP (astroneuron-like) or undifferentiated cells (negative for β-tubulin III and GFAP or S100β)

	Undifferentiated cell (%)	Astroneuron-like (%)	Astrocyte-like (%)	Neuron-like (%)
PLL 2% serum	56.3 ± 8.3	6.3 ± 3.6	13.2 ± 6.8	24.1 ± 4.7
PLL 2% serum + dBcAMP	18.6 ± 3.9	26.4 ± 12.9	9.0 ± 4.9	45.9 ± 6.2
PLL Serum Free	79.9 ± 9.4	2.6 ± 1.4	6.4 ± 3.6	10.8 ± 4.2
PLL Serum Free + dBcAMP	35.6 ± 4.1	10.7 ± 0.6	25.3 ± 3.4	28.4 ± 7.7
FN 2% serum	49.6 ± 4.0	17.4 ± 1.5	17.7 ± 6.5	15.1 ± 2.8
FN 2% serum+ dBcAMP	33.1 ± 6.2	14.1 ± 2.3	28.2 ± 2.01	24.5 ± 5.9
FN Serum Free	65.8 ± 7.5	5.2 ± 1.2	12.9 ± 0.1	15.9 ± 6.1
FN Serum Free + dBcAMP	22.4 ± 4.5	11.4 ± 7.6	7.8 ± 7.0	58.2 ± 6.1

Numbers show percentage of cells per unit square on the pattern

densely packed and uniform monolayers of protein patterns to be obtained. Our parallel studies on the fabrication of protein microarrays by non-contact piezoelectric deposition (Ceriotti et al. 2009) showed that the amount of the protein remaining on the pattern may depend upon the applied technology. During the spotting procedure, when increasing the starting concentration of the protein solution used for spotting, what finally remains on the surface after rinsing reaches a saturation level which is always lower than the mass density printed by microcontact printing reported in this study. However, cells recognize small amount of ECM proteins and are able to attach to surfaces containing small concentrations of ECM. That was confirmed by Ceriotti and coauthors (2009) for fibronectin spotted at various concentrations on protein microarray platforms. By using different cellular matrix proteins and polyaminoacid peptides in a various geometric layouts and defined time schedules we have already shown that microfabrication technology methods used in the study of our group (microcontact printing and non-contact piezoelectric spotting) allowed defined spatio-temporal stimuli (Ruiz et al.

2007, 2008a,b, Ceriotti et al. 2009) to be presented to HUCB-NSC.

The *in vitro* interaction of cells with the surrounding micropatterned environment has already been investigated on cell array platforms developed on various kinds of treated surfaces and with many different cellular models. They included ECM protein microarrays for probing cellular rat hepatocyte and mouse embryonic stem cell differentiation (Flaim et al. 2005), kidney embryonic or murine fibroblast adhesion properties (Kuschel et al. 2006), human neural precursor cells differentiation in presence of signaling molecules (Soen et al. 2006) and human mesenchymal stem cell-polymer interaction studies (Anderson et al. 2005).

The fibronectin – extracellular matrix protein and polyaminoacid poly-L-lysine were used to in this study to distinguish between specific – receptor mediated, and non-specific, caused by electrostatic forces cell-surface interactions. The morphology of HUCB-NSC attached to fibronectin was spread and flattened compared to rounded, loosely attached cells on PLL patterns. Uniform poly-L-lysine coatings have been used over the years to allow the attachment of cells for
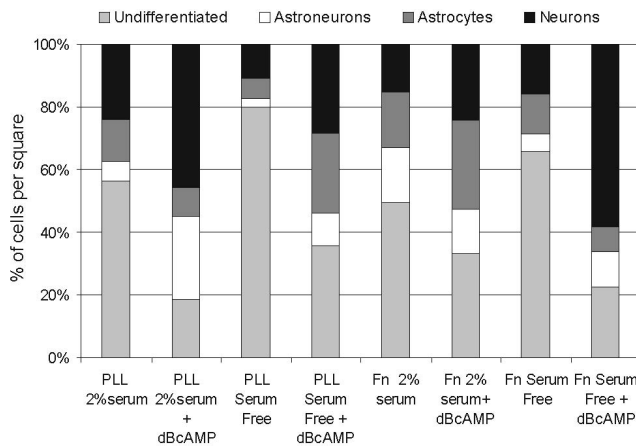
Fig. 8. Histogram shows distribution of undifferentiated, astro-neuronal, neuronal and astrocytic cells in control and differentiated HUCB-NSC cultures grown 7 DIV on PLL and fibronectin patterns in media with or without serum supplementation. Acquired phenotypes are shown as a % of cells expressing cell type specific marker per one unit (120 per 120 μm square) of active patterned surface.

observation and experimental purposes (Mazia et al. 1975). The polycationic poly-L-lysine molecules adsorb strongly to various solid surfaces, presenting cationic sites where the anionic sites on cell surfaces may bind. We have already shown in previous studies that adsorption of cells to PLL through anionic–cationic interactions allows cell patterning, cell movement and ability to differentiate within the constrained pattern (Ruiz et al. 2008a,b). Here we present quantitative data indicating statistically larger number of HUCB-NSCs attaching per pattern/unit as compared to the fibronectin microarrays. HUCB-NSC attached to PLL arrays are rounded and non-differentiated. Additionally our observations confirm the expression of nestin – the intermediate filament of neurally committed, but non-differentiated stem cells (not shown). This was found for the PLL patterns in serum and serum-free conditions after 1 or 7 days of culture, however the number of cells attached in serum-deprived medium in both time points (see Fig. 4, Fig. 5 and Table I) is significantly higher. The HUCB-NSCs attached to PLL pattern in serum free condition are rounded and of small size, thus more cells can be trapped on the same surface unit. The morphology of these cells resembles pluripotent – like cells found in human umbilical cord blood (McGuckin et al. 2006, Habich et al. 2006, Kucia et al. 2007, McGuckin and Forraz 2008), including HUCB-NSC (Jurga et al. 2006, Domanska-Janik et

al. 2008). In the present study phenotypic distribution of control cell population attached for one week to PLL pattern reveal as much as 80% of undifferentiated cells, which are lineage negative for neuronal and astrocytic markers. This population is now under our investigation for the expression of markers specific for pluripotent stem cells.

All these data indicate that poly-L-lysine microarray can serve as a platform to immobilize and maintain neural stem cells attached to the surface in their non-differentiated stage. Varying serum conditions may control the number and early neural commitment of attached non-differentiated cells (Fig. 6A,C, Fig. 7A,C, Fig. 8 – PLL/Serum Free; PLL/2% serum conditions).

In this study we show for the first time that neural stem cells can be immobilized to the surface and maintained non-differentiated in culture over a long period.

To our knowledge, no studies have been published showing that human neural stem cells can be grown and maintained over extended period in culture, as attached to the surface and in their non-differentiated stage. Floating 3D neurospheres are recognized as the main source of non differentiated neural stem cells maintained in culture. They derive from either embryonic stem cells (Pomp et al. 2008) or from tissues of various origin, for example: human fetal brains (Caldwell et al. 2001, Yang et al. 2003, Kim et al. 2006), bone marrow (Sanchez-Ramos 2002, Suzuki et al. 2004, Yang et al. 2008) or cord blood (Buzanska et al. 2005, 2006a) and contain the population of neural stem cells expressing nestin or early committed progenitors expressing early neuronal (β-tubulin III) or astrocytic (GFAP) markers. However, upon attachment neurospheres allow stem cells to spread out, migrate and differentiate.

Possibility for immobilization of non-differentiated neural stem cells to the surface by bio-functionalized microarray platforms, as shown in our study, may exert a great practical impact allowing new ways for their investigation and application.

Spontaneous and induced differentiation of HUCB-NSC on PLL and fibronectin was also accompanied by the presence of neural stem cells undergoing intermediate stage of development – "astroneuron-like" cells, co-expressing β-tubulin III and GFAP. The number of this cells was increased in serum containing medium (Table I). Recently, Draberova and colleagues (2008) demonstrated existence of cells co-expressing GFAP

and β-tubulin III *in vitro* and *in vivo* during human neurodevelopment and questioned the validity of β-tubulin III as a marker of neuronal cells at the early stages of development. It is most likely, that the cells co-expressing β-tubulin III and GFAP are before fate decision, whether to attain neuronal or astrocytic lineage and this may depend upon environmental clue. Double staining of β-tubulin III and GFAP was performed in our study, to identify such cell population and to exclude possible mistake of accounting all β-tubulin III expressing cells as committed to the neuronal lineage.

The presence of dBcAMP in the medium lowered the number of attached cells to fibronectin and PLL patterns, however this difference was only significant for PLL pattern in serum-free conditions. In this study dBcAMP was applied 24 h after cell plaiting, thus had no influence on cells attachment or spreading on adhesive substrates. By inducing differentiation, dBcAMP allows to remain on the PLL pattern only those not numerous cells, which have been firmly attached and started to grow protrusions. An extensive loose of the cell number on PLL pattern induced by dBcAMP in serum-free conditions may be than explained by the primary detachment of loosely adhered rounded cells, followed by their apparent death.

The effect of dBcAMP on the differentiation of HUCB-NSC attached to PLL was dependent upon serum conditions. In serum free conditions, though HUCB-NSC remained rounded and non-differentiated (from their morphology on contrast phase images), they already showed commitment both to neuronal and astrocytic phenotypes in more than 20% of population (Fig. 6D, Fig. 7D, Fig. 8 and Table I). However, extended protrusion formation by neuronal cells was observed only in the presence of serum (Fig. 6B, Fig. 7B) and in the minority of committed cells further indicating that PLL alone is not optimal for extended neuronal differentiation as an attachment substrate for HUCB-NSC. dBcAMP induced advanced neuronal differentiation of HUCB-NSC was observed on PLL previously, but only when PLL was additionally covered with laminin (Sun et al. 2005, Buzanska et al. 2006a,b).

The fibronectin, in contrast to poly-L-Lysine allowed for specific, receptor mediated attachment of HUCB-NSC and the cells in both serum-free and serum control condition revealed flattened and speeded morphology similarly to the behavior of many other mammalian cell types when adhering to fibronectin (Longhurst and Jennings 1998). This different cell adhesion behavior is consistent with the expression profile of HUCB-NSCs (Buzanska et al. 2006a). Neuraly committed cells express β1, α1 and α3 integrin subunits which together form fibronectin and laminin (β1 with α3) receptors (Peltonen et al. 1989).

The differentiating capacity of HUCB-NSC attached to fibronectin was significantly higher than in PLL attached cells, but only in serum free conditions (Fig. 7, Fig. 8 and Table I). Such results are in agreement with findings that differentiation of neural stem cells may be driven by growth factor deprivation (Widera et al. 2007). Serum withdrawal-related induction of differentiation may stand in contrast with our observation that in control cultures without cyclic AMP on both PLL and fibronectin there was significant increase of undifferentiated stem cell number in serum free conditions. Thus application of serum-free without appropriate stimulation for differentiation by neuromorphogens and growth factors may not be enough for sufficient differentiation of HUCB-NSC and is only an additional factor promoting this process.

The reliance of most *in vitro* cell cultures on undefined foreign components for their propagation, that is, feeder cells and serum, carries the potential risk of contamination with animal human-derived pathologies. Development of defined serum free media for stem cell culture is widely investigated (Richards et al. 2008, Tsuji et al. 2008). Our results showed that serum free conditions can be applied for successful maintenance and differentiation of neural stem cells.

Tailoring geometry of the biomolecule arrays influenced the way of HUCB-NSC differentiation on PLL and fibronectin patterns, by guiding neural cell protrusion outgrowth and allowing contacts of neuronal cells between isolated patches. It remains, that reassembling natural niche conditions for stem cell growth and development requires reconstruction of the environmental architecture and spatial connection between the cells and extracellular matrix. Proper communication between lineage related stem cells and their progeny is essential for correct development. However, their properties and responses to environmental changes (including toxins) are changing during development. For example, a specific pluripotent stem cells' attribute is the expression of Oct4 transcription factor and lack of connexin 43 expression. On the contrary, neurally committed progenitors successively stop expressing Oct4 and start to produce connexin 43, forming gap junctions which enable not only paracrine but also

direct, cytoplasmic exchange of information between cells (Davila et al. 2004, Trosko 2006). HUCB-NSC cultures resemble situation where lineage related neural stem cells coexist in the same niche at different stages of their development (Buzanska et al. 2002, 2006a,b). Furthermore, these stages, are characterized by specific molecular signature corresponding to the expression of related genes in stem cells in human embryo development (Buzanska et al. 2006a). The possibility to immobilize HUCB-NSCs at different developmental stages to well defined environment on microarray pattern opens new opportunity for investigation of the mechanisms of neural development.

CONCLUSIONS

We have shown, that patterning of adhesion molecules in arranged arrays allows for attachment and differentiation of the neural stem cells derived from human umbilical cord blood. We have indicated that serum conditions and type of the microprinted biomolecule determine the mode of attachment and fate decisions of stem cells. One of such decisions is the maintenance of non-differentiated stage by neural stem cells when attached to the surface of poly-L-lysine microppatterns. On the other hand attachment to fibronectin and serum depravation promote neuronal differentiation. Tailoring the geometry of the pattern has also an important impact on the cellular arrangement and mode of differentiation.

The Human Umbilical Cord Blood Neural Stem Cell line in combination with "intelligent" array of biomolecules on microarray platforms may serve as a model system applicable for studying mechanisms of neural stem cell development and for *in vitro* screening of the putative toxic effects of adverse environmental compounds.

Acknowledgements

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