Proliferation capacity of cord blood derived neural stem cell line on different micro-scale biofunctional domains

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Physical interactions of cells with the adhesive substrates of the microenvironment as well as the presence of the soluble growth factors are important for the proliferation capacity of neural stem cells. We have used biofunctionalized surface domains microcontact printed with either synthetic polyaminoacid poly-L-lysine or extracellular matrix (ECM) component such as fibronectin, to study the proliferation capacity of human umbilical cord blood-derived neural stem cells (HUCB-NSC). The proliferation measured by the expression of Ki-67 protein was accompanied by the investigation of the cell morphology under the transmission and scanning electron microscopy in different culture time, plating densities of cells and medium condition (serum-free or 2% of FBS). The poly-L-lysine domains of defined micro-scale area promoted the presence of round, loosely attached Ki-67-positive cells, while fibronectin domains of the same size allowed appearance of flattened, strongly attached cells with more differentiated phenotype. These results were in agreement with the non-specific, electrostatic type of interaction between cell and substrate on poly-L-lysine and integrin receptor-mediated specific adhesion on fibronectin. In this report we have described *in vitro* culture conditions, which allow for immobilization of the non-differentiated and highly proliferating population of neural stem/progenitor cells to the biofunctionalized surface. The microarrays with bioactive domains allocating non-differentiated and proliferating neural stem/progenitor cells may find application for drug and chemicals toxicology screening of diverse factors influencing neural development.

Key words: biomaterials, fibronectin, microcontact printing, neural stem cells, poly-L-lysine, proliferation

INTRODUCTION

Neural stem cells persist in the mammalian brain in the specific regions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. Their developmental decisions in vivo were shown to be dependent on reciprocal interactions with the components of neural stem cell niche, consisting of the lineage related cells (radial cells type B, transit-amplifying progenitors, type C cells), soluble factors and the extracellular matrix components arranged in 3D matrix structure as well as blood vessels and nerve terminals (Kazanis et al. 2010). The

mode of interaction between the cells (e.g. the presence of the gap junctions) and the cell/matrix interface relationship (e.g., nanosensing of the ECM by the cell membrane integrin receptors) were shown to be important for the in vivo cellular fate decisions (Pompe et al. 2007, Trosko et al. 2010). Biophysical cues of the niche are converted into biochemical signals that either allow self renewal and undifferentiated stage or commit the cell to a specific lineage. However, *in vitro* conditions for the maintenance of neural stem cells in different developmental stages and controlling their fate decisions are still to be determined.

Since the dynamic stem cell niche is difficult to mimic in its entirety, we have investigated in this study the influence of the several individual cues such as different adhesive molecules in biofunctional domains, but also various plating densities of cells and serum

content on the proliferation of the human umbilical cord blood-neural stem cells (HUCB-NSC). HUCB-NSC is non-transformed cell line, obtained in our laboratory (Buzanska et al. 2006), with a stable growth rate, the ability to self renew and to differentiate into neuronal, astrocytic and oligodendroglial lineages. Depending on the culture conditions, the cells can be harvested at different stages of neural commitment and differentiation. Thus HUCB-NSC represents a promising model for studying the interactions between the microenvironment (e.g., matrix adhesive biomolecules, the culture soluble factors and possible toxic compounds) with the neural stem cells in order to investigate cellular responses to the varying niche composition (Buzanska et al. 2009b).

For that purpose, we decided to test in vitro HUCB-NSC proliferation capacity in varying microenvironment by creating cell growth platforms with bioactive domains of defined micro-scale area, containing biomolecules similar to those found in natural stem cell niche. We have used microcontact printing technique which ensures simple, low cost and reproducible way of preparing such biofunctionalized surface arrays. Platforms with components of ECM/polycationic surrogates designed in spatial patterns allow a better control of cell behavior by physical and molecular interactions and enable investigating stem cell developmental processes (Ruiz et al. 2008, Guilak et al. 2010).

In this study, bioactive domains were designed to present insoluble factors mimicking different type of cell/extracellular matrix interactions. For that purpose, we have used synthetic polymeric poly-L-lysine (PLL) and extracellular matrix protein fibronectin (FN). Polycationic poly-L-lysine is known to bind cells by non-specific, electrostatic mechanism: positively charged PLL binds to negatively charged cell membrane (Rao et al. 2009); in contrast, ECM protein fibronectin (FN) which is affecting cell migration during gastrulation (Darribere and Shwarzbauer 2000), and is present when the stem cell fate is established, induces specific integrin-mediated cell adhesion mechanisms (Reilly and Engler 2010).

Different adhesive domains (PLL and FN) used for cell culturing in various densities in serum and serum-free conditions were applied in this study to HUCB-NSC to examine HUCB-NSC proliferation. Concomitantly, scanning and transmission electron microscopy allowed studying the difference in the physical interaction of the cells with the surface of adhesive domains and its important impact on cell morphology.

In this report we present microenvironmental conditions allowing for *in vitro* maintenance of highly proliferative, non-differentiated population of neural stem cells as immobilized to the surface.

METHODS

Cell culture

Human umbilical cord blood-neural stem cell (HUCB-NSC) line, obtained from non-hematopoietic fraction of cord blood (Buzanska et al. 2006), was cultured in DMEM/F12, 2% fetal bovine serum (FBS, Gibco), supplemented with insulin-transferrin-selenium (ITS 1:100, Gibco), antibiotic-antimycotic solution (AAS, 1:100, Gibco), at 37°C, 5% CO_2 and 95% humidity. Under these conditions HUCB-NSC grow as mixed population composed of the adherent neural progenitors and loosely attached or free-floating non-differentiated. When cells reached 80–90% confluency, cultures were harvested with trypsin-EDTA solution (Trypsin/EDTA 0.025%, Gibco) and propagated in 1:4 ratio. The cell medium was changed in growing cultures twice a week.

Preparation of microcontact printed patterns

Stamps made of polydimethylsiloxane (PDMS) silicone elastomer casted on silicon master were used for the direct printing of fibronectin and poly-L-lysine on anti-fouling surface of Petri dishes. The anti-fouling/anti-adhesive surface was obtained by vapor deposition of plasma polymerized polyethylene oxide-like (PEO) using plasma-enhanced chemical vapor. Complete description of the method can be found in Bretagnol and coauthors (2006) and Ruiz and colleagues (2008). Poly-L-lysine FITC-labeled diluted at 25 µg/ml in carbonate buffer (100 mM $NaHCO_3$, pH 8.5), and fibronectin (Sigma Aldrich) diluted at 42 µg/ml in printing buffer (100 mM acetate at pH 5.5 mM EDTA, 0.01% Triton-X 100, and 0.1% glycerol), were used as inking solution. The PDMS stamps were ultrasonicated in ethanol for 5 minutes and cleaned with mild O_2 plasma (200W, 1.2 torr, 30 seconds) before inking. PLL or FN was inked at room temperature on PDMS for 15 min and 45 min, respectively, then the excess solution was removed

and the stamps were dried in a N_2 stream. The inked stamps were put in contact with the Petri dish covered with PEO-layer for 10 minutes. Before seeding cells, the patterned surfaces were UV treated/sterilized for 15 min.

HUCB-NSC proliferation assay

In order to examine cell proliferation HUCB-NSC cells were seeded on the patterns with poly-L-lysine or fibronectin printed on Petri dishes (3 cm diameter). The cells were plated at three different densities: low (L) (1×10^4 cells/cm^2), intermediate (M) ($2,5 \times 10^4$ cells/cm^2) and high (H) (5×10^4 cells/cm^2) in serum (DMEM/F12, 2% FBS, ITS, AAS) or serum free condition (DMEM/F12, B27 1:50, EGF 20 ng/ml, AAS). After overnight incubation, the non-adherent cells were removed and adherent cells were cultured for 2, 4 or 7 days. At these time points of culture the immunocytochemical analysis of cells was performed in terms of Ki-67 and β-tubulin III expression.

The proliferation rate was calculated as the percentage of cells expressing Ki-67 per total cell number present on the square.

Immunocytochemistry

For immunocytochemistry, HUCB-NSC grown on fibronectin or poly-L-lysine patterns were washed with PBS and fixed for 15 minutes with 4% PFA after 2, 4 and 7 days of culture. After 15 min of permeabilization with 0,1% Triton X-100 cells were blocked for one hour with 10% NGS. Primary monoclonal antibodies: anti-human Ki-67 IgG1 (1:500, NovoCastra) or neuronal class III β-tubulin IgG2b (1:1000, Sigma) were applied overnight. After washing with PBS the following secondary antibodies AlexaFluo 546 goat anti mouse IgG1 (Invitrogen) or AlexaFluo 488 goat anti mouse IgG2b (Invitrogen) were applied for 60 minutes at room temperature. Cell nuclei were contrastained with Hoechst 33342 (1:1000, Molecular Probes) for 15 min and samples were closed in mounting solution (FluoroMount, Invitrogen). As a control for specific immunostaining the primary antibodies were omitted from the procedure. To obtain detailed images of the cells an Axiovert 25 fluorescence microscope (Carl Zeiss GmBH, Jena, Germany, http://zeiss) was used. Images were captured by AxioCamMRC5 camera.

Electron microscopy analysis

Electron microscopy analysis was performed on HUCB-NSC seeded on glass slides covered with either poly-L-lysine (25 µg/ml) or fibronectin (42 µg/ml) and cultured for 48 hours in 2% serum or serum free medium.

Scanning electron microscopy studies

For scanning electron microscopy studies, HUCB-NSC were fixed in 1% paraformaldehyde and 1,25% glutaraldehyde in cacodylate buffer for 2 h. Then the cells were postfixed in 1% OsO_4, and dehydrated with grades series of ethyl alcohol followed by acetone. After dehydration procedure cells were dried in a critical-point dryer and coated with gold before observation by scanning electron microscopy (JEOL JSM-6390LV).

Transmission electron microscopy studies

For transmission electron microscopy studies HUCB-NSC were fixed in 1% paraformaldehyde and 1.25% glutaraldehyde in cacodylate buffer pH 7.4 for 20 h followed by postfixing in a mixture of 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide $K_4[Fe(CN)_6]$. Then, the cells were processed for transmission electron microscopy and analyzed in a JEOL 1011.

Statistical analysis

For statistical analysis the data were collected from three independent experiments. Each variant of experimental condition: poly-L-lysine and fibronectin substrates, three different time points of cell culture, various plating densities and two different medium conditions (serum-free and 2% serum) were considered as the independent probes. The mean number of cells *per* square and standard deviation (SD) were calculated in 30 squares *per* each experimental variant. The proliferation rate was defined as the percentage of Ki-67 positive cells per square (100 µm side) in relation to the total number of the cells/square. The statistical analysis was performed using ANOVA one-way analysis of variance, Bonferroni's multiple comparison test (GraphPad Prism 3.0). Significance of difference (*p*-value, for *p*<0.01) was established for comparison

of the proliferation rate between serum and serum free and between poly-L-lysine and fibronectin groups.

RESULTS

Proliferation test of HUCB-NSC

To examine the effect of diffrent biomolecules on proliferation of HUCB-NSC, we cultured these cells on the surface patterned with either extracellular matrix protein: fibronectin (FN) or polyaminoacid poly-L-lysine (PLL) printed as separate domains on cell repelent, non adhesive surface of PEO-like film (Fig. 1). Such pattern enables to capture cells on the defined squares of 100 μm side with a 120 μm distance between the squares.

HUCB-NSC cultured on poly-L-lysine domains

The total number of cells per square on PLL domains was dependent on the starting plating density of cells and rised gradually with time of cell culture from the level 28 ± 8.7 and 42 ± 8.3 at day 2nd reaching the level of 69 ± 9.7 and 70 ± 9.7 cells/domain on day 7th at intermediate (M) and high (H) plating density of cells respectively. The significant difference in the number of cells per square in different culture medium conditions were visible at day 7th in the intermediate (M) and high (H) plating density of cells, (69 ± 9.7 cells *per* square in serum-free conditions as compared to 44.3 ± 10 cells *per* square in 2% serum, Fig. 2A).
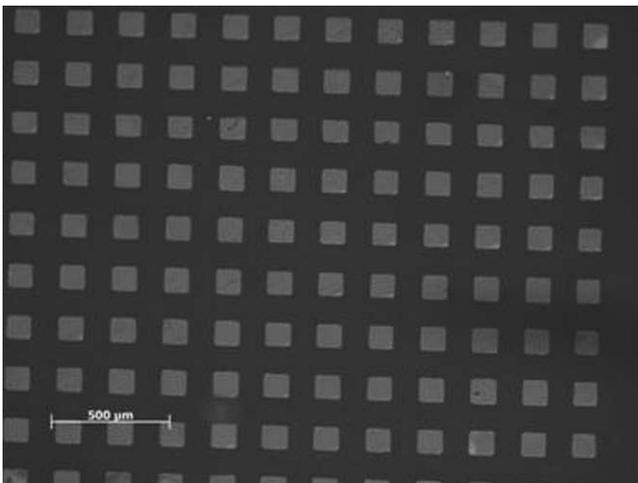

Fig. 1. Layout of poly-L-lysine pattern on PEO-like surface which shows a pattern of squares of 100 μm side separated by 120 μm distance. To visualize the quality of printing, poly-L-lysine was conjugated with FITC. Scale bar: 500 μm.

In contrast to the gradual changes of the total number of cells, there was no significant diffrence in proliferation rate of cells growing on PLL in serum-free conditions, when different experimental time points were compared. However, in the presence of serum, the rate of proliferation for intermediate (M) and high (H) densities was significantly higher at the day 7th of culture as compared to day 2nd and 4th (Fig. 2A, C).

On PLL domains, the proliferation rate of HUCB-NSC was usually higher in the absence of serum than in medium containing 2% serum. However the significant difference between the serum and serum-free medium ($p<0.01$) at various time of observation depended upon plating densities of cells: in intermediate (M) plating density of cells at day 2nd, in low (L) and high (H) plating density of cells at day 4th and in low (L) plating density of cells at day 7th (Fig. 2C). Increasing proliferation rate associated with higher plating densities of cells was observed on PLL at day 2nd, both for serum (L: $36.7 \pm 5.3\%$, M: $42.6 \pm 7\%$, H: $45.9 \pm 6\%$) and serum-free medium (L: $23.6 \pm 13.1\%$, M: $56.2 \pm 5.6\%$, H: $58.6 \pm 12\%$) (Fig. 2C). Such correlation was not observed at day 4th and 7th of cell culture, with the exeption for low density at day 7th, where generally lower proliferation rate was observed as compared to intermediate and high plating densities of cells.

HUCB-NSC cultured on fibronectin domains

The total number of cells *per* square on FN domains does not change significantly during 7 days of culture starting from 24.1 ± 6.3 and 30.6 ± 7.4 up to 41.4 ± 7.4 and 55 ± 5.2 cells/domain for intermediate and high plating density of cells, respectively. Different content of serum in the medium (serum-free or 2% serum) did not significantly changed the total number of cells on fibronectin domains during 7 days of culture (Fig. 2B).

There was no significant difference in proliferation rate of HUCB-NSC cultured on fibronectin domains in 2% serum and serum-free conditions during 7 days of experiment (Fig. 2D).

Variable plating density of cells generally did not influence proliferation rate of HUCB-NSC growing on FN domains. Significant difference in proliferation rate of FN attached cells was observed only in one case: at fourth day in the lowest plating density of cells ($20.8 \pm 10.2\%$ in the case of serum presence and $50.1 \pm 17.1\%$ in serum-free conditions). This was corresponding to the very low number of cells/square (com-

pare Fig. 2B with 2D), suggesting possible non-specific estimate at this exact time point.

Comparison of HUCB-NSC proliferation rate on poly-L-lysine versus fibronectin domains

The HUCB-NSC cells revealed lower proliferation rate on FN domains than on PLL domains for all investigated groups in 2nd, 4th and 7th day of culture (DIV – days *in vitro*). We have found that in the serum-free condition, the proliferation rate was higher on PLL domains (e.g., at day 4th in low density: $68.6 \pm 8.9\%$) than on FN ($22.7 \pm 6.9\%$), in all observed plating densities of cells and time of culture. Specifically the statistically significant differences between PLL and FN domains are shown for the samples: M DIV2nd, H DIV2nd ,M DIV4th, H DIV 4th, L DIV7th ($p<0.001$, Fig.

2C, D). In the presence of serum, higher proliferation rate of HUCB-NSC cultured on PLL as compared to FN domains was also shown, however statistically significant difference appeared at L DIV4, M DIV7 ($p<0.001$) and M DIV4 ($p<0.01$).

Immunocytochemical analysis revealed that on PLL domains, many Ki-67-positive, round non-differentiated cells were present (Fig. 3A, B). This indicates that PLL domains promoted immobilization to the surface of HUCB-NSC cells with high capacity for proliferation (in both serum and serum-free conditions). However, the presence of serum stimulated neural commitment of some cells attached to PLL (more β-III tubulin-positive cells in Fig. 3A as compared to Fig. 3B). In contrast to PLL, the HUCB-NSC observed on FN were flattened, strongly adherent, occupying almost all the square surface with a lower number of
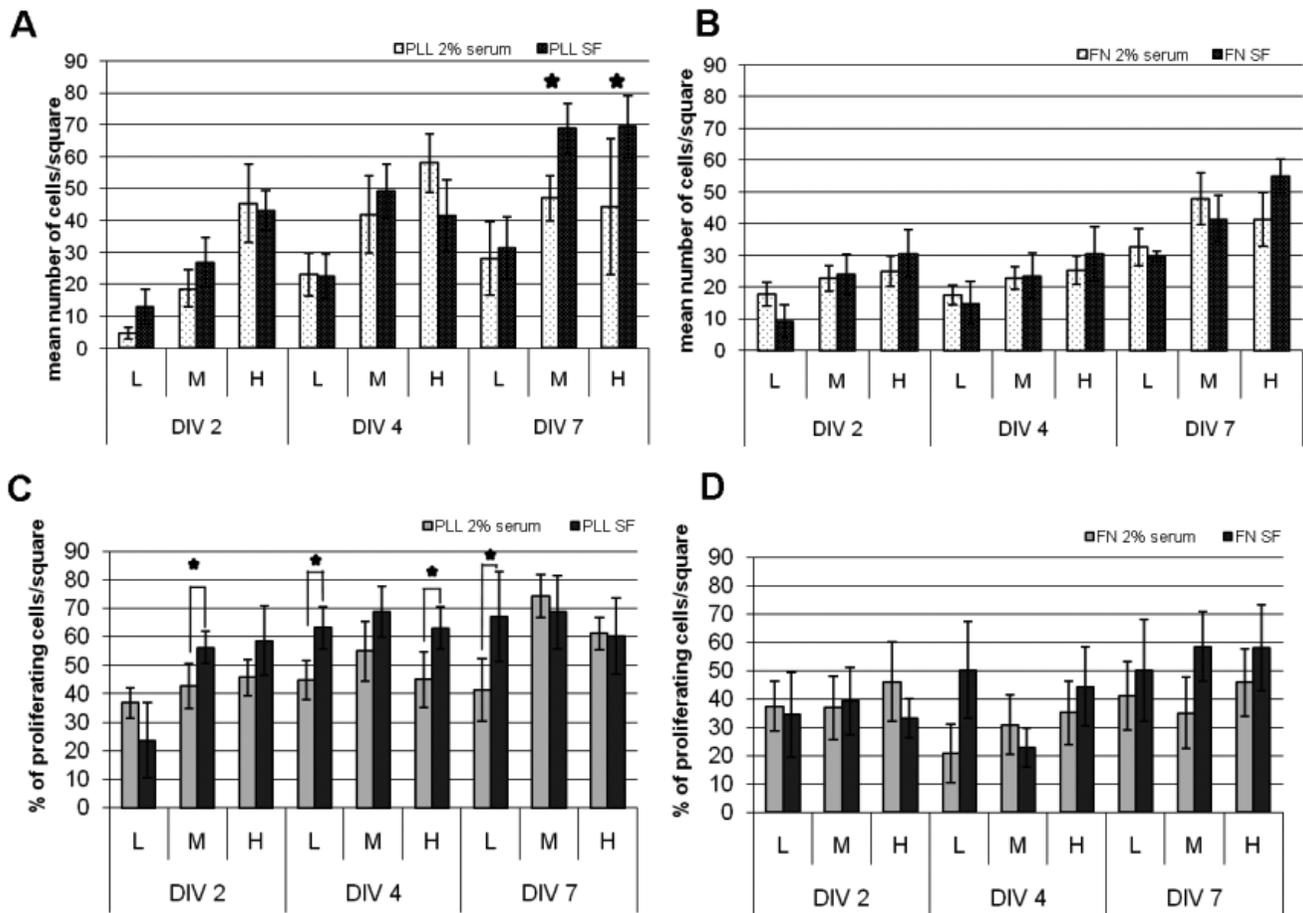

Fig. 2. Histogram of the total number of HUCB-NSC cells on domains printed with poly-L-lysine (PLL, A) and fibronectin (FN, B) and proliferation rate of cells persist on poly-L-lysine (C) and fibronectin (D) squares, in three starting plating densities: low, (L: 1×10^4 cells/cm²), intermediate (M: $2,5\times10^4$ cells/cm²) and high (H: 5×10^4 cells/cm²) and cultured in the presence of 2% of serum, or in serum-free (SF) medium after 2, 4 and 7 days *in vitro* (DIV). Asterisks show statistically significant differences between indicated pairs (values presented as means ± SD, from three experiments, $p<0.001$).

cells *per* square (Fig. 3C, D as compared to Fig. 3A, B). In both serum and serum-free conditions, cells were neurally commited (expression of β III tubulin-positive cells in Fig. 3C, D) on FN; however the serum-free conditions induced outgrowth of neuronal protrusions (Fig. 3D), thus promoting further differentiation of HUCB-NSC cells. Figure 3 represents HUCB-NSC from the same plating density of cells on PLL and FN domains at serum-free and 2% serum conditions and shows the higher number of attached and proliferating cells on PLL than on FN (e.g., 49.2 ± 8.3 as compared to 23.5 ± 7 cells *per* square at the fourth day of culture respectively, compare Fig. 2C and D) in all investigated experimental variants.

Electron microscopy investigation

The scanning and transmission electron microscopy was performed in order to visualize the three dimentional and ultrastructural morphology of HUCB-NSC
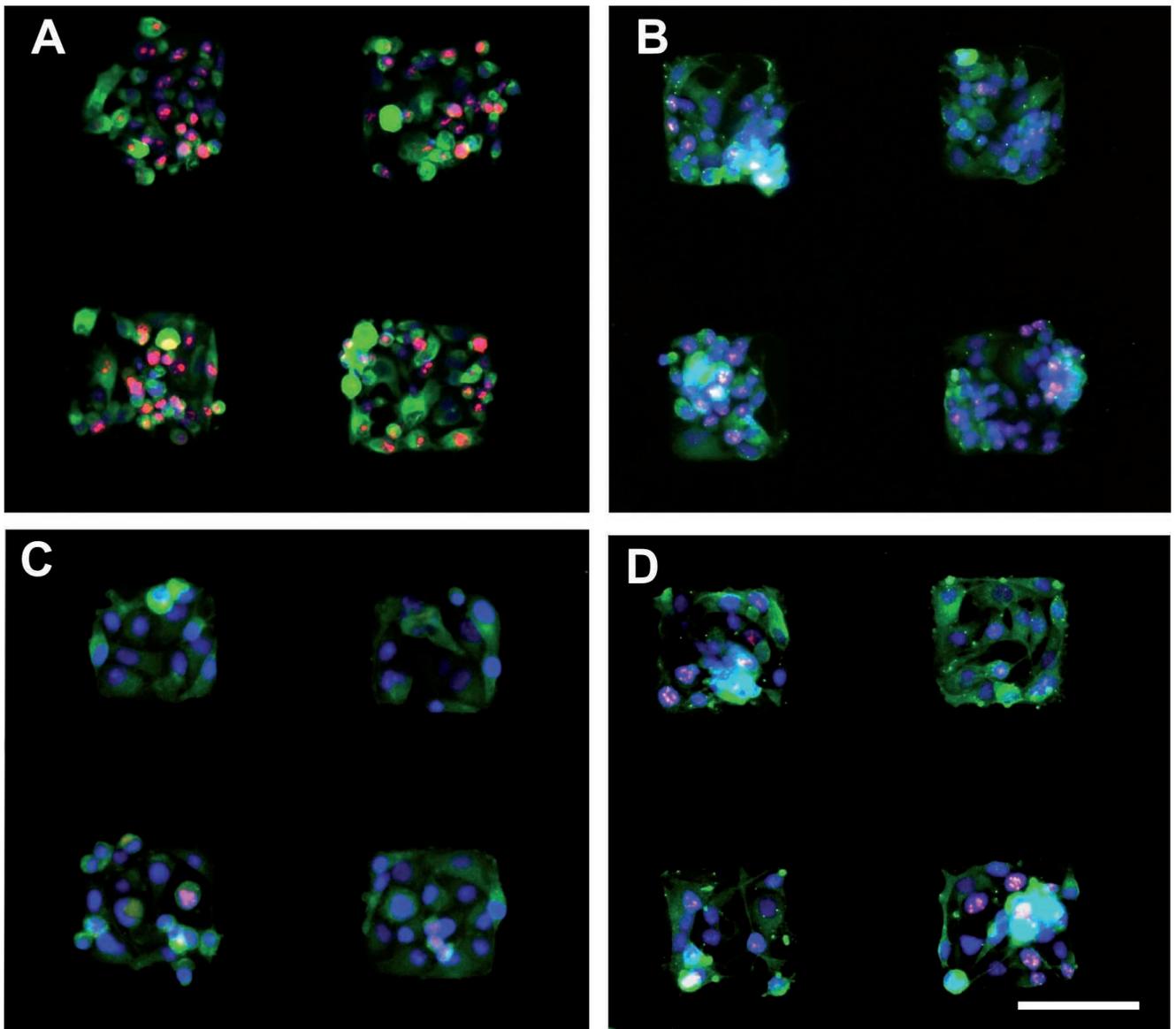

Fig. 3. Immunofluorescence images of HUCB-NSC cells plated on either poly-L-lysine (A,B) or fibronectin (C,D) patterns at 2.5×10^4 cells/cm^2 plating density in 2% of serum (A,C) and serum free (B,D) medium at day 4. Cells grown on poly-L-lysine show higher proliferation rate (Ki-67-positive red dots in blue stained nuclei) than on fibronectin. The β-III tubulin-positive cells are presented in green. Cell nuclei are contra-stained with Hoechst (blue). Scale bar: 100 μm.

in different culture conditions. Such analysis showed cell/cell and cell/substrate interactions, as well as the changes in the cellular ultrastructure induced by diffrent culture conditions.

HUCB-NSC cultured on poly-L-lysine domains

We have confirmed that the non-specific type of adhesion on PLL resulted in mostly undifferentiated type of HUCB-NSC morphology. The small and round cells, not firmly attached (Fig. 4A, B), poor in organelles cells (Fig. 5A), with small area of cell-surface and cell-cell connections/contacts but rich in microspikes/filopodia were mostly visible on PLL samples in the absence of serum (Fig. 4 and 5A, B). Transmission electron microscopy images indicated the presence of chromosomes during cell metaphase (Fig. 5B asterisk) and the vicinity of fibres that might correspond to intermediate filaments. The scanning electron micro-

scope revealed that cells grown on PLL domains exhibited relatively weak adhesion to this substrate (Fig. 4A, B). During the experimental processing, the cells were more fragile for the treatment, not tightly connected and easily disintegrated.

HUCB-NSC cultured on fibronectin domains

On the contrary, HUCB-NSC growing on FN adhered strongly to the substrate, were tightly arranged, flattened (Fig. 4C, D), with visible junction between cells (Fig. 5C, D arrows) but with fewer microspikes/filopodia, especially under low serum conditions (Fig. 4C). However, fibronectin attached cells cultured in serum-free conditions were comparatively less flattened than in 2% serum conditions and displayed outgrowth of long protrusions (Fig. 4D). The cytoplasm of the cells grown on FN revealed numerous intracellular organelles, such as Golgi apparatus, highly developed
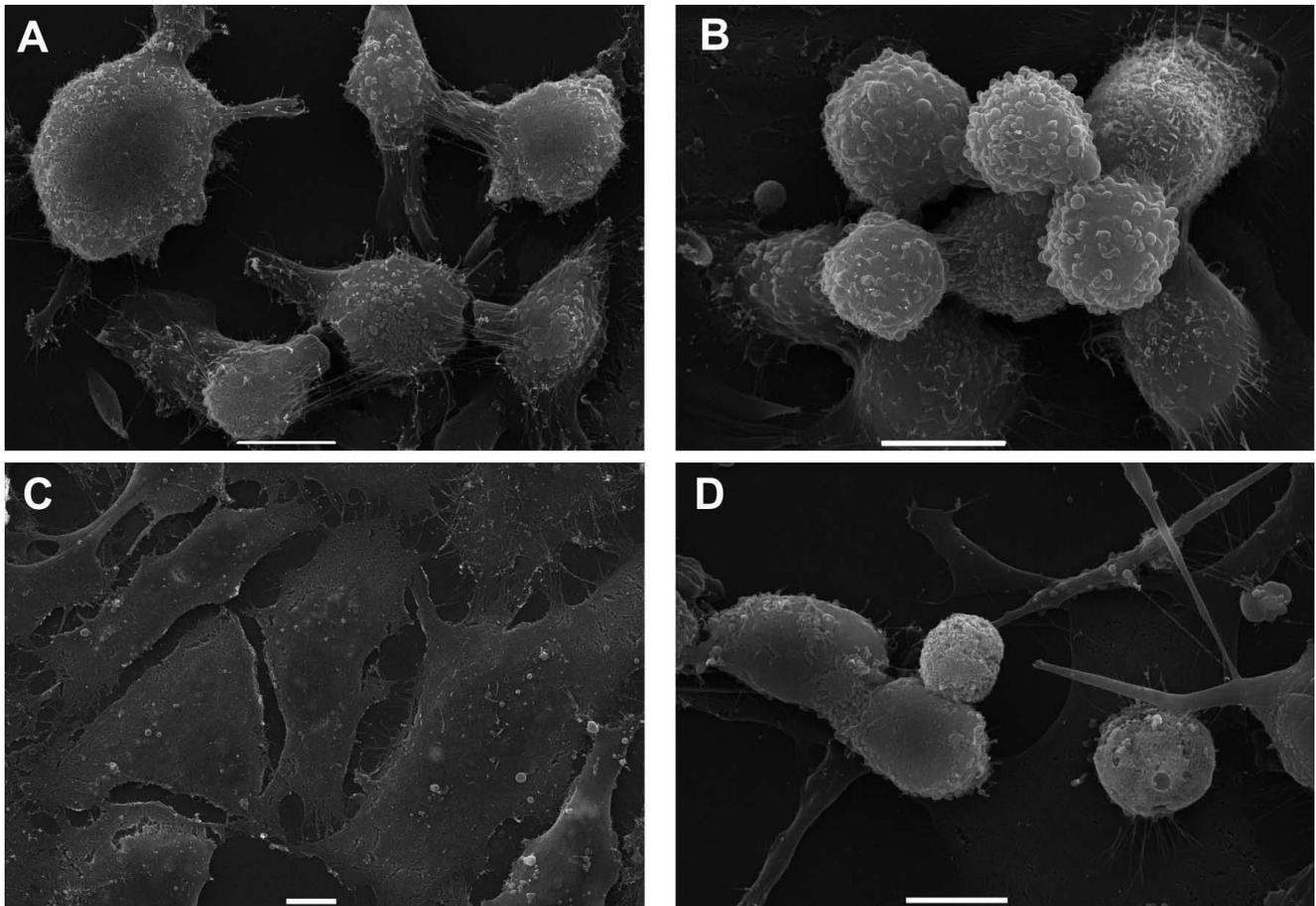

Fig. 4. Scanning electron microscopy images of HUCB-NSC cells plated on poly-L-lysine (A,B) and fibronectin (C,D) in 2% of serum (A,C) or serum free medium (B,D). Note the flattened phenotype of fibronectin attached cells in in the presence of serum (C) and the change of the shape and outgrowth of neuronal protrusions in the serum free conditions (D). Scale bar: 10 μm.

endoplasmic reticulum and dividing mitochondria (Fig. 5C, D). Electron microscopy images of FN as compared to PLL grown cells indicate more advanced stage of cell differentiation.

DISCUSSION

Different biofunctional domains, varying culture medium conditions and cell plating densities were investigated to assess proliferation capacity of HUCB-NSC. Adhesive substrates such as poly-L-lysine and fibronectin were arranged as a pattern confining exact size and shape of the biofunctional domains. This was performed by microcontact printing technique, previously reported to be a suitable method allowing spa-cio-temporal stimuli to be presented to HUCB-NSC (Ruiz et al. 2007, 2008, 2009, Buzanska et al. 2009a).

Cell / biomaterials interactions and HUCB-NSC proliferation

In this study, we show that the balance between proliferation and differentiation of HUCB-NSC on PLL domains is shifted towards maintaining the division capacity, while on FN the differentiation pathway is promoted. Fate decisions of neural stem cells derived from human cord blood have already been reported to be influenced by different types of adhesive substrates (Ceriotti et al. 2009, Buzanska et. al 2009, 2010, Sypecka et al. 2009, Szymczak et al. 2010).
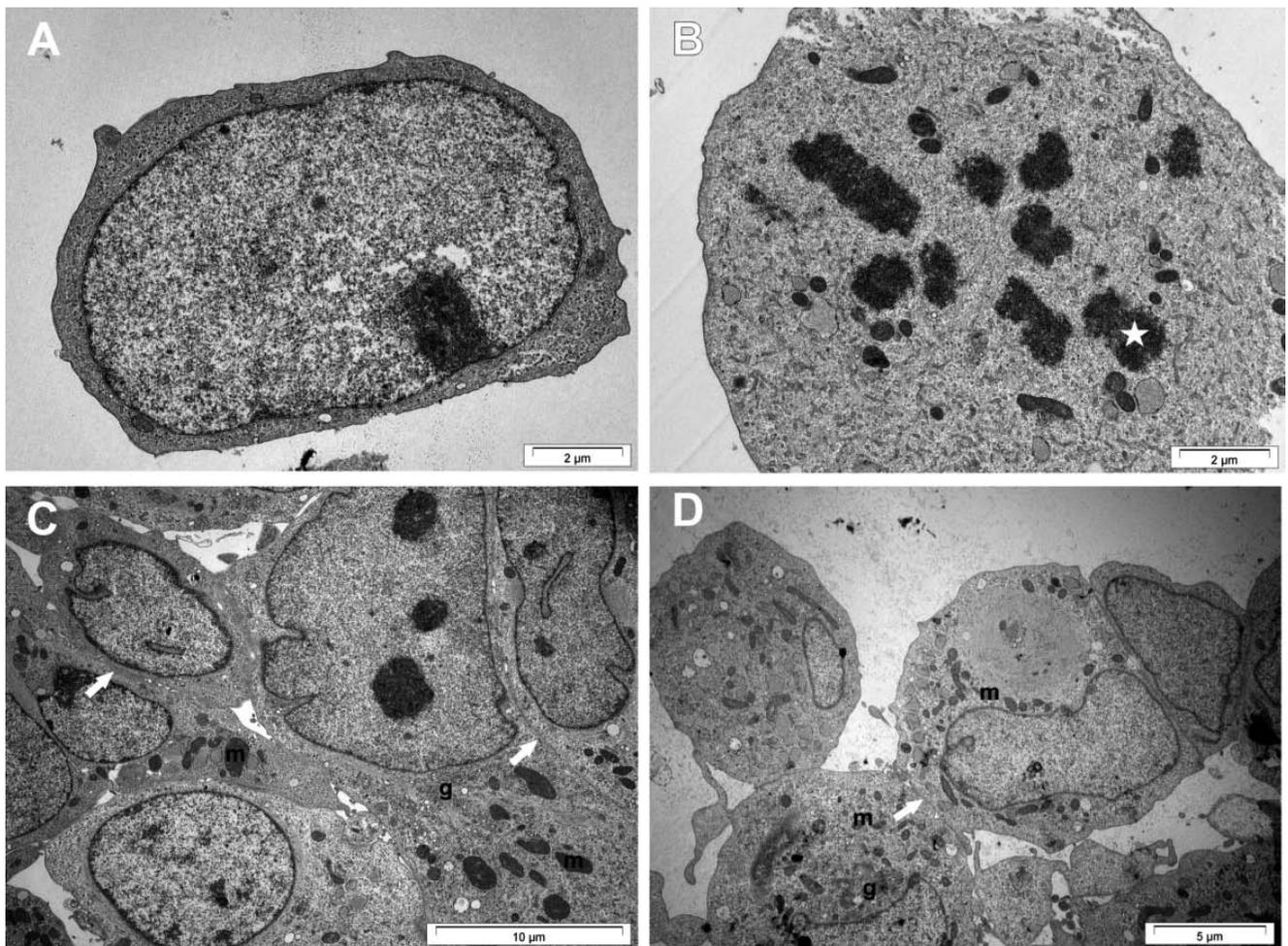

Fig.5. Transmisson electron microscopy images of HUCB-NSC cells plated on poly-L-lysine (A,B) and fibronectin (C,D) in 2% of serum (A,C) or serum free (B,D) medium. On poly-L-lysine, cells are loosely arranged, have big nucleus (A) or are during cell division, where the nuclear membrane has disappeared and the chromosomes during metaphase are highly condensed and individually distinct (B asterisk). The tight contact between cells is visible on fibronectin (C,D arrows), as well as many intracellular organelles (mitochondria-m, Golgi apparatus-g; C,D).

Observed differences in the adhesion profile and morphology of HUCB-NSC are likely attributed to the property of interaction on the cell membrane / biomaterial interface different on PLL and FN domains. Poly-L-lysine, which is a non-native polypeptide comprised of repeated, positively charged lysine sequence, attracts the negatively charged cell membrane and creates non specific, electrostatic bond formation (Yavin and Yavin 1974, Rao et al. 2009). However, PLL at longer time of cell culture can promote the adsorption of medium proteins (Heggins and Banker 1998) that leads to secretion of ECM components by the cell, then receptor-mediated adhesion and further mechano-responses (Rao et al. 2009) such as partial spreading and differentiation. That explains the sporadic appearance of spread and growing protrusions of cells on PLL at longer times of incubation, specifically appearing in the presence of serum. In contrast to PLL, FN acts through more specific interactions. The RGD domain (Arg-Gly-Asp) found in ECM proteins (laminin, collagen and fibronectin) have been identified as a minimum cell recognition sequence that can mediate adhesion of many cell types, including neurons (Hersel et al. 2003, Rao et al. 2009). In the fibronectin molecule, in addition to the RGD domain, a second integrin binding site, the ''synergy'' site comprised of the sequence PHSRN (Pro-His-Ser-Arg-Asn), also participates in cell-ECM recognition (Hattori et al. 2009, Stabenfeldt et al. 2010). The fibronectin increases migration and survival of primary NSCs (Tate et al. 2002) and differentiation of HUCB-NSC (Buzanska et al. 2009); moreover, this effect is enhanced in the presence of neurotrophins, like NGF or NT-3 (Nakajima et al. 2007, Silva et al. 2009). In our study, HUCB-NSC cells on fibronectin domains were stimulated for differentiation while keeping relatively high proliferation rate. Data from *in vivo* observation of developing mammalian brain revealed the correlation between appearance of transient amplifying and migrating neural progenitor cells. This was followed by the extracellular matrix reorganization from laminin to fibronectin based type and accompanied by so-called "integrin switch" on the cellular membrane (Stabenfeldt et al. 2010).

In theory, control of the cells by the adhesive substrate is not only dependent on its composition but also on multiple other physical mechanisms, such as ECM elasticity, geometry at the micro- and nanoscale and mechanical signals derived from substrates to the cell (Guilak et al. 2009). The restricted shape or size of surface pattern can exert a direct effect on the obtained cell phenotypes. In this report cells are confined to the domains of precise shape (square of 100/100 μm) and thus have very limited and defined area for attachment and spreading. In this study such defined PLL domains allowed for immobilization to the surface of proliferating and non-differentiated HUCB-NSC, while our previous observations on non-limited area covered by PLL (on multi-well plates or culture bottles) revealed ability of HUCB-NSC to differentiate and grow neuronal protrusions (Buzanska et al. 2006, Sypecka et al. 2009, Szymczak et al. 2010). This strongly suggests, that micro-scale area limitation of PLL domains is crucial for fate decision regarding proliferation/differentiation developmental switch. The area limitations and borders of cell attachment domains were shown by other groups to influence directly cellular fate. The MSCs cultured on smaller islands covered with fibronectin committed to adipocyte differentiation whereas on the bigger ones differentiated into osteoblastic lineage (Marklein and Burdick 2010).

The ECM, being a crucial component of the stromal microenvironment, functions as a scaffold for tissue organization, encapsulating and releasing growth factors and chemokines, thus regulating cell behavior features, such as survival, proliferation, migration and differentiation (Daley et al. 2008, Marastoni et al. 2008, Hu et al. 2009). This knowledge can be crucial in developing/engineering artificial ECMs for guiding embryonic or somatic stem cells after transplantation into the different organs (Guilak et al. 2009).

Cell-cell contact and HUCB-NSC proliferation

Plating density appeared to be an important factor for the stimulation of proliferation of HUCB-NSC on both PLL and fibronectin adhesive domains. We have revealed that plating density stimulates HUCB-NSC cells for the proliferation up to the certain threshold – cells seeded at intermediate density have significantly higher proliferation rate than low density. However, further increase in seeding density (to high density) did not influence the proliferation rate. Furthermore, in the high seeding conditions on PLL, the proliferation rate has even tendency to decrease. Similar correlation of seeding density to proliferation rate on different biomaterial surfaces was observed by Heng and coworkers (2010) for progenitors of endothelial cells derived from human umbilical vein. Such effect was coupled to the change in gene expression profile of endothelial pro-

genitor cells. Different mechanical signals including forces generated by cell-matrix and cell-cell contacts were shown to be involved in regulation of developmental processes like motility, proliferation, differentiation and survival (Janmey and Miller 2010).

Serum conditions and HUCB-NSC proliferation

The elimination of serum in the stem cell culture is an important issue because neural stem cell based research often aims in further application for human therapy. Such application requires elimination of any animal-origin compounds (xeno-free conditions) during the *in vitro* culture. Serum used for culture is a cocktail of not well-defined factors essential for an *in vitro* expansion and carries the potential risk of contamination with animal-derived pathogens. Additionally, serum containing medium may direct cells into not controlled differentiation. To avoid this all, defined serum-free media for stem cell culture are proposed (Richards et al. 2008, Tsuji et al. 2008) also for culturing of neural stem cells derived from cord blood and keep them in non-differentiated stage (Buzanska et al. 2006). However, neural stem cells growing in serum free conditions are usually non adherent population of floating cells difficult for studying their developmental processes such as adhesion, proliferation or migration. Thus, it was important to establish conditions for concomitant maintaining of neural stem cells in serum free medium in their non-differentiated state, but stably immobilized to the surface. We have achieved this goal applying PLL functional domains to immobilize to the surface of HUCB-NSC (Buzanska et al. 2009a and this study).

The proliferation rate of HUCB-NSC was significantly higher on PLL as compared to FN regardless serum conditions. Thus, according to our study, different serum conditions exerted profound effect on the attachment and differentiation of HUCB-NSC on different biomaterial surfaces, but not on the proliferation rate. Furthermore, the observed strong adherence of HUCB-NSC to FN domains in both serum and serum-free conditions promoted intracellular changes, characteristic for differentiation: cytoplasm was more densely packed with organelles and the long protrusions were formed. Recently, similar feature was shown by Meinertzhagen and coauthors (2009) as characteristic for differentiating neuronal cells. We have previously demonstrated that the serum-free condition in the presence of extracellular matrix components as well as dBcAMP can promote the differentiation of HUCB-NSC (Buzanska et al 2009). In this paper Ki-67-positive cells were present on fibronectin domains both in 2% serum and serum-free conditions and the presence of serum had no significant influence on the proliferation rate of HUCB-NSC and their ability for differentiation. This observation is in agreement with well-documented capacity of the committed and already differentiating neural progenitors for extensive proliferation both *in vivo* and *in vitro* (Encinas and Enikolopo 2008, Buzanska et al. 2009, Peltier et al. 2010, Tucker et al. 2010).

CONCLUSIONS

Neural stem cells derived from human cord blood have been cultured on the PLL and FN patterns in order to investigate their proliferation capacity at different microenvironmental conditions. Choice between the proliferation *versus* differentiation pathways of HUCB-NSC was attributed to the property of interaction on the cell membrane/biomaterial interface different on PLL and FN domains. We have shown that PLL domains of defined micro-scale adhesive area allow for maintaining highly proliferating population of neural stem cells in their non-differentiated state, as immobilized to the surface. Changes in the serum content in the culture medium and various plating densities of cells did not change this effect.

These studies conducted on confined adhesive pattern of microengineered cell growth platforms are giving more insight to the mechanisms of cellular decision on the proliferation/differentiation developmental switch.

REFERENCES

Bretagnol F, Lejeune M, Papadopoulou A, Hasiwa M, Rauscher H, Ceccone G, Colpo P, Rossi F (2006) Fouling and non-fouling surfaces produced by plasma polymerization of ethylene oxide monomer. Acta Biomaterialia 2: 165–172.

Buzanska L, Jurga M, Stachowiak EK, Stachowiak M K, Domanska-Janik K (2006) Neural stem-like cell line derived from a nonhematopoietic population of human umbilical cord blood. Stem Cells Dev 15: 391–406.

Buzanska L, Ruiz A, Zychowicz M, Rausher H, Ceriotti L, Rossi F, Colpo P, Domanska-Janik K, Coecke S (2009a) Patterned growth and differentiation of human cord blood-derived neural stem cells on bio-functionalized surfaces. Acta Neurobiol Exp (Wars) 69: 1–14

Buzanska L, Sypecka J, Nerini-Molteni S, Compagnoni A, Hogberg HT, del Torchio R, Domanska-Janik K, Zimmer J, Coecke S. (2009b) A human stem cell-based model for identifying adverse effects of organic and inorganic chemicals on the developing nervous system. Stem Cells 27: 2591–601.

Buzanska L, Zychowicz M, Ruiz A, Ceriotti L, Coecke S, Rauscher H, Sobanski T, Whelan M, Domanska-Janik K, Colpo P, Rossi F (2010) Neural stem cells from human cord blood on bioengineered surfaces--novel approach to multiparameter bio-tests. Toxicology 30: 35–42.

Ceriotti L, Buzanska L, Rauscher H, Mannelli I, Zychowicz M, Gilliland D, Hasiwa M, Bretagnol F, Ruiz A, Bremer S, Coecke S, Colpo P, Rossi F (2009) Fabrication and characterization of protein arrays for stem cells patterning. Soft Matters 5: 1–12.

Daley WP, Peters SB, Larsen M (2008) Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci 121: 255–264.

Darribere T, Schwarzbauer JE (2000) Fibronectin matrix composition and organization can regulate cell migration during amphibian development. Mech Dev 92: 239–250.

Encinas JM, Enikolopo G (2008) Identifying and quantitating neural stem and progenitor cells in the adult brain. Methods in Cell Biology 85: 243–272

Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS (2009) Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2: 17–26.

Hattori A, Hozumi K, Ko JA, Chikama T, Oomikawa K, Kato J, Ishida K, Hoshi N, Katagiri F, Kikkawa Y, Nomizu M, Nishida T (2009) Sequence specificity of the PHSRN peptide from fibronectin on corneal epithelial migration. Biochem Biophys Res Commun 6: 346–350.

Heggins D, Banker G (1998) Primary dissociated cell cultures. In: Culturing nerve cell (G. Banker, K. Goslin, eds). MIT-Press, Cambridge, Great Britain, pp. 37–78.

Heng BC, Bezerra PP, Preiser PR, Law SK, Xia Y, Boey F, Venkatraman SS (2010) Effect of cell-seeding density on the proliferation and gene expression profile of human umbilical vein endothelial cells within ex vivo culture. Cytotherapy Dec 20. [Epub ahead of print].

Hersel U, Dahmen C, Kessler H (2003) RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 24: 4385–4415.

Hu J, Deng L, Wang X, Xu XM (2009) Effects of extracellular matrix molecules on the growth properties of oligodendrocyte progenitor cells *in vitro*. J Neurosci Res 87: 2854–62.

Janmey PA, Miller RT (2011) Mechanisms of mechanical signaling in development and disease. J Cell Sci Jan 1: 9–18.

Kazanis I, Lathia JD, Vadakkan TJ, Raborn E, Wan R, Mughal MR, Eckley DM, Sasaki T, Patton B, Mattson MP, Hirschi KK, Dickinson ME, French-Constant C (2010) Quiescence and activation of stem and precursor cell populations in the subependymal zone of the mammalian brain are associated with distinct cellular and extracellular matrix signals. J Neurosci 21: 9771–9781.

Marastoni S, Ligresti G, Lorenzon E, Colombatti A, Mongiat M (2008) Extracellular matrix: a matter of life and death. Connect Tissue Res 49: 203–206.

Marklein RA, Burdick JA (2010) Controlling stem cell fate with material design. Adv Mater 12: 175–189.

Meinertzhagen IA, Takemura SY, Lu Z, Huang S, Gao S, Ting CY, Lee CH (2009) From form to function: the ways to know a neuron. J Neurogenet 23: 68–77.

Nakajima M, Ishimuro T, Kato K, Ko IK, Hirata I, Arima Y, Iwata H (2007) Combinatorial protein display for the cell-based screening of biomaterials that direct neural stem cell differentiation. Biomaterials 28: 1048–1060.

Peltier J, Agrawal S, Robertson MJ, Schaffer DV (2010) *In vitro* culture and analysis of adult hippocampal neural progenitors. Methods Mol Biol 621: 65–87.

Pompe T, Keller K, Mothes G, Nitschke M, Teese M, Zimmermann R, Werner C (2007) Surface modification of poly(hydroxybutyrate) films to control cell-matrix adhesion. Biomaterials 28: 28–37.

Rao SS, Han N, Winter JO (2009) Adhesion molecule-modified biomaterials for neural tissue engineering. Front Neuroengineering 2: 6.

Reilly GC, Engler AJ (2010) Intrinsic extracellular matrix properties regulate stem cell differentiation. J Biomech 5: 55–62.

Richards S, Leavesley D, Topping G, Upton Z (2008) Development of defined media for the serum-free expansion of primary keratinocytes and human embryonic stem cells. Tissue Eng Part C Methods 14: 221–232.

Ruiz A, Ceriotti L, Buzanska L, Hasiwa M, Bretagnol F,Ceccone G, Gilliland D, Rauscher H, Coecke S, Colpo

P, Rossi F (2007) Controlled micropatterning of biomolecules for cell culturing. Microelectronic Engineering 84: 1733–1736.

Ruiz A, Buzanska L, Gilliland D, Rausher H, Sirghi L, Sobanski T, Zychowicz M, Ceriotti L, Bretagnol F, Coecke S, Colpo P, Rossi F (2008) Micro-stamped surfaces for the patterned growth of neural stem cells. Biomaterials 29: 4766–4774.

Ruiz A, Zychowicz M, Buzanska L, Mehn D, Mills CA, Martinez E, Coecke S, Samitier J, Colpo P, Rossi F (2009) Single stem cell positioning on polylysine and fibronectin microarrays. Micro and Nanosystems 1: 50–56.

Sypecka J, Dragun-Szymczak P, Zalewska T, Domańska-Janik K (2009) Laminin promotes oligogliogenesis and increases MMPs activity in human neural stem cells of HUCB-NSC line. Acta Neurobiol Exp (Wars) 69: 37–45.

Szymczak P, Wojcik-Stanaszek L, Sypecka J, Sokolowska A, Zalewska T (2010) Effect of matrix metalloproteinases inhibition on the proliferation and differentiation of HUCB-NSCs cultured in the presence of adhesive substrates. Acta Neurobiol Exp (Wars) 70: 325–536.

Silva A, Pereira J, Oliveira CR, Relvas JB, Rego AC (2009) BDNF and extracellular matrix regulate differentiation of mice neurosphere-derived cells into a GABAergic neuronal phenotype. J Neurosci Res 87: 1986–1996.

Stabenfeldt SE, Carson Brown A, Barker TH (2010) Engineering ECM Complexity into Biomaterials for Directing Cell Fate. Stud Mechanobiol Tissue Eng Biomater 2: 1–18.

Tate MC, Shear DA, Hoffman SW, Stein DG, Archer DR, LaPlaca MC (2002) Fibronectin promotes survival and migration of primary neural stem cells transplanted into the traumatically injured mouse brain. Cell Transplant 11: 283–295.

Trosko JE, Kang KS (2010, *in press*) Stem cells in toxicology: fundamental biology and practical considerations. Toxicol Sci [Epub ahead of print].

Tsuji Y, Yoshimura N, Aoki H, Sharov AA, Ko MS, Motohashi T, Kunisada T (2008) Maintenance of undifferentiated mouse embryonic stem cells in suspension by the serum- and feeder-free defined culture condition. Dev Dyn 237: 2129–2138.

Tucker ES, Lehtinen MK, Maynard T, Zirlinger M, Dulac C, Rawson N, Pevny L, Lamantia AS (2010) Proliferative and transcriptional identity of distinct classes of neural precursors in the mammalian olfactory epithelium. Development 137: 2471–2481.

Yavin E, Yavin Z (1974) Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated surface. J Cell Biol 62: 540–546.