

Effect of matrix metalloproteinases inhibition on the proliferation and differentiation of HUCB-NSCs cultured in the presence of adhesive substrates

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Cell adhesion to extracellular matrix (ECM) generates intracellular signals that modulate cell survival, proliferation, migration and differentiation. Because of its heterogeneous nature, ECM has the potential to induce unique responses that are composition-dependent. One approach to study the effect of ECM signals on cell development, independently on signals from other extracellular sources, has been to deprive cells of serum and to analyze the influence of specific ligands. In the current work we determine the potential of different ECM proteins (fibronectin, laminin, collagen) on the proliferation and differentiation of human umbilical cord blood-derived neural stem cells (HUCB-NSCs) cultured in serum-free conditions. The effect of tested ECM components on the above processes might be associated with the particular pattern of their proteolysis. In this context enzymes that are responsible for the modification of ECM proteins are of particular pertinence. Matrix metalloproteinases (MMPs) represent a family of enzymes known to play role in the modification of ECM and by this can change the cell-ECM substrate interaction, required for cell development. In an effort to elucidate the participation of MMPs in the proliferation and differentiation HUCB-NSCs were cultured in the presence or absence of MMPs inhibitors – GM6001 and doxycycline. Our results show that addition of the above inhibitors interfered with both the proliferation and differentiation of progenitor cells toward the neuronal lineage. This effect depends on the adhesive ECM substrate and is most pronounced in the presence of fibronectin and laminin. In conclusion, our results suggest that MMPs modulate interaction between HUCB-NSCs and their environment and therefore might be an important component in neurogenesis-associated processes.

Key words: astrocytes, extracellular matrix, *in situ* zymography, neurons, oligodendrocytes, stem cells

INTRODUCTION

Neural stem-like cells derived from non-hematopoietic fraction of HUCB, due to their stable growth rate, the capacity for self renewal, the maintenance of differentiating potential into all neuronal lineages and ability for long-lasting culturing *in vitro* (Jurga et al. 2006) might serve as a potential source for transplantation therapy in neurological disorders and also for tissue engineering, and drug screening. Any effective cells replacement therapy would require the basic understanding of mechanisms governing their *in vitro*

proliferation and efficient differentiation into a particular neural phenotype. Increasing evidence suggests that ECM plays pivotal roles in regulating stem cell differentiation, as well as in cell migration and proliferation during embryonic development (Suzuki et al. 2003, Flaim et al. 2005, Kihara et al. 2006).

ECM is a macromolecular complex comprising largely of collagens, non-collagenous glycoproteins, proteoglycans, and glycosaminoglycans. These are secreted substances which are assembled locally into an organized network to which cells can adhere. Cell adhesion to ECM generates intracellular signals that modulate cell survival, proliferation, migration of neural precursor cells and differentiation *in vitro* (Giancotti and Ruoslahti 1999, Kearns et al. 2003) and *in vivo* (Murase and Horvitz 2002). Because of its heterogeneous nature,

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Received 03 October 2010, accepted 26 November 2010

ECM also has the potential to tailor unique responses that are composition-dependent (Yarwood and Woodgett 2001, Andressen et al. 2005). One approach to study the effect of ECM signals independently of signals from other extracellular sources has been to deprive cells of serum and to analyze the effect of specific ligands.

The function of ECM in developmental processes may be associated with particular patterns of proteolysis of extracellular matrix. In this context enzymes that are responsible for the modification of proteinaceous structures are of particular pertinence. Among the proteases the matrix metalloproteinases represent a family of enzymes known to play an important role in the modification of all ECM elements through the processing of matrix proteins and by this influence cell behaviour. MMPs are expressed abundantly in neural stem cells isolated from the human nervous system (Frolichsthal-Schoeller et al. 1999) and according to Mannello and coauthors (2006) they have regulatory roles during proliferation and differentiation of neural precursor cells in the embryonic development of the mouse brain.

In the current study we sought to determine the potential of native to the brain purified ECM proteins: fibronectin, laminin, collagen and a non-ECM positively charged polymer poly-L-lysine on the proliferation and differentiation of human cord blood-derived neural stem cells (HUCB-NSC) cultured in serum-free conditions. In an effort to elucidate the involvement of metalloproteinases (MMPs), we have tested the effect of their inhibitors – GM6001 and doxycycline on the above development-associated processes.

METHODS

Reagents

For the immunocytochemistry of HUCB-NSCs, the following primary antibodies applied (final dilution and source) were: mouse monoclonal anti-Ki67 (Novocastra Lab Ltd., Newcastle, UK, 1:100), mouse monoclonal anti-TuJ1 (Covance, Emeryville, CA, 1:500), mouse monoclonal anti-MAP2 (Sigma, St Louis, MO, 1:500), and mouse monoclonal anti-galactocerebroside (GalC) (Chemicon, Temecula, CA, 1:200). All ECM human components and poly-L-lysine were from Sigma. As mentioned in the Results section, some experiments were conducted with the following pharmacological agents added to the culture medium: broad-spectrum inhibitors of MMP – 1,10-O-phenanthroline (Merck, Whitehouse Station, NY, 1 mM), and GM6001 (a peptidyl hydroxamate, Sigma-Aldrich Co, 25 μ M), a non-selective MMPs inhibitor -doxycycline (Sigma-Aldrich, St Louis, MO, 60 μ M), an inhibitor of serin proteinases - Pefabloc SC [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride] (Roche Appl Sci, Mannheim, Germany, 5 mM) and the furin inhibitor Dec-RVKR-CMK (Calbiochem, San Diego, CA; 50 μ M).

GM6001 was prepared as 1 mM stock solution in DMSO. All the other agents were dissolved in PBS. For each agent, a corresponding diluting solution was used in these experiments as control.

Culture and treatments of HUCB-NSCs

Neural-like stem cell line derived from human umbilical cord blood (HUCB-NSC) (Buzanska et al. 2002) was cultured as a mixed population of committed adherent progenitors and free-floating undifferentiated cells in medium: F12/DMEM + 2% FBS + ITS medium (Gibco, Paisley, UK) in stabilized conditions of 37°C and 5% CO₂ in a fully humidified atmosphere. The pooled fractions of adherent and floating HUCB-NSCs were seeded at a density of 10⁴ cells/cm² onto glass plates coated with one of the ECM components dissolved in PBS: fibronectin – FN (10 μ g/ml), laminin1- LN (20 μ g/ml), or collagen IV - COL (10 μ g/ml), and conventional coating factor - poly-L-lysine - PLL (10 μ g/ml in PBS). Prior to seeding fibronectin, laminin, and collagen remained on culture dishes overnight at 4°C without air drying and the excess of substrate was then removed and plates rinsed with warm PBS. Following cell adhesion, the standard medium was replaced with the serum-free equivalent, either with or without inhibitor of MMPs – GM6001 or doxycycline. HUCB-NSC cultures were left for 14 days *in vitro* (DIV) to grow and differentiate under the given conditions.

Culture growth

To assess the effect of the substrates on the overall growth of HUCB culture identical numbers of viable cells were plated on the different substrate-coated coverslips. At the designated times of culture (4, 8, or 14 DIV) in the presence or absence of inhibitors, the standard medium was discarded and cells incubated (3 h at 37°C) with a medium containing Alamar Blue (Promega Corp., Madison, WI). Fluorescence was read

using a MultiScan Ascent FL (LabSystems Oy, Helsinki, Finland) spectrofluorimeter (by excitation wavelength 545 nm/emission 590 nm), and its level (proportional to the number of viable cells present on the multiwell plates) was converted to the number of surviving cells.

Immunocytochemistry

The cell cultures were fixed for 20 min with 4% PFA diluted in PBS. A blocking solution, containing 10% normal goat serum in PBS, was applied for 1 h at 25°C (room temperature -RT). The capacity of HUCB-NSCs to generate neurons and glia was examined through application of specific antibodies against neuronal (TuJ1, MAP2), oligodendroglial (GalC) and astrocytic (GFAP) markers. Cell proliferation was evaluated using anti-Ki67 determining cells in the mitotic cycle. Immunoreaction with the primary antibodies was carried out overnight at 4°C. Cells were rinsed with PBS and then incubated for 1 h at RT with an appropriate secondary antibody conjugated to Alexa Fluor-546 (1:1000, Molecular Probes). Controls for specificity of immunostaining were processed with either the primary or the secondary antibody excluded. Cell nuclei were visualized using 30 min incubation at RT with 5 μ M Hoechst 33258 (Sigma). The labeled cells were examined under fluorescence and confocal microscope. Images were captured and processed as described above.

Analyses of HUCB-NSCs differentiation

Neurons, astrocytes and oligodendrocytes were counted manually. To quantify the percentage of cells expressing a specific marker in any given experiment the number of positive cells in the whole population was determined, relative to the total number of Hoechst-positive non-apoptotic nuclei. In a typical experiment, 5000 cells were counted per marker and were expressed as a percentage of total cells determined by counting Hoechst-stained nuclei.

In situ zymography

In order to localize activity of matrix gelatinases, MMP-2 and MMP-9 in HUCB-NSCs, we have conducted *in situ* zymography according to the method described by Ogier and coworkers (2006). HUCB-NSCs cultured on glass cover slips were incubated

for 3 h at 37°C in a humid dark chamber in reaction buffer containing 50 μ g/ml of FITC-labeled DQ-gelatin (Invitrogen Molecular Probes, Eugene, OR) that is quenched intramolecularly. Gelatin-FITC cleavage by tissue metalloproteinases (gelatinases) releases peptides whose fluorescence is representative of proteolytic activity. The sections were rinsed in PBS and fixed in cold 4% paraformaldehyde for 20 min., then mounted in fluorescent mounting medium (Dako) and observed using fluorescence microscopy. To confirm that the proteolytic activity is attributable to MMPs, some sections in each experiment were incubated in the above conditions with a broad spectrum inhibitor of metalloproteinases, 1mM 1,10-O-phenantroline. Fluorescence was visualized using an Axiovert 25 fluorescence microscope (Carl Zeiss, Jena, Germany) and confocal microscope. Images were captured on the Videotronic CCD-4230 camera, and processed by Axiovision image analysis system. All images subjected to direct comparisons were captured at the same exposure and digital gain settings to eliminate confounds of differential background intensity or false-positive fluorescent signals across sections.

Statistical analysis

All values are given as mean \pm SEM. Differences between means were determined using one-way analysis of variance (ANOVA) followed by posthoc Bonferroni test. Statistical significance was deemed to be present if $p < 0.05$.

RESULTS

Growth, proliferation and differentiation of HUCB-NSCs in the presence of adhesive substrates

To assess the effect of substrates on the overall growth of HUCB-NSC cultures, we plated equivalent numbers of viable cells on coverslips coated either with one of the ECM-component (FN, LN, COL) or on poly-L-lysine (PLL), and assayed the total number of living cells over time. We refer to the increase in the number of cells as "cell expansion". Quantitation of HUCB-NSCs cultured for 14 days showed that greater number of cells were present on FN-coating matrices than on the other tested substrates (Fig. 1A). Cells in fibronectin increased in

number by ~14-fold compared to ~8-10-fold elevation observed in the presence of the remaining substrates. It is worth noting that cells expansion cultured on LN or COL was not different from that growing on PLL.

We also examined the influence of the tested adhesive substrates on the kinetics of HUCB-NSCs proliferation. For this purpose we used a marker of dividing cells, Ki67. As shown in Fig. 1B most intensive proliferation rate was seen between 4 and 8 days. During the next 6 days in culture the number of dividing cells did not show significant changes. The highest proliferation rate was also observed in the presence of FN, where the proportion of Ki67-positive cells reached approximately 72% of the entire population. In the case of culture growing on LN or PLL the amount of labeled cells accounts for 60% of population. However, the absolute number of

immunopositive cells was significantly higher on LN, compared to PLL, which served as a positive control ($p < 0.001$). The lowest rate of proliferation was observed on the presence of COL (40% population). The number of dividing cells attached to this substrate was very close to that found in the presence of PLL.

To determine whether tested adhesive substrates effect differently the pattern of differentiation, HUCB-NSC cells were stained with specific antibodies against neuronal (TuJ1, MAP-2), oligodendroglial (GalC) and astrocytic (S100beta) markers. Cell counts at 14 DIV showed that the attachment of cells to fibronectin leads to the highest absolute number of TuJ1-positive cells (4.8×10^4 cells/cm²), compared to average 2.8×10^4 cells/cm² detected in culture grown on other tested substrates. Likewise, preponderant number of mature

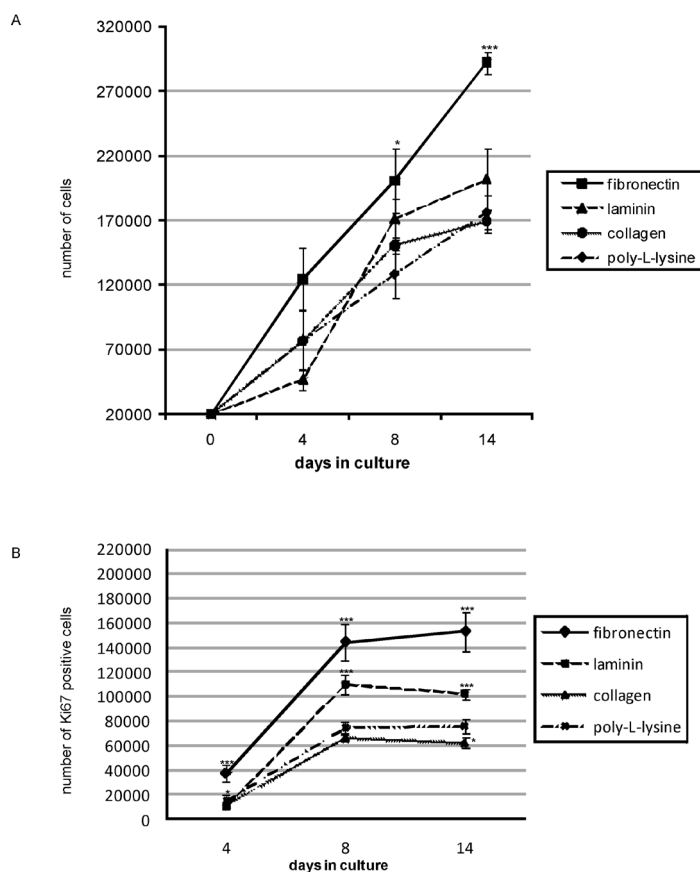


Fig 1. The growth and proliferation of HUCB-NSCs in serum-free conditions.

Identical numbers of HUCB-NSCs were plated on coverslips coated either with different ECM components or with poly-L-lysine and grown 14 days in serum-free medium. A) Graph shows an average number of surviving cells after 4, 8 and 14 days in culture. B) Graph shows the rate of HUCB-NSCs proliferation expressed as a number of Ki67-immunopositive cells.

The results (mean values \pm SEM) represent five independent experiments. Asterisks indicate a statistically significant difference versus poly-L-lysine, which served as a positive control. * $p < 0.05$; *** $p < 0.001$.

MAP2-positive neurons was as well found in fibronectin-treated culture (5.8×10^4 cells/cm²), whereas fewer cells expressing MAP2 were generated on laminin, poly-L-lysine and collagen (3.8×10^4 /cm²; 2.8×10^4 /cm²; and 2.7×10^4 /cm², respectively (Fig. 2). The amplification of neuronal cells in the presence of FN is probably due to greater cell density. Despite the differences in absolute number of cell phenotypes, their proportion in relation to the entire population remained on the similar level. In all cultures about 32% of cell population was immunopositive for TuJ1, and 36% were stained for more advanced neuronal marker – MAP2. Because neurons are considered postmitotic cells, the effect on their number is not likely to be due to enhanced proliferation of neurons once formed, but instead is likely to be related to the generation of new neurons from

HUCB or their increased survival on substrates once differentiated.

Oligodendrocytes and astrocytes (GALC and S100beta immunopositive) constitute the minor fraction of differentiated cells, and account for roughly the same low proportion (10% and 9%, respectively) of the cell population.

The effect of MMP inhibitor GM6001 and doxycycline on the growth, proliferation and differentiation of cultured HUCB-NSCs

Figure 3 shows the endogenous activity of matrix metalloproteinases assayed by *in situ* zymography in HUCB-NSCs growing on fibronectin. As depicted in Fig. 3 (A,B,C) the activity of MMPs determined over time (at 4, 8 and 14 DIV, respectively) corresponds to the

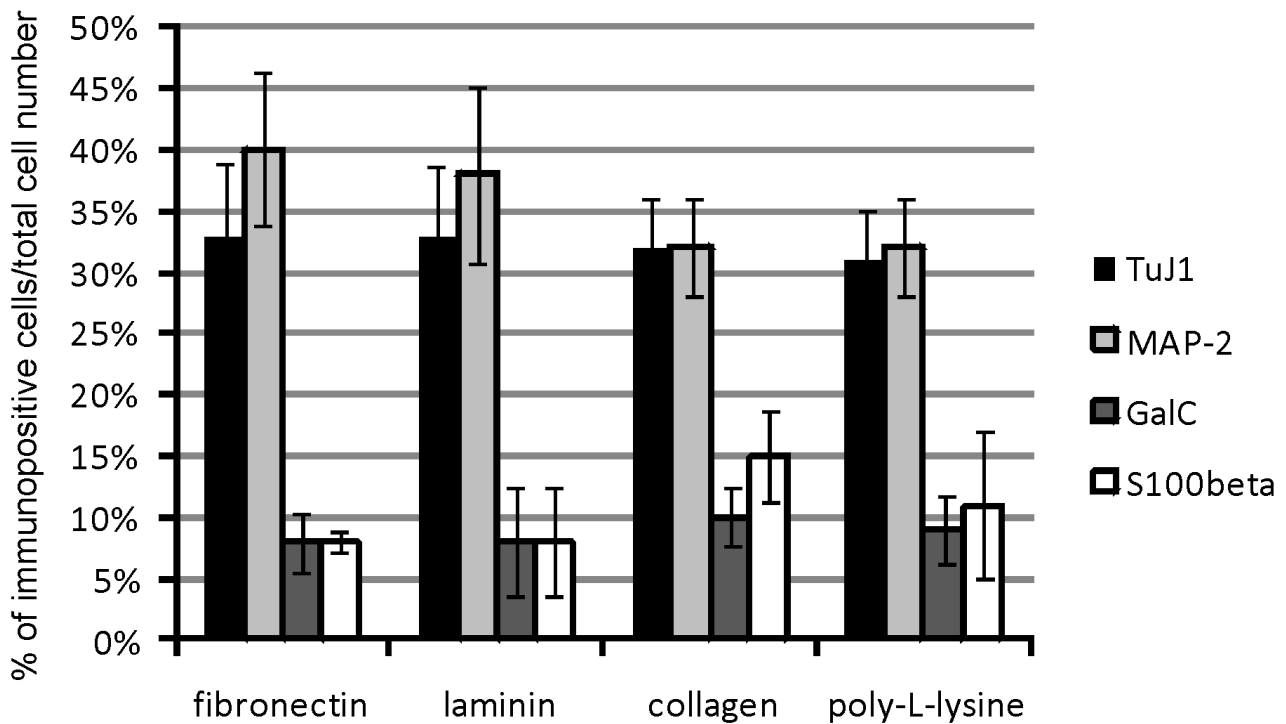


Fig.2. Differentiation of HUCB-NSCs in serum-free conditions. Identical numbers of HUCB-NSCs were plated on coverslips coated either with different ECM components or with poly-L-lysine and grown 14 days in serum-free medium. Graph presents the percentages of immunolabelled cells relative to the entire population that are present in the culture. The results (mean values \pm SEM) represent five independent experiments. Note the lack of differences in the percentages of cell phenotypes in HUCB-NSCs cultured in the presence of tested adhesive substrates.

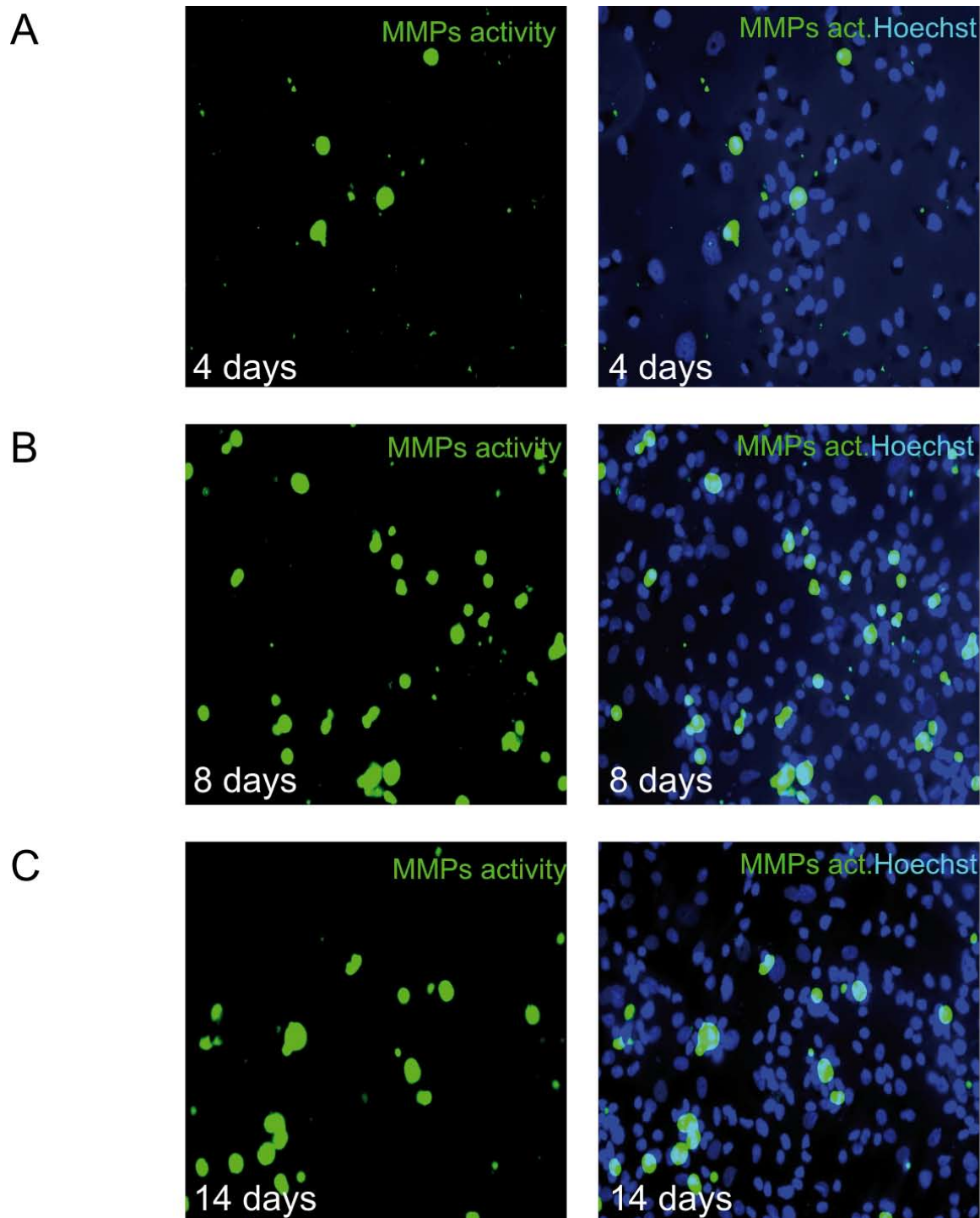


Fig.3. Activity of MMPs in HUCB-NSCs . Identical numbers of HUCB-NSCs were plated on fibronectin-coated coverslips and grown in serum-free medium. Activity of MMPs was assayed by *in situ* zymography in culture growing 4 (A), 8 (B) and 14 (C) days (Left panel) Cell nuclei were counterstained with Hoechst (right panel). The activity of MMPs expressed as a green fluorescence signal.

rate of cell growth. Addition of GM6001 (25 μ M) or doxycycline (60 μ M) to the culture medium abolished the gelatinolytic activity of MMPs (Fig. 4) and reduced the number of living cells attached to all substrata tested by about 45% and 60%, respectively (Fig. 5). However, despite the diminished proportion of alive to the total cells number, inhibitors-treated cultures showed a stable growth rate. The further way in which responses to GM6001 and doxycycline were manifested was significant suppression of the proliferation potential (Fig. 6). The relative number of Ki67-positive (in relation to surviving) cells growing on the tested substrata was reduced to average 43% in the presence of GM6001. Even more profound decrease (to about 25%) was caused by addition of doxycycline. The effect of MMPs inhibition on the percentage of proliferating HUCB-NSCs does not seem to depend on the ECM component.

We next tested whether these inhibitors might affect the pattern of cells differentiation. The observations presented here indicate that GM6001 as well as doxycycline added to the culture medium altered the profile of differentiation. Inhibitors treatment was associated with inhibition of neuronal cells generation, and promotion of oligodendrocytes and astrocytes in laminin-, fibronectin- and collagen attached cells (Fig. 7). As shown in the graph the percentage of TuJ1- and MAP2-positive neurons was reduced respectively by a factor of ~ 3 and ~ 2 (to 11% and 20%) in the cultures treated with GM6001 and to 9% and 13% (by a factor ~ 3.5 and ~ 3) after treatment with doxycycline. Concomitantly, the percentage of cells expressing the astroglial and

oligodendroglial markers (S100 beta and GalC), was higher, and reached above 30% and 22% respectively. In contrast, there was no change in differentiation of HUCB-NSCs growing on poly-L-lysine.

In addition, we checked whether the described above effects are related to the MMPs inhibition. For this purpose we have added to the culture medium the serin proteinase inhibitor Pefabloc (5 mM), as well as furin inhibitor Dec-RVKR-CMK (50 μ M). We did not find an influence of these agents on the above processes (results not shown).

DISCUSSION

Our study show that developmental processes of HUCB-NSCs cultured in serum-free conditions depend on the particular adhesive substrate. This finding is substantiated by the work of others using a variety of cell systems (Drago et al. 1991, Andressen et al. 2005, Mruthyunjaya et al. 2010). The current data also demonstrate that matrix metalloproteinases might participate in the above processes as the timing of their activation in HUCB-NSCs correlates with the acceleration of stem cells proliferation and their further differentiation. Expansion and proliferation of HUCB-NSCs are all enhanced predominantly on fibronectin containing matrices compared with other ECM components tested as well as with poly-L-lysine. Collagen and poly-L-lysine occurred to be less effective matrices mediating cells division. This is consonant with the studies of Ali and coauthors (1998), which showed that collagen inhib-

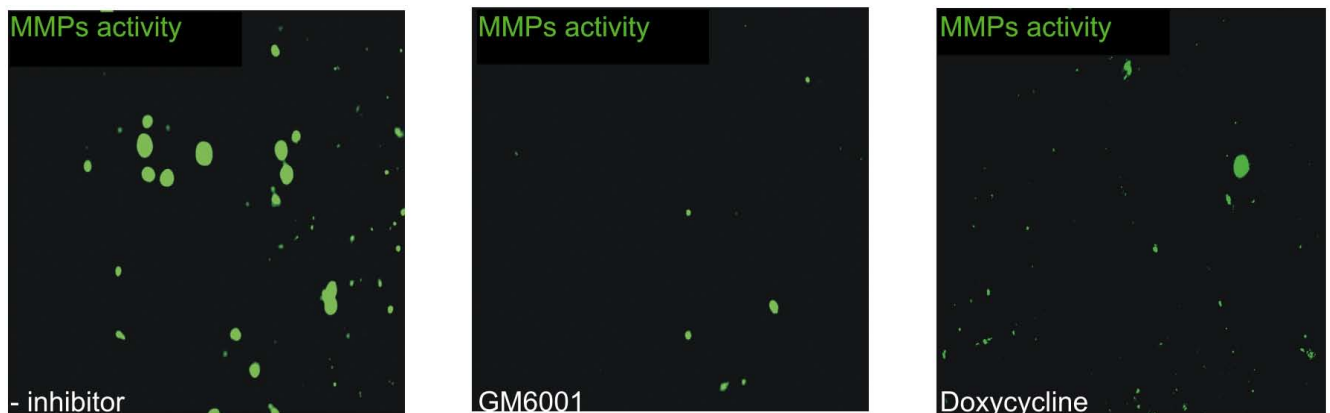


Fig. 4. Effect of GM6001 and doxycycline on MMPs activity in HUCB-NSCs. Identical numbers of HUCB-NSCs were plated on fibronectin-coated coverslips and grown 8 days with or without MMPs inhibitors - GM6001 (25 μ M) and doxycycline (60 μ M). Photomicrographs show inhibition of MMPs activity by GM6001 and doxycycline. The activity of MMPs expressed as a green fluorescence signal.

ited cell proliferation. The same proportion of Ki67-positive cells found in COL- as well as in PLL-treated cultures did not confirm the established rule that cell-substrate interaction varied from those solely based on charge to those mediated by binding to ligands (Flanagan

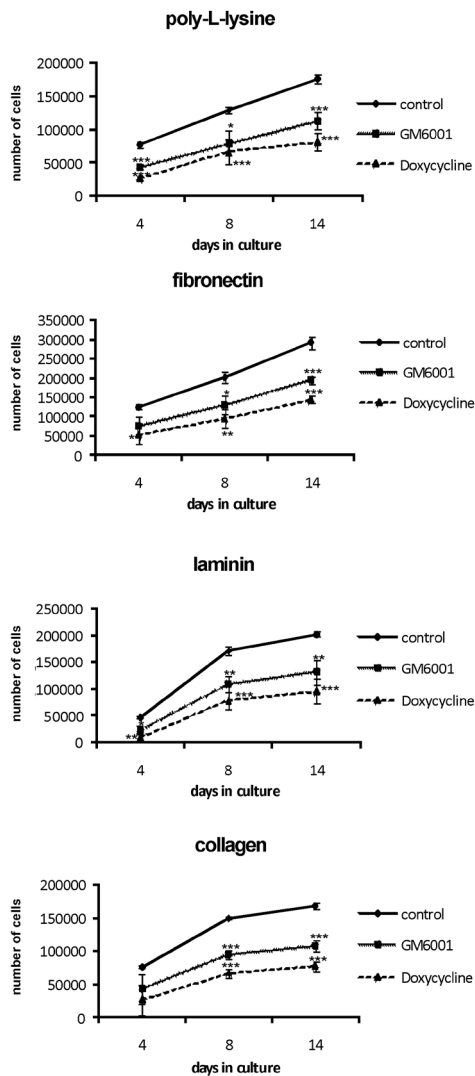


Fig.5. Effect of GM6001 and doxycycline on the growth of HUCB-NSCs. Identical numbers of HUCB-NSCs were plated on coverslips coated either with different ECM components or with poly-L-lysine and cultured in serum-free medium without (control) or with MMPs inhibitors - GM6001 (25 μ M) and doxycycline (60 μ M). Graph shows an average number of surviving cells after 4, 8 and 14 days in culture. The addition of inhibitors to the incubation medium decreased the number of surviving cells in all experimental conditions. The results (mean values \pm SEM) represent five independent experiments. Asterisks indicate a statistically significant difference versus control. * p <0.05; ** p <0.01; *** p <0.001.

et al. 2006). Therefore, considering the effect of PLL, it is tempting to suggest that cell development could be regulated, at least in part, independently on cell adhesion. This might be due to the secretion by the cells of some ECM compounds and promoted cell development, but this deduction remains to be unproven.

As was already demonstrated previously in our lab (Buzanska et al. 2002) upon growth factors withdrawal HUCB-NSCs differentiate spontaneously into the major brain cell types. The results of the current work indicate that all tested proteins participate in the decision of neuronal differentiation and phenotypic specification. The percentages of HUCB-NSCs differentiated toward neuronal fate were almost the same independent on the present substrate. The amplification of absolute number of neurons observed in FN-treated culture was probably due to the higher cell density found on this coating. In opposite to the stimulating effect of fibronectin found in the current study, it has been proven that laminin is the most permissive substrate in proliferation and differentiation of embryonic stem cell lines and cortical neural progenitors (Drago et al. 1991, Andressen et al. 2005, Flanagan et al. 2006). It means that different cell types may have their own favored ECM for development, depending probably on repertoire of specific receptors expressed on their cell surface.

Unfortunately, neither the mechanism mediating the HUCB-NSCs proliferation nor differentiation triggered by different ECM are well known. The proper response of extracellular signals which play a crucial role in cell development-associated processes is possible due to an array of integrin receptors on the cell surface and hence capable of triggering a cascade of events instructive for cell proliferation and differentiation (Flanagan et al. 2006, Chen et al. 2007). It was found that this signaling pathways interact with those downstream of growth factor receptors (Yamada and Even-Ram 2002). Recently published data implicate involvement of MAP kinase pathway (Campos et al. 2004, Tate et al. 2004). The fact that proliferating HUCB-NSC cells are responsive to ECM proteins suggests that they have acquired the appropriate receptors and these are likely to be members of integrin receptor family. In addition, different substrate can transduce signals through preferential activation of multiple integrin-mediated pathways. In agreement with this assumption remain data indicating that integrin-mediated signaling events triggered by cell adhesion to LN

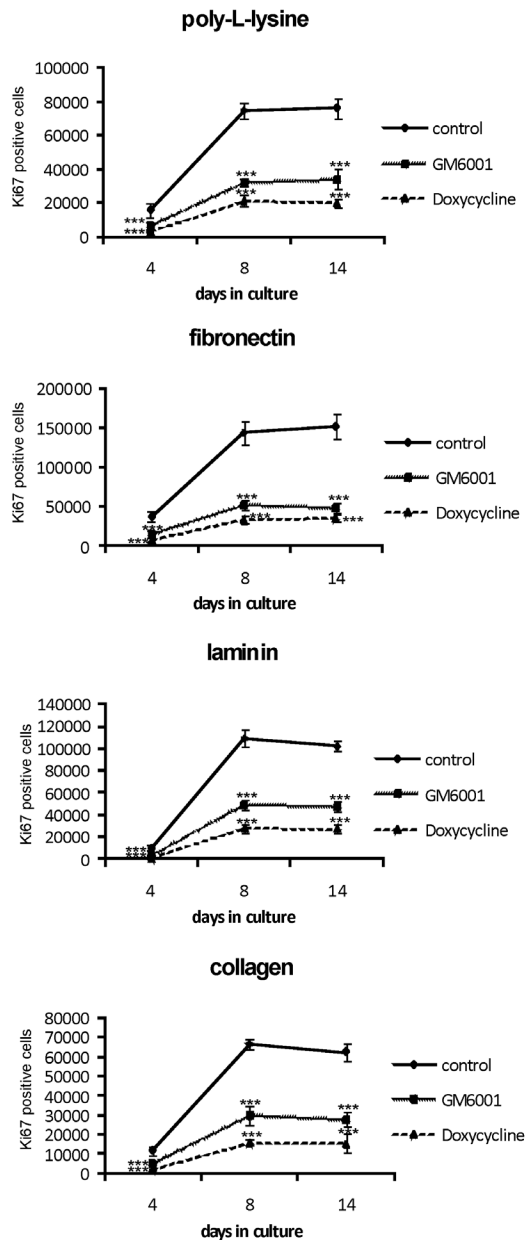


Fig. 6. Effect of GM6001 and doxycycline on the proliferation rate of HUCB-NSCs. Identical numbers of HUCB-NSCs were plated on coverslips coated either with different ECM components or with poly-L-lysine and cultured in serum-free medium without (control) or with MMPs inhibitors - GM6001 (25 μ M) and doxycycline (60 μ M). Graph shows the rate of HUCB-NSCs proliferation expressed as a number of Ki67-immunopositive cells. The addition of inhibitors to the incubation medium decreased the number of Ki67⁺ cells in all experimental conditions. The results (mean values \pm SEM) represent five independent experiments. Asterisks indicate a statistically significant difference versus control. *** p <0.001.

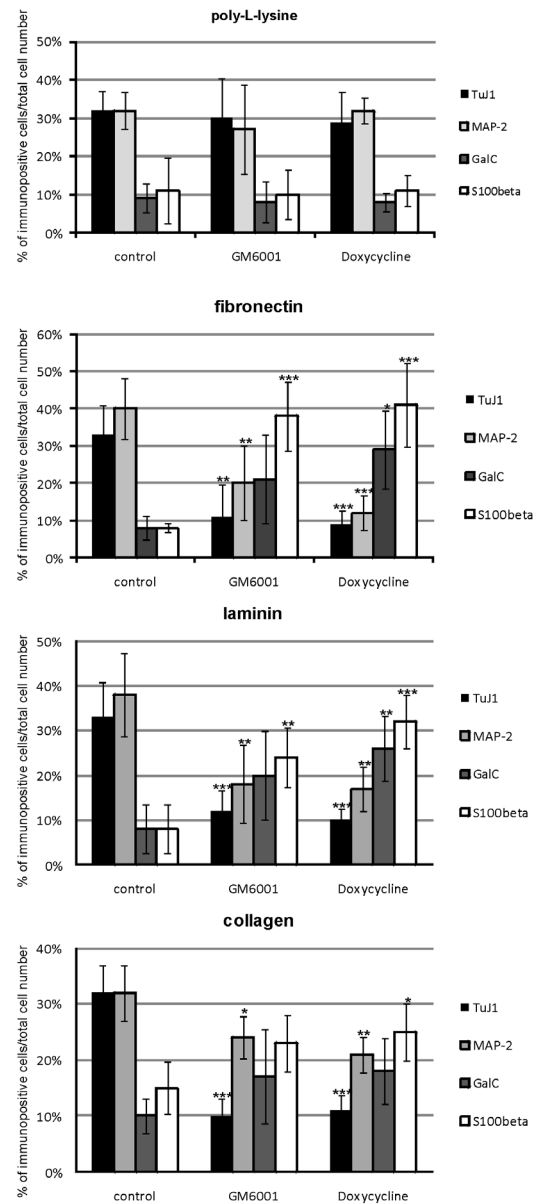


Fig. 7. Effect of GM6001 and doxycycline on the differentiation of HUCB-NSCs. Identical numbers of HUCB-NSCs were plated on coverslips coated either with different ECM components or with poly-L-lysine and grown 14 days in serum-free medium without (control) or with MMPs inhibitors - GM6001 (25 μ M) or doxycycline (60 μ M). The graph presents the percentages of immunolabelled cells relative to the entire population that are present in the culture. Note the decrease of the relative numbers of cells presenting neuronal antigens and simultaneous elevation of the proportion of glial cells in the presence of GM6001 and doxycycline only in the cultures grown on ECM components. The results (mean values \pm SEM) represent five independent experiments. * p <0.05; ** p <0.01; *** p <0.001.

are quite different from those triggered by adhesion to FN (Gu et al. 2002).

There is now ample evidence that external environmental signals must integrate with intrinsic molecular machinery to control the fate choices of individual cells. In this context, enzymes that modify the extracellular matrix and cell adhesion molecules are particularly interesting. The matrix metalloproteinases are one such group of proteinases known to play important roles in the ECM remodeling required for developmental processes. In the course of our study we observed a dynamic evolution of MMP activity that accompanies the progression of proliferation and differentiation of stem/progenitor cells into mature neurons. In our previous work we found activity of MMPs in different phenotypes of HUCB-NSCs - in immature and differentiated neurons, as well as in astrocytes and oligodendrocytes (Szymczak et al. 2009, Sybecka et al. 2009). The relationship between activation of MMPs and generation of developed neuronal cells may suggest a casual link between these processes. These data are in general agreement with the already reported regulatory roles of MMPs during proliferation, differentiation and migration of neural precursor cells isolated from human and rodents central nervous system (Mitra et al. 2005, Mannello et al. 2006, Bovetti et al. 2007, Barkho et al. 2008). The involvement of MMPs in the development of HUCB-NSCs is strongly supported by our further results showing that inhibition of endogenous MMPs activity in HUCB-NSCs growing on ECM substrates significantly reduced the absolute number of cells, as well as their proliferation and differentiation toward neuronal lineage. Simultaneously, the number of oligodendrocytes and astrocytes was elevated compared to the culture in the absence of inhibitors. The decrease efficiency of neural progenitors to differentiate into neurons and promotion of astrocytes number caused by acute reduction of MMPs was also reported by Barkho and coworkers (2008). However, it should be taken into account that the reduced number of mature and immature neurons (TuJ1- and MAP-2 positive, respectively) might likely be related to a lower level of generation thereof, and/or their decreased survival. Unlike neurons, astrocytes are not terminally postmitotic, so some of the increase in their proportion could be due to increased proliferation. Further support to stress the importance of MMPs in neurogenesis as compared with other proteinases stems from the failure of serine proteinase and furin inhibitors (Pefabloc and Dec-RVKR-

CMK) to modulate this process. On the other hand, partial preservation of HUCB-NSCs proliferation in the presence of metalloproteinase's inhibitor – GM6001 or doxycycline, suggests that the neurogenesis-associated processes are not entirely dependent on MMP activity.

Despite accumulating evidence concerning the participation of MMPs in progenitor cells development at present it is not possible to define precisely which of their pleiotropic functions are linked directly to this phenomenon. The most probably scenario and consistent with the established function of MMPs is proteolytic remodeling of the ECM and/or the modulation of other guidance molecules (Nagase and Woessner 1999) needed for progenitor migration. Consistent with this notion, there remain emerging *in vitro* and *in vivo* data pointing to regulatory roles of MMPs in neuroblast migration across tissue matrices (Tsukatani et al. 2003, Lee et al. 2006, Wojcik et al. 2009) as well as during proliferation and differentiation of neural precursor cells after ischemia in rodents (Barkho et al. 2008, Lu et al. 2008). The cleavage of ECM may trigger intracellular signaling that leads to changes in neural progenitors. In addition, the proteolysis-mediated modification of the physical interactions between cell and ECM proteins may uncover cryptic sites of liberate soluble fragments that promote migration (Gianelli et al. 1997, Xu et al. 2001). In the light of the above data it became obvious, that during neurogenesis of HUCB-NSCs, when progenitor cells generate the neurons and glia, altered cell-cell and cell-ECM interaction induced by different degree of inhibition may have profound consequences. However it is not clear which of the suppressed function of MMPs is responsible for the decrease of cells number, for the decline of proliferation rate and altering the pattern of differentiation of HUCB-NSCs. The inhibition of MMPs activity present in neuronal nuclei may change the mechanism responsible for cell growth and expression of genes involved in cell cycle progression (Yarwood and Woodgett 2001, Mannello et al. 2006).

CONCLUSION

In conclusion, our results suggest that metalloproteinases modulate interaction between HUCB-NSCs and extracellular matrix and therefore might be an important component in neurogenesis-associated processes in HUCB-NSCs culture. Further studies are necessary to determine which integrins and downstream signaling

events mediate particular behaviour of HUCB-NSCs. An understanding of the influence of ECM components and their integrin ligands on human HUCB-NSCs may lead to therapies utilizing exogenous neural stem cells.

ACKNOWLEDGEMENT

This work was supported by MSHE grant 0154/B/P01/2010/38.

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