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Aggregation-promoted expansion of neuraly committed human umbilical cord blood progenitors *in vitro*

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Human umbilical cord blood (HUCB) has been established as a promising source of hematopoietic as well as various non-hematopoietic stem cell populations. This offers numerous advantages of HUCB stem/progenitor cells for therapies, however *in vitro* conditions that contribute to long term propagation of proliferating undifferentiated cells have not yet been established. Here we evaluate culture conditions promoting spheroid aggregates/neurospheres formation which, together with serum withdrawal and mitogenes treatments in strictly defined media, maintain population of HUCB progenitor cells in undifferentiated and dividing state exhibiting neurogenic potential *in vitro*. Our results indicate that formation and maintenance of three-dimensional aggregates enhanced by cell culture rotating motion, is crucial for high and prolonged expression of genes and proteins characteristic for cord blood stem cells and their further neural commitment.

Key words: cell aggregation, cord blood, pluripotency, stem cells

INTRODUCTION

It is generally accepted that cord blood is an attractive source not only of hematopoietic but also various non-hematopoietic stem cell populations (Forraz et al. 2004, Leor et al. 2006), which have the ability to express markers and morphologies of other cell types within the same mesodermal germ layer such as bone (Rosada et al. 2003), smooth muscle, and skeletal muscle (Jazedie et al. 2009). Moreover, many research papers have shown different subsets of cord blood cells differentiating also into endothelial and neuroepithelial directions (Zhang et al. 2006, Chua et al. 2009). HUCB derived cells under defined culture conditions can attain phenotypes of multipotent neural progenitors (Buzanska et al. 2002, McGuckin et al. 2004, Buzanska et al. 2006, Ali et al. 2009) and even matured neurons (Sun et al. 2007) exhibiting action potential *in vitro* (Jurga at al. 2009). Furthermore the expression of some marker genes and proteins characteristic for pluripotent cells, like Oct3/4, Sox2 and Nanog have been demonstrated in these cells (Habich et al. 2006). Thus, it is plausible to suggest that the most primitive stem cells residing in HUCB express pluripotency similar to embryonic SCs. But even if cord blood truly contains a subset of cells resembling pluripotent, embryonic-like precursors with respect of their multilineage differentiation potential, there are still obstacles to expanding them *in vitro* and to produce their progeny in quantities needed for therapeutic applications. The reason is that CB progenitors, like any other adult SCs, loose their "stemness" ability to indefinitely self-renewal and growth *in vitro*. On the other hand there is more and more data showing almost unlimited plasticity of stem cells residing in their body niches which can be activated by still poorly recognized condition *in vitro* and even *in vivo* (Bjornson et al. 1999, Theise and Wilmut 2003, Herzog et al. 2003, Tropel et al. 2006, Jablonska et al. 2010). It is also not clear whether exposure to such morphogenic factors would make HUCB progenitors disregard their hematopoietic identity due to epigenetic reprogramming (de- or trans-differentiation) or whether they stimulate direct expansion of preexisting, small pluripotent cell subpopulation, positively selected during purification procedures. Such phenotypic conversion which may involve both genetic and epigenetic mechanisms, although mechanically not yet

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clear, can be correlated with typical molecular switches from pluripotent-like cell pattern to that characteristic of more committed precursors and at last, for mature neuronal cells. Above paradigm of correlative studies between gene expressions, phenotypic transition and cell proliferation/differentiation potential has been applied in this work for defining the most permissive culture condition for producing neural HUCB derivatives for their clinical applications.

Development of standards to effective and repetitious HUCB cell propagation as well as methods of direct expansion of particular lineages according to therapeutic demands, are the main goals of numerous recent studies (McGuckin et al. 2004, Sun et al. 2007, Greschat et al. 2008). Cord blood-derived cells , which are easily accessible, well tolerated after transplantation and with more primitive molecular characteristic resulting in their great flexibility, might be highly appreciated for further preclinical studies.

METHODS

Isolation of HUCB-MNC^{CD34-} cell population

Human cord blood was obtained from full-term deliveries by puncturing umbilical cord stub. Written informed consent with the terms of the ethics committees of the Hematology and Blood Transfusion Institute and Medical Research Institute was obtained from all mothers. Within 12 h after collection a Ficoll/Hypaque density gradient was performed to obtain the mononuclear cells fraction (HUCB-MNC). CD34-negative cell isolation was then achieved via immunomagnetic sorting using mouse monoclonal anti-human CD34 antibody and MACS CD34 MicroBeads (MilteneyBiotek) following the manufactures instructions. HUCB-MNC^{CD34-} cells were suspended in PBS (Gibco) and part of cells was taken directly for PCR analysis and the other part was cultured as described below.

HUCB-MNC^{CD34-} cell culture in serum media

The isolated HUCB-MNC^{CD34-} cells were suspended in DMEM/F12 (Gibco) medium supplemented with 30% fetal bovine serum (FBS, Gibco) and placed in plastic bottles (25cm^2) in a density of 10^7 cells/ml. After 24h serum concentration was reduced to 10%. Thereafter, cell culture was continued to grow in stabilized conditions of 37ºC, 5% CO_2, in a fully humidified atmosphere for 21 days *in vitro* (DIV) with half of media being changed twice a week. Additionally half of the culture flasks were put on rotary shaker and culture in rotation condition (40 rpm).

HUCB-MNC^{CD34-} cell culture in serum free media

The isolated HUCB-MNC^{CD34-} cells were suspended in serum free medium containing DMEM/F12 (Gibco) and mitogenes like EGF and bFGF in concentration 40 µg/ml and 20 µg/ml respectively. Cell culture was continued to grow in stabilized conditions of 37ºC, 5% CO_2, in a fully humidified atmosphere for 21 days *in vitro* (DIV) with half of media being changed twice a week. Additionally after 24 h in static condition half of the culture flasks were put on rotary shaker and culture in rotation condition (40 rpm).

Differentiation of HUCB-MNC^{CD34-} derived from static culture *in vitro*

HUCB-MNC^{CD34-} cultured for 14 days in DMEM/F12 medium supplemented with 10% FBS at 37ºC, 5% CO_2 in static conditions were trypsinized and plated in DMEM/F12 (Gibco) medium containing 10% FBS, at density of 5×10^4 cells/cm^2 on poly-L-lysine-coated cover slips placed in 24-well tissue culture plates (2 cm^2). To promote differentiation 0.5 mM all-trans-retinoic acid (RA, Sigma, St. Louis, MO, USA) and BDNF (Sigma) at concentration of 10 ng/mL were added. After 1 week the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes and washed twice with PBS.

Differentiation of HUCB-MNC^{CD34-} derived from rotation culture *in vitro*

Floating spheroid aggregates from rotation conditions were isolated and placed on poly-L-lysine-coated cover slips (5 - 10 aggregates per well) in 24-well tissue culture plates (2 cm^2). Than aggregates were cultures in standard conditions to attach to the surface. To promote differentiation 0.5 mM all-trans-retinoic acid (RA) and BDNF at concentration of 10 ng/mL were added. After 1 week the cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes and washed twice with PBS.

Table I

Primers for quantitative real-time PCR.

GENE (PROTEIN)	PRIMERE SEQUENCE FORWARD REVERSE	PRODUCT LENGTH (bp)
POU5F1 (Oct3/4)	5'-GTA TTC AGC CAA ACG ACC ATC -3' 5'-CTG GTT CGC TTT CTC TTT CG -3'	176bp
Nanog	5'-CCT GTG ATT TGT GGG CCT G -3' 5'-GAC AGT CTC CGT GTG TGA GGC AT -3'	128 bp
ZEP42 (Rex1)	5'-GCA CAC TAG GCA AAC CCA CC-3' 5'-CAT TTG TTT CAG CTC AGC GAT G-3'	177 bp
Sox2	5'-GTA TCA GGA GTT GTC AAG GCA GAG -3' 5'-TCC TAG TCT TAA AGA GGC AGC AAA C -3'	142 bp
Nestin	5'-TGG CTC AGA GGA AGA GTC TGA-3' 5'-TCC CCC ATT TAC ATG CTG TGA-3'	148 bp
NF200	5'-GAG GAA CAC CAA GTG GGA GA -3' 5'-TTC TGG AAG CGA GAA AGG AA -3'	144 bp
MAP2	5'-AGA CCA CCA TTG ACG ACT CC -3' 5'-TCT CCG AGC TTC CTT TTC AG -3'	131 bp
βactin	5'-TTC TAC AAT GAG CTG CGT GTG -3' 5'-GGG GTG TTG AAG GTC TCA AA -3'	162 bp

Gene detection by Real Time PCR

Total RNA was isolated from HUCB-MNC^{CD34-} after 1, 7, 14 and 21 DIV using the RNAasy Mini Kit (Qiagen Inc). cDNA was synthesized using 1μg of mRNA, 200 units of SuperScript RNAse HŻ Reverse Transcriptase in its associated buffer, 1 μg of oligo-dT, 400 μM of dNTPs and 40 units of RNAseout. Real time PCR was carried out using SYBR Green PCR Master Mix and ABI Prism 7500 Sequence Detection System (Applied Biosystems). Relative expression values (RQ value) were measured for each sample using the delta-delta Ct method. All samples were normalized due to its average Ct values *versus* Ct values for a housekeeping gene (βactin). Also gene's average PCR efficiency was took into account (Pfaffl 2001). Moreover, the gene dissociation curves were performed in each case to confirm the specificity of reactions. Primer sequences (100–200 bp) used for the Real time PCR are shown in Table I.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were blocked for 1 h at room temperature with 10% normal goat serum/0,1% Triton X-100/PBS and then incubated overnight with primary antibodies at 4ºC. The list of primary antibodies and their working dilutions are shown in Table II. After three washes in PBS, cells were exposed to goat anti-mouse IgG1, IgG2a, IgG2b or goat anti-rabbit IgG (HDL) – Alexa 488 or Alexa 546 conjugated secondary antibodies for 60 minutes at RT in the darkness. The adjacent cells, omitting the primary antibodies, served as negative controls. Cell nuclei were stained with Hoechst 33258 (Sigma) for 20 min. After final wash the slides were mounted in Fluoromount-G (Southern Biotechnology Association, Galveston, TX).

Table II

Primary antibodies and their working dilutions.

Antigen	IMMUNOGLOBULIN TYPE/WORKING DILUTIONS	COMPANY
Oct4	MonoIgG1 1:50	Chemicon MAB 4305
Nestyna	MonoIgG1 1:50	R&D Systems Nr Kat.: MAB1259
NF-200	MonoIgG1 1:400	Sigma Nr Kat.: N0142
β-Tubulina III (TUJ1)	MonoI2a 1:500	Covance Nr Kat.: MMS-435P
MAP2	MonoIgG1 1:1000	Sigma Nr Kat.: M4403
GalC	MonoIgG3 1:100	Chemicon MA 342
Ki67	MonoIgG1 1:100	Novocastra Nr Kat.: NCL-L-Ki67-MM1
O4	MonoIgM 1:100	Sigma Nr Kat.: 07139
CD133/1 (AC133)	MonoIgG1 1:50	MACS 130-090-422
S100 β	PoliRabbit H+L 1:1000	Swant Nr Kat.: 37
Doublecortin (N-19)	PoliGoat H+L 1:500	Santa Cruz Sc-8067
GFAP	PoliRabbit H+L 1:300	Cappel Nr Kat.: 10555

Cell counting and statistics

For quantification of the percentage of cells positive for a specific marker in any given experiment, the number of immunopositive cells was determined in relation to the total number of Hoechst-labeled nuclei. In a typical experiment a total of 300 cells were counted per marker. Quantification of marker expression in floating cells/aggregates was performed on cytospined preparations, and counted with the support of Axiovision v. 4.5 (Carl Zeiss) software. Statistical comparisons were made using a one-way ANOVA test followed by Dunnett's test from Graph Pad Prism 5.0.

RESULTS

Maintenance of floating cell aggregates by rotary culture

Our previously observation indicated that formation of cell aggregates in the dense cultures containing 10^7 cells/ml medium, correlates well with inhibition of HUCB cell differentiation (Habich et al. 2006). Persisted maintenance of floating aggregates in culture media can be achieved by employing rotary movement of culture dishes. To study the effect of culture rotation on aggregates formation and cell behavior, 10^7 cells/ml were suspended in culture medium and cultured by 24 hours (Fig. 1) in stationary culture. Figure 1 shows that promoted formation of cell aggregates in the dense culture rapidly induces enhanced expression of typical set of pluripotent (Oct3/4, Sox2, CD133) and neuroectodermal (Nestin, GFAP) genes and proteins. Then the cultures were divided to keep initial cell density and the flasks were placed on orbital shakers for the next 12 days of rotation culture. The cells were grown in serum and serum free media as described in Methods section and monitored under phase contrast microscopy. During this time the aggregates retained floating unlike that in stationary culture, where almost all aggregates become adherent after one week observation (Fig. 2A). After about two weeks, despite of constant rotary motion, the most of cell aggregates started to adhere to the bottom of culture dishes but still were keeping their compact, round structure. Then the single cells started to migrate out from the center of these round cell aggregates successively forming a monolayer (Fig. 2B). Those cells which stayed in the center kept round shaped, while migrating cells differentiate and acquired cigar-like form. In the third week of rotation culture adherent aggregates become smaller and migrating differentiated cells become majority. Also we observed clusters of small round cells growing on the big, flat, egg-like cells, probably observed by others, so called "feeding cells". The rotary cell cultures were growing maximum up to two months, than becoming sparse with the number of undifferentiated cells dropping down sharply (Fig. 2C).

Introduction of rotation movement into the cell culture allowed increase in number of cells and their aggregates and delayed their adhesion and differentiation. This modification also extended the time of culture *in vitro* comparing to stationary condition.

Influence of rotary movement on cell proliferation

After 3 days the cultured HUCB cells were intensively proliferating independently from employing culture conditions. Percentage of cells positive for cell cycle specific (Ki67) marker protein exceeded 80% (Fig. 3) and remained significantly high up to 7 days in culture. After one week in stationary culture amount of Ki67-positive cells decrease rapidly reaching 20% (Habich et al. 2006). Although withdrawal of serum made this pro-

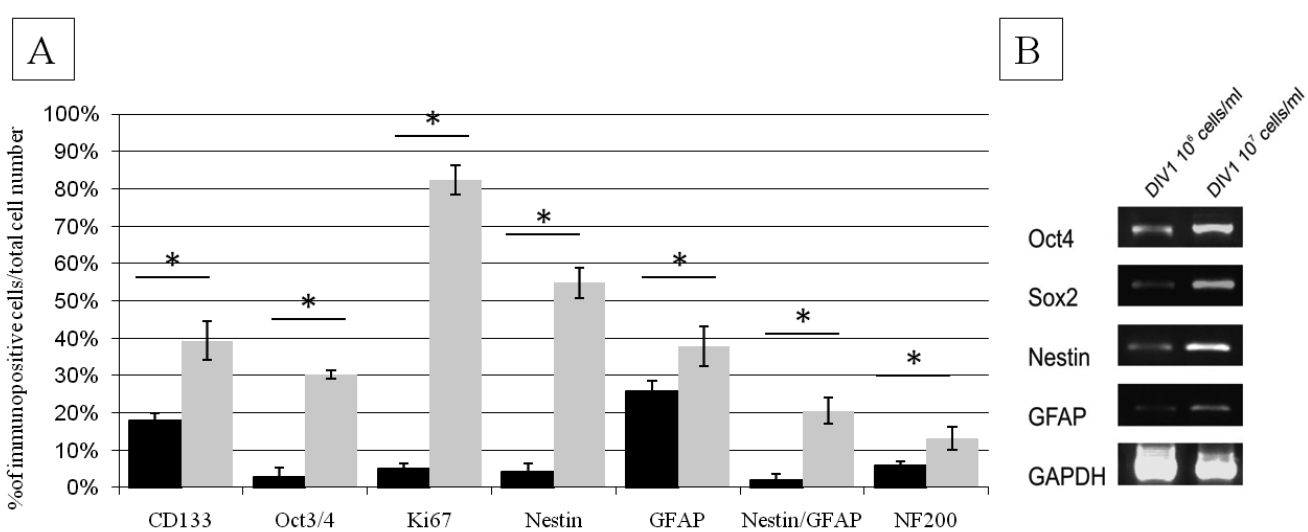

Fig. 1. Initial cell culture density influences on protein (A) and gene (B) expression after 24 hours in culture *in vitro*.

cess goes slower, after 21 days there were almost no proliferating cells also in this culture condition. Under rotation, in contrast, the level of Ki67-positive cells kept high significantly longer. Probably such extension of proliferation was related to the presence of long lasting, floating cell aggregates. Even after 21 days the level of Ki67-positive cells remained significantly higher than that observed in stationary conditions (Fig. 3).

Influence of rotary movement on phenotype acquisition

As we have reported previously, freshly isolated HUCB-MNC^{CD34-} fraction does not express any gene or protein markers for neural lineage-specific progenitors. Instead, they expressed Oct3/4, Sox2, Nanog and Rex1, the approved treads of pluripotent stem cells. Even more, during the first week of culture the level of these markers show tendency to increase regardless of culture conditions. However, the most significant increase was observed in cells cultured in media devoid of serum and kept under rotary movement (Fig. 4). Withdrawal of serum and maintenance of floating aggregates in constant motion allowed the extended Oct3/4 expression remains at high level even for two weeks in culture (Fig. 4). In contrast, in stationary culture expression level of Oct3/4, one of the most important pluripotency gene, was dramatically decreasing and after two weeks any expression signal was observed. Also the level of Nanog, Sox2 and Rex1, the other genes characteristic for pluripotency, were significant higher in the cells from rotary than from stationary cultures (Fig. 4). Similar pattern of expression demonstrated Nestin, neuroectoderm progenitor cells marker. In the stationary cultures level of Nestin expression increasing during first week but further cell culture led to rapid lowering of its level. In contrast, rotary culture allow to further increase of Nestin, continuing this till the second week of culture. In cells cultured in serum-free rotating condition Nestin expression remained high for the longest time and even after 21 days was still observed (Fig. 4). Next, the conversion of HUCB cells to neural phenotypes was monitored by NF200 and MAP2 expression, respectively. After 7 days a significant increase of NF200, the neurofilament characteristic for early neural cells, was observed in all culture conditions. The higher increase however was in cells from stationary than rotary culture. What is important, in the same culture also substantial MAP2 expression was observed. In contrast there was no such induction under rotary conditions (Fig. 4).

This observation of specific gene expression strongly correlates with phenotypic changes in appropriate cultures. Cells in cultures under rotation movement not only remained longer in tight clusters but also reminded undifferentiated and proliferating (Ki67-positive). After two weeks of culture number of Nestin (92.36% ± 3.75%, Fig. 5A), GFAP (44.03% ± 2.35%) and NF200 (50,61 ± 5,22%, Fig. 5B) positive cells in rotation conditions were similar to that found in static cultures. Also percentage of cells expressing doublecortin, a marker of migrating neuroblasts, was relatively high (Fig. 5D). But because of a long lasting maintenance of aggregate structures due to rotation movement and serum withdrawal, even after three weeks the number of cells positive for early neural markers was rather high with Nestin (65,13% ± 5.65%) and GFAP (48.81% ± 0.44%) of total cell number. At the same time, the cells positive for markers of neurons (βTubulinIII 5.80% ± 3.69%) astrocytes (s100β 5.04% ± 1.36%) and oligodendrocytes (O4 1.89% ± 0.04%) appeared exclusively in stationary cultures, whereas in cultures with

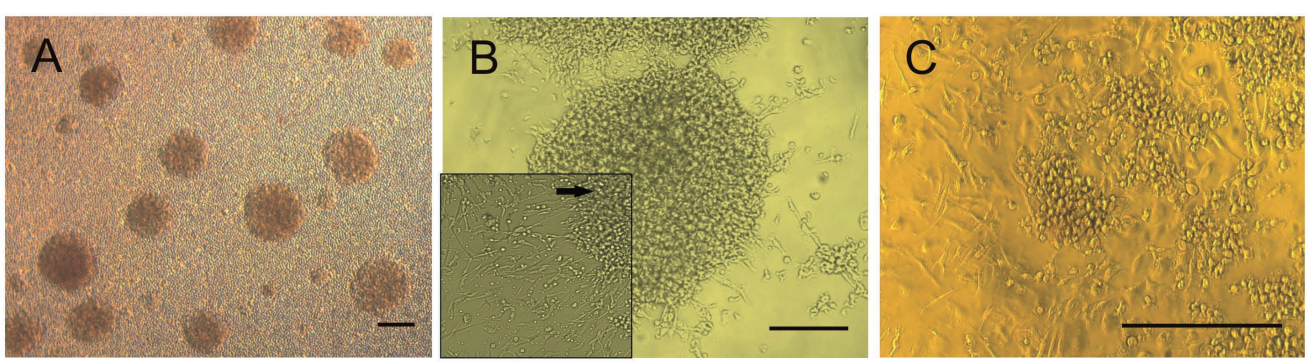

Fig. 2. Human umbilical cord blood stem cells HUCB-MNC^{CD34-} culture in rotation serum free conditions. Floating spheroid cells aggregates (A); cells migrating from the center of the aggregate (B, black arrow shows the aggregate center); undifferentiated small round cells growing on more differentiated cells (C). Scale bars = 50 μm.

rotation movement, even after 21 day βTubulinIII exerted only 2.02% ± 0.90% and any astrocyte or oligodendrocyte markers were find at all (Fig. 5C).

Our observation of neuron specific marker expression like Nestin and NF200 (Fig. 5A, B) as well as proliferation intensity (Ki67) suggest that the aggregates may serve as a niche- like structures for maintenance of undifferentiated cells. Loosing direct cell-cell contacts by migration cause inhibition of proliferation and subsequent cell differentiation.

HUCB cell neural differentiation *in vitro*

As shown previously the rotation cultures conditions promoting aggregates formation support maintenance of undifferentiated neural progenitors in culture *in vitro*. Despite, that described earlier aggregates after 2-3 weeks adhere to the surface, undifferentiated progenitors remains in the center of clusters. Thus to investigate if this compaction of the cells can influence the ability to further neural differentiation, cells aggregates were differentiated in medium supplemented with RA and BDNF. After 7 days in the presence of those neuromorphogenes the undifferentiated cells migrated intensively out of aggregates and acquired long, bipolar shapes. The number of round, Nestin positive cells remaining in the center of the clusters decreased, and the percentage of cells differentiating neurally and positive for NF200 (60.84% ± 3.07%, Fig. 5E), βTubulinIII (58.19% ± 4.28%, Fig. 5F) and Doublecortin (89.43% ± 1.50%) markedly increased as comparing with cultures not treated with neuromorphogenes (Fig. 6). However, only small percent of cells was positive for oligodendrocyte marker GalC (5.04% ± 1.39%, Fig. 5E) as well as for advanced differentiated neuronal marker - MAP2 (Fig. 5F).

The rotation movement and aggregates formation cause increase of neuronal cell differentiation after neuromorphogenes treatment (βTubulinIII 58.19% ± 4.28%) comparing with stationary cultures (18.50% ± 5.05%). What equally important, the cells from stationary cultures differentiated more eagerly toward oligodendrocytes, with GalC positive cells consisting even 21.64% ± 1.56% of total cell number.

DISCUSSION

Previous results revealed that cord blood contains subpopulation of undifferentiated ES-like cells which under defined culture conditions attain phenotypes of multipotent neural progenitors (Habich et al. 2006, McGuckin et al. 2009, Kucia et al. 2007). In the present study we have examined influence of the 3D- cell aggregate formation on proliferation and long lasting culture of undifferentiated stem/progenitor cells *in vitro*.

Freshly isolated HUCB-MNC fraction negatively selected for CD34 antigen and cultured in aggregates promoting conditions revealed higher number of undifferentiated progenitor cells belonging to so called "side population" than cells from non-aggregating cultures (Habich et al. 2006). Further experiments performed in this work, revealed that cells in aggregates, especially that from serum free cultures, remained longer undifferentiated and their expression of stemness genes, like Oct3/4, Nanog and Sox2 is increased comparing to single cells culture. Moreover proliferation potential of aggregating cells is significantly higher than single cells.

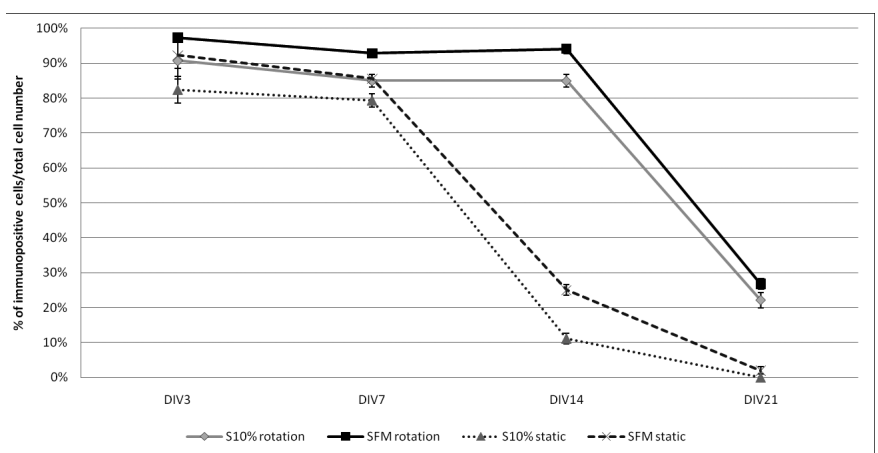

Fig. 3. The rate of proliferation (Ki67 positive cells) of HUCB-MNC^{CD34-} culture *in vitro* for 0-21 days in four different type of culture conditions – comparison between static and rotation culture conditions.

What is interesting, the most intense phenotypic changes were noticed on the edges of the aggregates whereas the centre remained undifferentiated. This effect was also observed previously in umbilical cord blood neural stem cells line HUCB-NSC. Buzanska and coworkers described that in serum free conditions cells from this line form floating aggregates with characteristic zonal structure resembling neurospheres (Buzanska et al. 2006, Jurga et al. 2006). It is assumed that this zonal structure of aggregates/neurospheres creates the essen-

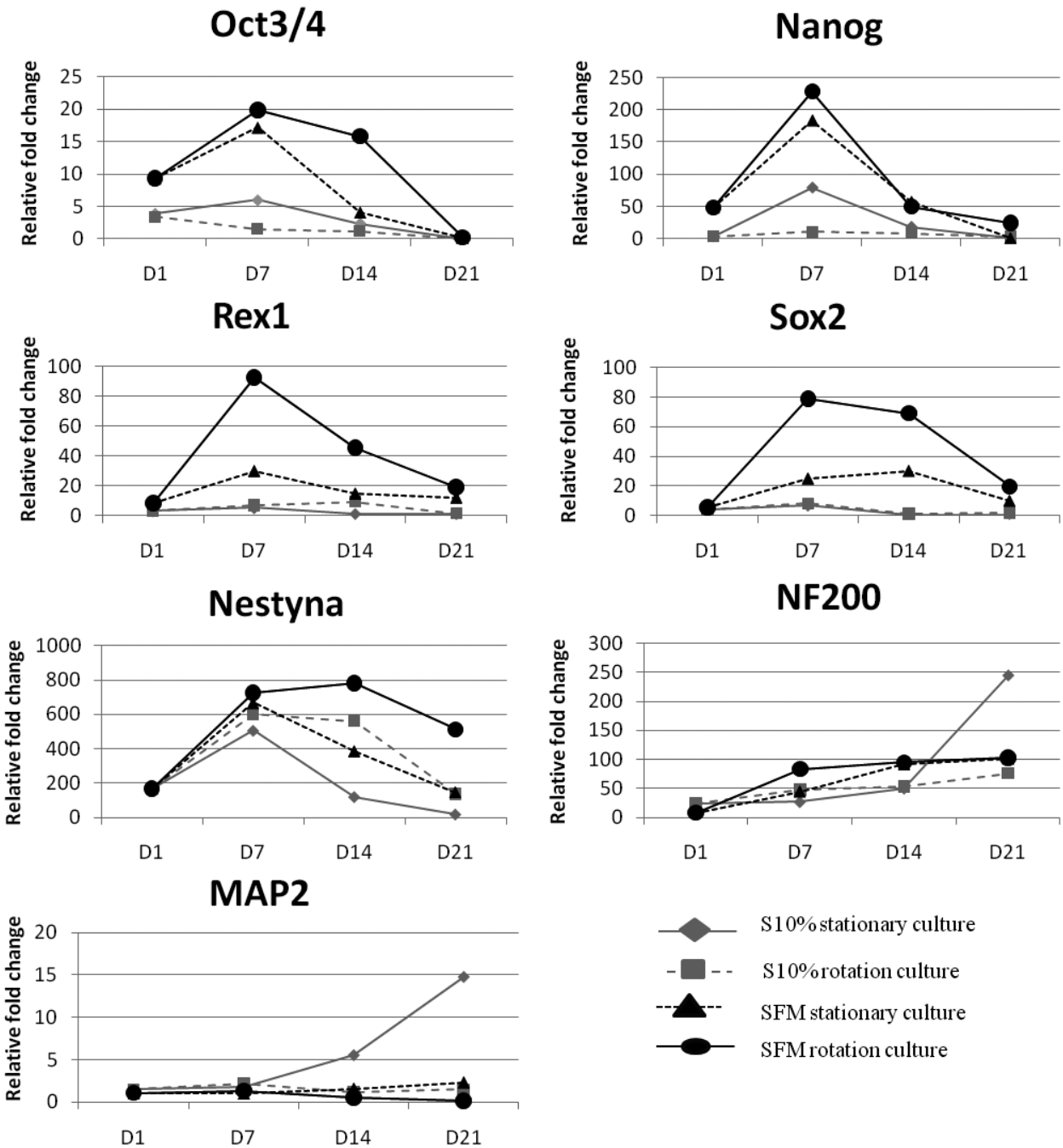

Fig. 4. Real Time PCR analysis of relative gene expression changes during 21 days of HUCB-MNC^{CD34-} culture *in vitro*. The graphs show relative fold change between controls sample and sample of interest, in static culture conditions comparing to rotation culture conditions.

tial microenvironment for maintenance of a constant number of undifferentiated stem cells. Presumably this phenomenon is caused by interactions between progenitor cells on different level of their neural commitment. This effect is also well described in tissue stem cells niches (Doetsch 2003, Fuchs et al. 2004). Unfortunately the three-dimensional aggregates *in vitro* adhere and differentiate spontaneously within a few days after their formation in HUCB-MNC cultures in contrast to neurospheres formed from NSC line. Although serum withdrawal can increase proliferation rate and maintenance of floating undifferentiated spheres though it doesn't stop further differentiation but only make this process go slower. Simultaneously with adhesion of aggregates the expression of stemness genes is inhibited together with activation of differentiation genes like NF200 and MAP2, what results in simultaneous dramatic decrease in stem/progenitor cells number. Thus formation and maintenance of spheroid aggregates in culture appears to be crucial for retaining HUCB in proliferative state.

Orbital rotary movement have been used to create spheroid aggregates from different cell types such as hepatocytes (Hamilton et al. 2001), neural stem cells (Mahoney and Saltzman et al. 2001), mouse embryonic stem cells and even glioblastoma cells (Witusik-Perkowska et al. 2010) since the constant circular motion can generally increase the efficiency of aggregates formation (Carpenedo et al. 2007). In order to increase aggregation and decrease adhesion of already formed spheroid aggregates HUCB-MNC^{CD34-} were culture in moderate rotation.

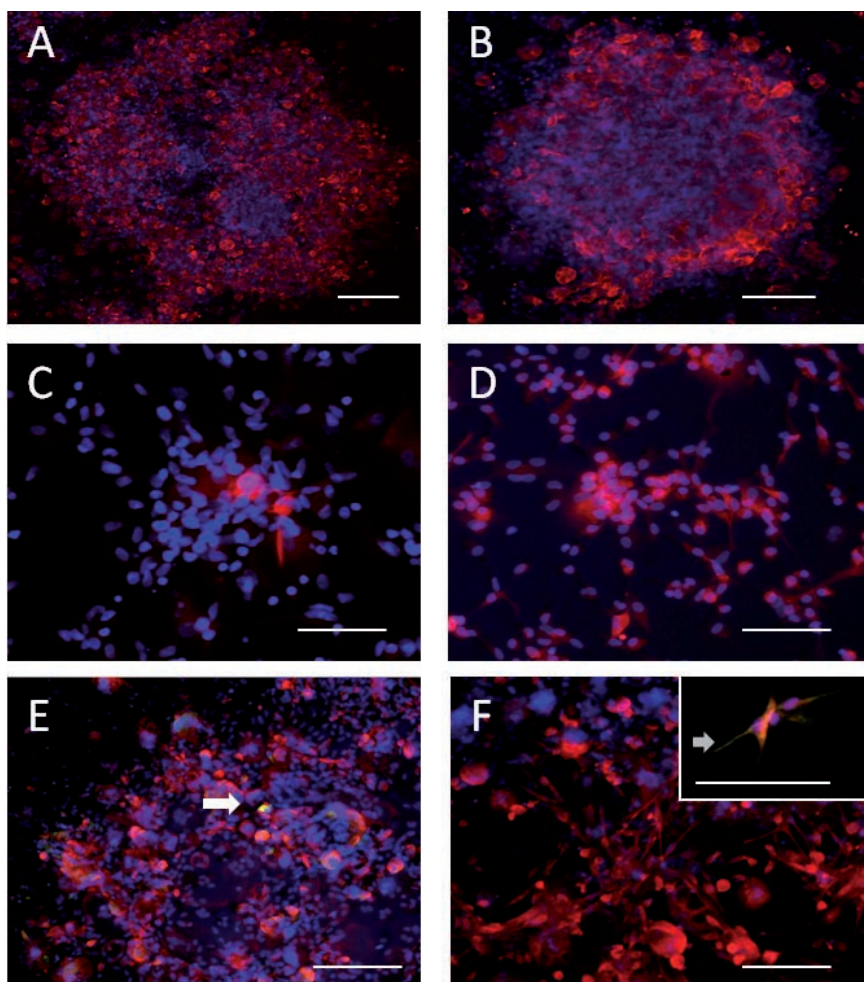

Fig. 5. Immunostaining of HUCB-MNC^{CD34-} aggregates in serum free rotation culture before and after neural differentiation *in vitro*. Cells after 14 days in serum free rotation culture expressing Nestin (A), NF200 (B) and after 21 days expressing βTubulinIII (C) and DCX (D). Immunostaining of neurally differentiated aggregates from serum free rotation culture *in vitro*. Double-staining for NF200 (red) and GalC (green) – white arrow (E), bipolar βTubulinIII (red) positive cells (F) and differentiated neuronal cells positive for βTubulinIII (red) and MAP2 (green) – green arrow (F - insert). Cells nuclei were stained by Hoechst 33252 (blue). Scale bars = 50 μm.

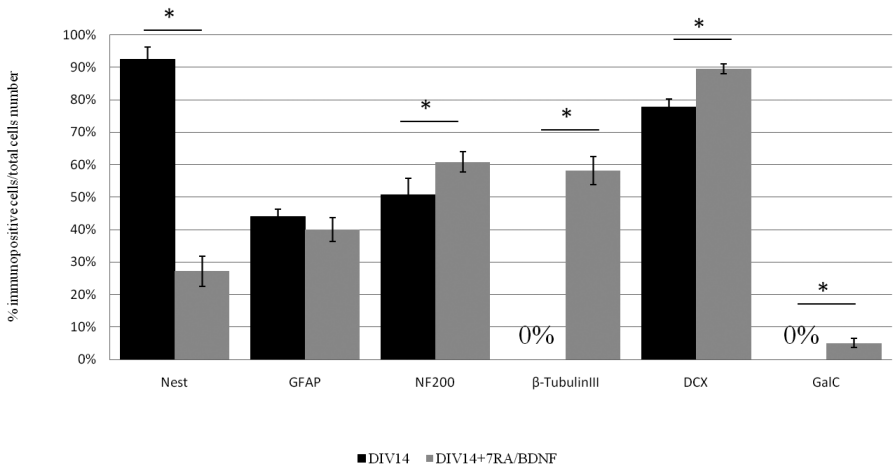

Fig. 6. Frequency of early neural marker expression in aggregates derived from 14-DIV HUCB-MNC^{CD34-} serum free rotation culture *in vitro* followed by 7 days culture in the presence of RA and BDNF. The results are expressed as the mean±s.d. of cell number from three independent cultures.

This modification of culture conditions allowed to initial increase of the spheres number and also facilitated aggregates to stay floating longer comparing to static culture conditions. The number of proliferating cells was also significantly higher in aggregating than in single cell cultures. Moreover spheroid structure influences not only on the number of proliferating cells but also on extension of a period that they can stay in proliferative state during culture *in vitro*. Stationary cultures exhibited the maximum of proliferation not longer than by 7 days. In contrast cells from cultures with aggregation promoted by rotation and serum withdrawal maintain high proliferation rate significantly longer, even more than two weeks. Furthermore maintenance of cells in spheroid structure caused extension of simultaneous expression of stemness (Oct3/4, Nanog) and neural progenitor genes. Probably these close cell to cell contacts characteristic for aggregates also cause inhibition in expression of genes characteristic for differentiation process, like NF200 and MAP2. This extended expression of stemness genes, especially Oct3/4, the key regulator of undifferentiated stem cells state, indicate that aggregating condition promotes maintenance of stem cells population.

Exposing these spheroid aggregates on neuromorphogenes, like RA and BDNF, cause differentiation of cells toward neural lineages. But comparing to static culture more cells from aggregates reveal the immunoreactivity for βTubulinIII and even MAP2, pointing out on differentiation toward neurons. In contrast. more cells from non-aggregating conditions exhibit glial features (GalC-positive cells).

Summarize the number of undifferentiated but neutrally committed progenitors was higher in rotating cultures due to intense proliferation (Ki67-positive cells) and slower rate of differentiation. Importantly, despite longer maintenance in undifferentiated state the cells from aggregation promoting conditions retained well their differentiation potential toward neurons.

This data and our previous experiments (Buzanska et al. 2006) indicates that there is possibility to obtain neutrally committed culture of stem/progenitors cells derived from human umbilical cord blood exclusively by altering environmental conditions without additional molecular modifications like gene transfers.

ACKNOWLEDGMENTS

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