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Neural commitment of cord blood stem cells (HUCB-NSC/NP): Therapeutic perspectives

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Abstract. Human umbilical cord blood (HUCB) is considered a promising source of neural progenitors capable of being used for cellular therapies in neurological disorders. Here we review briefly our work on the elucidation of mechanisms and development of practical standards as regards the selection, maintenance and use of cord blood derivatives for such purposes. Our results join those of other recent studies in suggesting strongly that, the generation of neural-like cells from tissue belonging to a different germ layer (such as a cord blood is) is most probably explained by reference to a discrete subpopulation of embryonic-like stem cells of pluripotent characteristics. Such cells identified in cord blood through their expression of specific genetic and protein markers can be expanded *in vitro* and directed toward neurally-committed progenitors differentiating further into more mature neuron-like or macroglia-like cell phenotypes. From this HUCB-derived neural progenitor fraction a novel neural-like stem cell line (HUCB-NSC) has been developed, and characterized in respect of *in vitro* and *in vivo* (post-transplantation) properties.

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INTRODUCTION

Umbilical cord blood can be a rich source of stem/progenitor cells, not only for hematopoietic, but also for other tissue-specific lineages. The fact that non-embryonic stem cells can and will perform such "miracle" functions is compelling, because it would seem to offer a way round the ethical problems associated with the use of embryonic stem cells, and because the therapeutic effects exerted in preclinical experiments by this particular kind of adult stem cell have been looking very promising. During the last few years, many consistent and incessant reports have been confirming *in vitro* cord blood cell conversion into different organ-specific cell phenotypes, including that of the central nervous system (Buzanska et al. 2002, Ha et al. 2001, Kogler et al. 2004, Sanchez-Ramos et al. 2001), as well as similar activity *in vivo* (Lu et al. 2002, Zigova et al. 2002). Concomitantly, the remarkable attenuation of symptoms achieved in experimental models of neuronal diseases (ALS, PD, HD, ischemia or spinal cord trauma) in response to cord blood infusion or transplantation has strengthened expectations as regards assessment of the therapeutic effects this kind of stem cell can afford. However, in spite of successful first clinical treatments (Bang et al. 2005, Mazzini et al. 2003), there remains much doubt in regard to both the effectiveness and the safety of using stem cells in general for human therapy. Thus, a detailed definition of the way in which cord blood-derived cells exert their neuroprotective functions, as well as an understanding of the exact mechanisms underpinning their change of destination, would be decisive in any further development of therapeutic strategy. However, the isolation and characterization of multipotent stem/progenitor cells from cord blood have been hampered by their relative paucity, as well as by the constant lack of stem-specific surface markers allowing for their prospective and controlled selection. As a result, successful strategy as regards the isolation of neural progenitors continues to rely mainly on poorly-defined physicochemical cell properties, such as adhesiveness to solid surfaces, density-dependent cell-to-cell contacts, sensitivity to mitogens and extracellular matrix protein signals and the promotion of neural differentiation by exogenously-provided neuromorphogenes and growth factors. Equally, defined mechanisms involved in decisions concerning the fate of cord blood cells are only beginning to be derived. A brief characterization of the procedures allowing us to select neural progenitors, as well as to develop an indefinitely self-renewing, neural-like stem-cell line (HUCB-NSC) from the human umbilical cord mononuclear fraction is presented and discussed here, in the context of cells' possible future utilisation in therapy.

METHODS

Isolation of the HUCB-NSC/NP cell population

Human cord blood was obtained from full-term deliveries by puncturing the 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. The mononuclear cells (HUCB-MNC) were isolated on a Ficoll/Hypaque density gradient within 12 h of collection. The total HUCB-MNCs were labeled with mouse monoclonal anti-human CD34 antibody (4°C, 30 min). Cells were subsequently labeled using MACS CD34 MicroBeads. CD34-negative cell isolation was then achieved *via* immunomagnetic sorting of the MiniMACS (MilteneyBiotek), following the manufacturer's instructions. After washing, HUCB-MNC^{CD34-} cells were suspended in PBS.

In separate experiments, the HUCB-MNC^{CD34-} cells were frozen in PBS medium containing 10% DMSO, with or without 50% FCS. The samples were stored in a deep-freezer at –70°C. Two weeks later, the cells were thawed rapidly in a 37°C water bath. Once washed, they were diluted in culture medium containing 30% FCS, and cultured for 24 h in stabilized conditions of 37°C and 5% CO_2 in a fully humidified atmosphere.

HUCB- NSC/NP cell culture

The isolated HUCB-NSC/NP cells were suspended in DMEM/F12 (Gibco) medium, as supplemented with 30% fetal bovine serum (FBS, Gibco) and placed in 24-well culture plates at a density of 7–8 × 10^6cells/ml. Then, after 24 h (the time needed for cells to form dense aggregates), serum concentration was successively reduced by exchanging half of the media for DMEM/F12 containing: 10% FCS, 5% FCS or 5%

FCS + EGF every second day. Thereafter, cell culture was continued to grow in stabilized conditions of 37°C and 5% CO_2 in a fully humidified atmosphere up to 7 days *in vitro* (DIV).

RT-PCR analysis

Total RNA was isolated from the CD34-negative HUCB mononuclear cell fraction after 7 days of culture, by using the RNAeasy mini-kit (Qiagen). Reverse transcription was achieved in 20 µl total volume using 1 µg of RNA. PCR was carried out in a 25 µl reaction using 1U Taq DNA Polymerase (Fermentas) and an identical amount of cDNA per reaction with 1 µM of forward (F) and reverse (R) primers. The PCR products were separated by electrophoresis on 1% agarose gel in 0.5 × TBE buffer, before being visualized with ethidium bromide staining. Gel images were acquired with a GelExpert 4.0.

Labeling of HUCB-NSC

HUCB-NSCs were transected transiently with pEGFP-N1 vector. Adherent and floating fractions of HUCB-NSC were collected by trypsinization. Approximately 10^6 cells were suspended in 400 µl of DMEM/F12 medium and placed in the electroporating cuvette. 20 µg of DNA pEGFP-N1 was then added. Electroporation was performed at 250 V and 950 µF, using a Gene Pulser II (Bio-Rad) electroporating system. Immediately after having received the electric pulse, the cuvette was placed on ice for 10–15 min to chill the cells. Cells were then moved to low-serum medium and incubated under standard culture conditions for 24 h, prior to transplantation to a hippocampal organotypic slice culture. Efficiency of transfection was about 30–35%, and EGFP protein was detected for up to 2 weeks without significant loss of fluorescence.

Organotypic hippocampal culture

Hippocampal slices were prepared from 7–10 day-old Wistar rats in line with the method of Stoppini (Stoppini et al. 1991), as slightly modified in our laboratory. After brief anaesthesia with Vetbutal (pentobarbital; Sigma), ice-cooled pups were plunged into 70% alcohol solution and decapitated with scissors, before their brains were quickly removed to ice-cold HBSS (Gibco). The desired brain areas were separated and cut into 400 µm slices using a McIlwain tissue chopper. Slices were transposed to Millicell-CM (Millipore) membranes, four slices on each. Millicell-CM membranes in 6-well plates were pre-equilibrated with 1 ml of culture medium (pH 7.2; DMEM 50%, HEPES, HBSS 25%, Horse Serum 25%, 2 mmol/L L-glutamine, 5 mg/ml glucose, 1% amphotericine B and 0.4% penicillin-streptomycin). Cultures were started in a horse serum-containing medium, which was gradually replaced between days 4 and 7 in culture by a serum-free, defined solution based on DMEM–F12 and including the N2A and B27 supplements. Culturing was took place in a moist atmosphere of air and 5% CO_2, at 36°C for 14–16 days (Gähwiler et al. 1997).

HUCB-NSCs were transplanted to hippocampal organotypic cultures cultured for 7 days, once medium had already been replaced by the serum-free equivalent. HUCB-NSCs transfected with the EGFP gene 24 h before transplantation were trypsynised (adherent fraction) and mixed with the floating fraction. Subsequently, approximately 10^5 of HUCB-NSCs were transplanted all over the surface of the HOC using a 10 µl micropipette. Slices were then cultivated for 1 week at 36°C, in an air + 5% CO_2 atmosphere of 100% humidity.

Immunocytochemistry

HUCB-MNC/NP PRIMARY CULTURES

The primary antibodies used in identifying cell phenotypes were: mouse monoclonal antibodies directed against human Oct 3/4; CD133; CD45; Nestin; GFAP; and Ki-67. Before incubation with primary antibodies, non-specific binding was blocked with 10% normal goat serum (NGS, Sigma) diluted with 0.1% Triton X-100 at RT for 60 min. The cells were then incubated with primary antibodies overnight at 4°C. After washing with PBS, cells were exposed to goat anti-mouse IgG1, IgG2a, IgG2b or goat anti-rabbit IgG (H+L)-Cy3 – FITC or Texas Red conjugated secondary antibodies for 60 min at RT in the darkness. The adjacent cells omitting the primary antibodies were served as negative controls. Cell nuclei were counterstained with 5 µM Hoechst 33258 (Sigma) for 20 min. After final washes, the slides were mounted in Fluoromount-G (Southern Biotechnology Association).

HIPPOCAMPAL SLICES

Hippocampal slices were fixed in 4% paraformaldehyde for 24 hours. After washing in PBS, slices were cut out from the membrane and moved to PBS in a 24-well culture plate. Free floating slices on the pieces of membranes were permeabilized with 1% Triton X-100 for 15 minutes and blocked with 5% NGS or 5% BSA (for anti-Nestin staining), diluted in PBS. All blocking media were applied for 1 hour at RT. The primary antibodies applied were: monoclonal antibodies anti Ki67 (IgG1, 1:100), anti MAP2 (IgG1, 1:1000), Nestin (IgG1, 1:50), TUJ1 (IgG2a, 1:1000), NF200 (IgG1, 1:400), GalC (IgG, 1:10), O4 (IgG, 1:10), and polyclonal antibodies anti GFAP (1:200), all over-night at 4°C. Secondary antibodies applied following a wash in PBS were: anti mouse IgG1 for MAP2, Nestin, NF200, anti mouse IgG2a TUJ, anti mouse IgG3 GalC, anti mouse IgM O4 and anti rabbit IgG-Cy3 GFAP. All secondary antibodies were conjugated with Texas Red and were applied for 60 min. Cell nuclei were stained with 5 mM Hoechst 33258 for 30 min. As a control, first antibodies were omitted during immunocytochemistry staining.

CELL COUNTING AND STATISTICS

For quantification of the percentage of cells expressing a specific marker in any given experiment, the number of positive cells was determined relative to the total number of Hoechst-labeled nuclei. In a typical experiment a total of 1000 cells were counted per marker. Quantification of marker expression in floating cells/aggregates was performed on cytospined preparations, and counted with the support of KS-300 (Zeiss) software. Statistical comparisons were made using a one-way Anova test followed by Dunnett's test from Graph Pad Prism 3.02.

RESULTS AND DISCUSSION

Transition of cord blood mononuclears toward proneural phenotypes

Our group resembles others in showing that cells derived from HUCB under defined *in vitro* conditions acquire neural features (Bicknese et al. 2002, Buzanska et al. 2002, McGuckin et al. 2004, Walczak et al. 2004, Zigova et al. 2002). However, it was not clear whether exposure to specific factors *in vitro* makes these cells disregard their hematopoietic identity due to overlapping genetic programs in the mechanism called transdifferentiation (Abkowitz 2002, Jiang et al. 2002, Korbling et al. 2002, Poulsonn et al. 2002, Terskikh et al. 2001), or whether they acquire new features due to directed expansion of a discrete, preexisting primitive pluripotent stem cell subpopulation (McGuckin et al. 2005). Thus, the shift of hematopoietic phenotype toward other lineages is still under discussion. Recently we have developed a method for the expansion and prospective selection of neural stem/progenitor-like cells from the HUCB non-hematopoietic (CD34- negative) mononuclear fraction (Habich et al. 2006). HUCB-MNCs negatively selected for CD34 antigens were cultured *in vitro* for up to 14 days. Phenotypic switching and changes in stem/neural cell gene and protein expression were evaluated up to this time point and after neuronal differentiation promoted by the presence of RA/BDNF for an additional 7 days.

The freshly-isolated HUCB-MNC^{CD34-} fraction does not express any gene or protein markers for lineage-specific progenitors. Instead, genes believed to be master regulators of the pluripotent stem state, i.e. Oct3/4, Sox2 and Rex1, have been expressed in this naive cord blood isolate. After 24 h of cell culture in the conditions specified in "Methods" section, comparative immunochemistry showed that the frequency of Oct3/4- and CD133-positive cells increased markedly, with a parallel increase in cell proliferation (Ki67 labeling) and with 10-fold enlargement of the "side population" (as measured by high Hoechst 33342 efflux) – which is the other approved stem/progenitor cells marker. Concomitantly, cultured cells start to form aggregates and further express pro-neural genes, i.e. enhanced Sox2, OTX1, Nestin, GFAP and NF200. It is of note that the expansion of SP cells and induction of proneural genes were not observed in parallel cultures grown in non-aggregating conditions (Habich et al. 2006). During the next few days, immunoreactions for β-tubulin III, MAP2, GFAP, S100ß, Doublecortin and GalC were induced with reciprocal lowering of all of the "stemness" markers. At this stage, cells successively adhered to the bottom, dispersed and became subject to a decreased proliferation rate (Ki67 expression). Additional treatments with neuromorphogenes or co-culturing with rat-brain primary culture induced further differentiation of these neural precursors toward more advanced neuronal phenotypes in a majority of cells (>80% of total cell number). However, gradual depletion of Oct3/4, Rex-1, Sox2 and

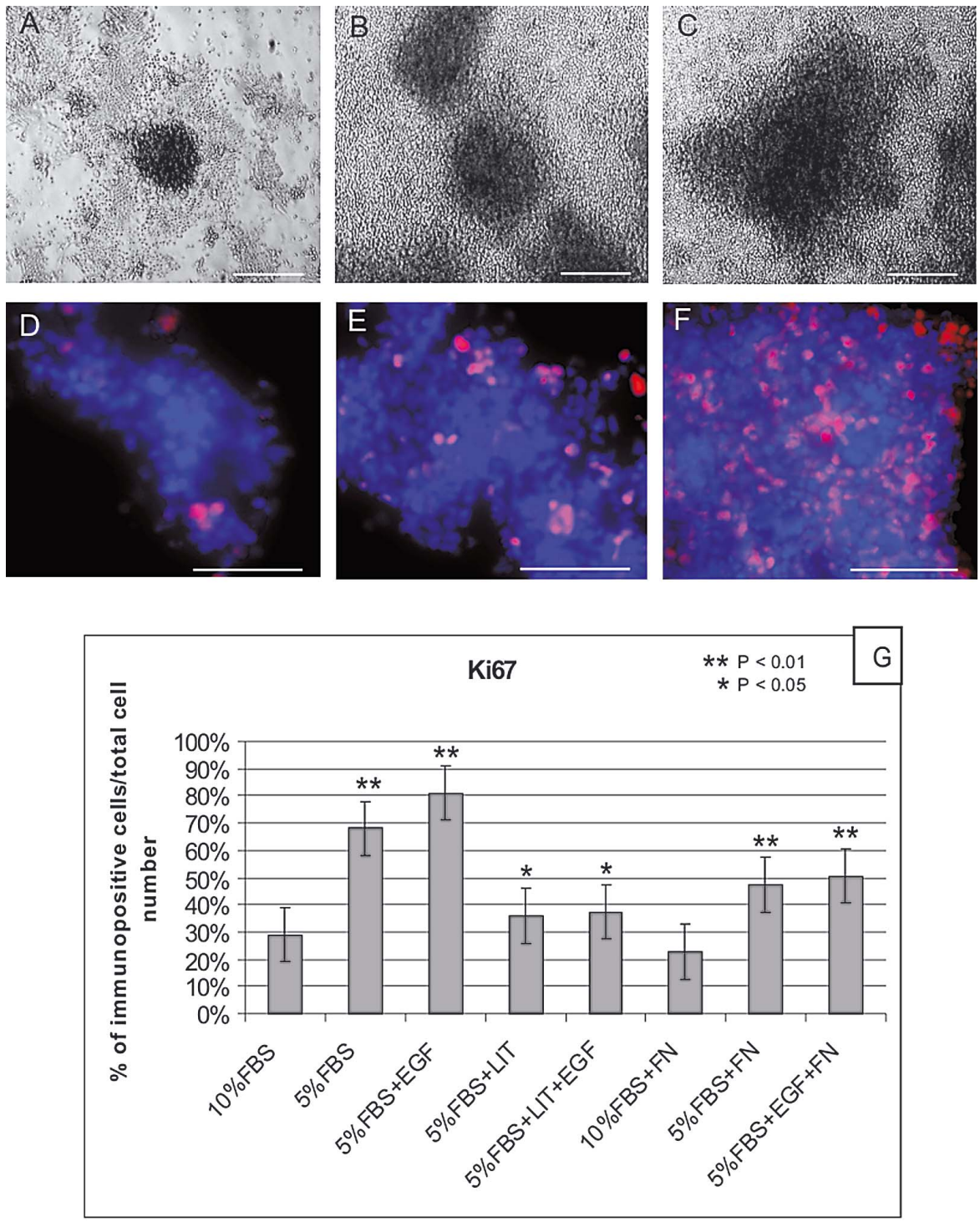

Fig.1. Visualization of HUCB-MNC/NP cultured for 7 days in different culture conditions. Phase contrast images of HUCB-MNC/NP cultured in: (A) 10% FBS + DMEM/F-12; (B) 5% FBS + DMEM/F-12; (C) 5% FBS + DMEM/F-12 + EGF. Immunostaining for Ki67 of HUCB-MNC/NP cultured in: (D) 10% FBS + DMEM/F-12; (E) 5% FBS + DMEM/F-12; (F) 5% FBS + DMEM/F-12 + EGF. Cells stained with Texas Red (red) were detected simultaneously and together with their nuclei revealed by use of Hoechst 33252 staining (blue). Scale bars are 100 μm. (G) The rate of HUCB-MNC/NP proliferation (percentage of Ki67-positive cells) cultured *in vitro* for 7 days in different culture conditions on plastic surfaces and fibronectin (FN)-coated coverslips.

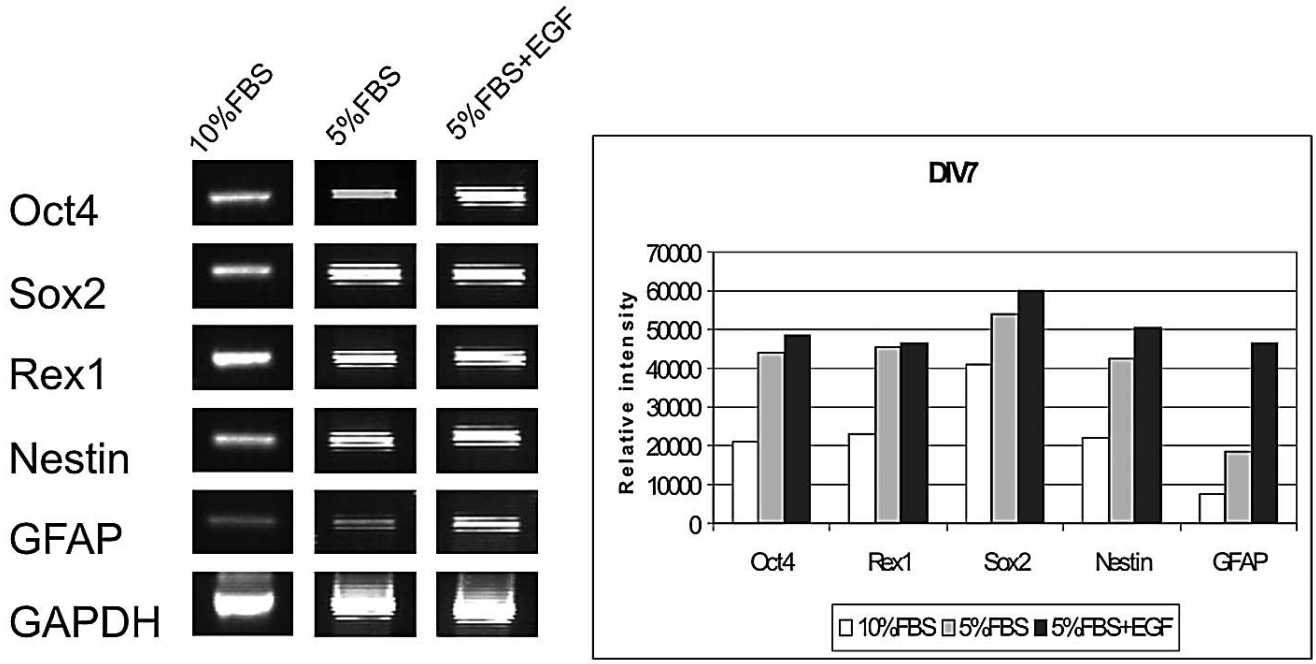

Fig.2. RT-PCR analysis of HUCB-MNC/NP cultured *in vitro* for 7 days (DIV7) in different culture conditions. Evaluated genes are shown (left panel) with representative gene-expression changes (right panel).

Ki67 clearly indicates the onset of domination by the relative proportion of newly-formed, neural cells over the uncommitted, self-renewing and proliferating stem/progenitors (HUCB-NS/P), leading to their successive depletion and dilution *in vitro*.

The ongoing experiments presented here are focused on further definition of the environmental (epigenetic) signals, which are able to enhance and/or prolong self-renewal and proliferation of HUCB-NP *in vitro*. The isolated cells were grown at a low concentration of FBS, and treated with EGF (40 ng/ml) or lithium (1 mM), or grown on fibronectin-coated surfaces. We have estimated the percentage of cells entering the cell cycle using Ki67 immunoreactivity analysis (Fig. 1), as well as concomitant measuring of the level of stem/proneural gene expression by PCR analysis (Fig. 2). The results show that a lowering of the FBS concentration to 5% plus the addition of EGF to the culture medium was able to prolong high expression of Oct 3/4, Sox2 and Rex1, as well as enhancing expression of nestin and GFAP genes. In contrast, lithium and fibronectin counteract the stem/neural progenitor-specific gene expression, leaving them at levels similar to that observed in 10% FBS medium alone.

Also assessed here is whether routine banking procedure for HUCB-MNCs could affect these cells' ability to express the pluripotent SC phenotype. As Fig. 3 shows, cryopreservation of HUCB-MNC did not affect the cells' viability (>80%), or the frequency of hemathopoietic progenitor cells phenotypes as assessed by CD45 expression. In contrast, this procedure almost completely depleted the population of cells expressing the most primitive SC phenotype markers (Oct 3/4, CD133). Surprisingly, the number of cells reacting positively with NSC markers such as nestin or GFAP did not change significantly, whereas the formation of early aggregates was substantially hampered in cryopreserved isolates. These data, if confirmed, may suggest that routine freezing is detrimental to the maintenance of the cell fraction directly responsible for HUCB-MNC pluripotency.

Overall, these studies – together with those reported in our recent paper (Habich et al. 2006) – allow for a conclusion that cord blood includes a minute subpopulation of primitive, uncommitted cells (embryonic-like progenitors), which can in a context-dependent manner expand and give rise to *in vitro* phenotypes belonging to a neural lineage. Moreover, the proliferation/expansion of this primitive cell subpopulation seems particularly sensitive to environments that are either positive (EGF) or negative (cryopreservation procedure).The precise definition of these will be a matter for further study.

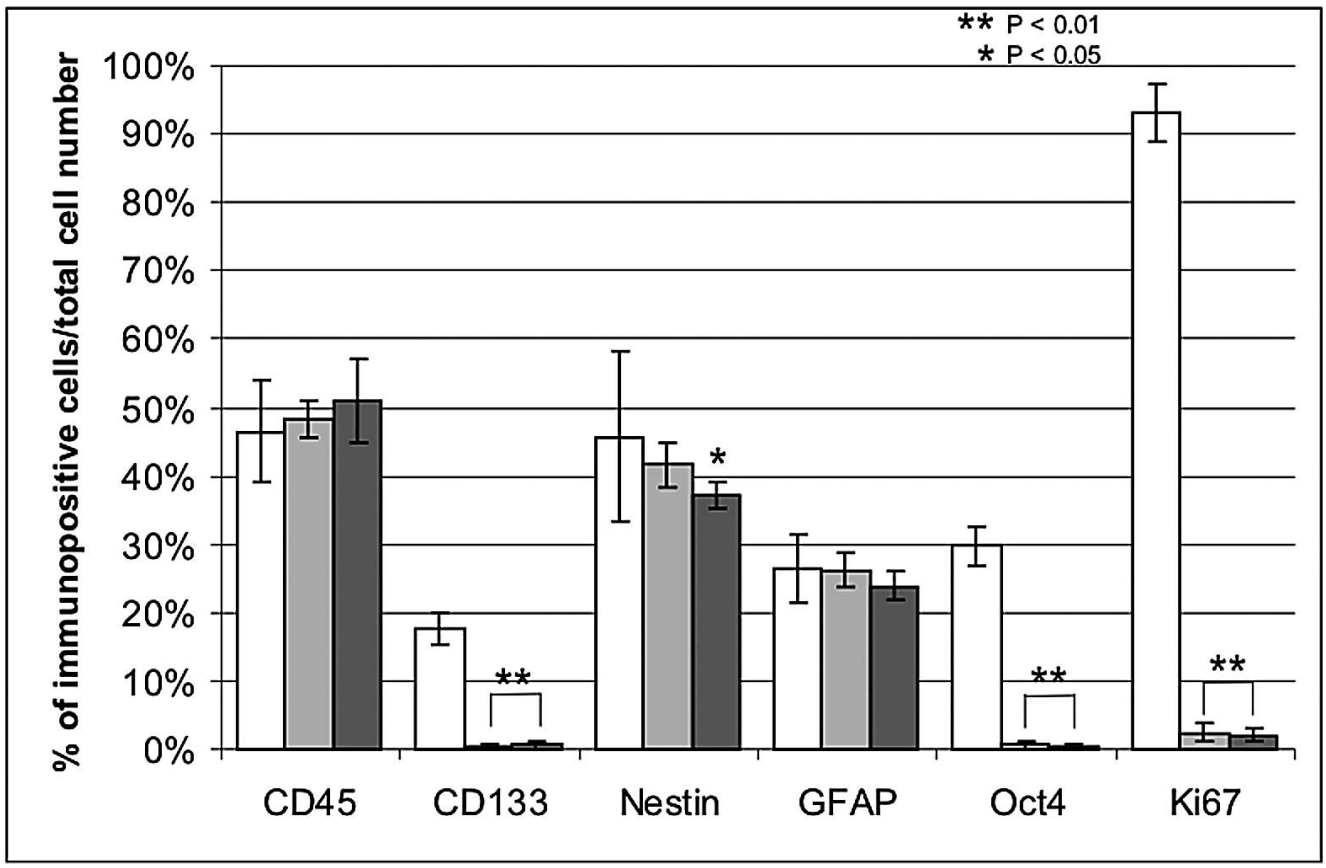

Fig.3. Immunohistochemical characteristic of control, not frozen (open bars) and cryopreserved HUCB-MNC populations after 24 h of culturing in conditions defined in "Methods". The cells were frozen in DMSO/PBS in presence (light green bars) or absence (dark bars) of serum.

In the more general aspect, it is worth recalling the hypothesis of Melton and Cowan (2004), which postulates that somatic SCs residing in the niches are not a part of the canonical developmental continuum of cell differentiation, but rather arise from a distinct population of pluripotent PG (primordial germ)- or ES (embryonic stem)-like cells. These cells would be separated from the main stream of organogenesis before germ-layer formation, and subsequently home to developmentally-specified tissue and organ niches. The remnants of these early events may persist into adulthood in the form of an embryonic-like stem cell population. This idea, if true, would be of much more than purely academic consequences. In line with it, the specification of tissue (adult) stem cells would be a stochastic phenomenon governed by the selection pressure provided by the specific environment of the niches, rather than the result of the emanation of an irreversible and deterministic (epi)genetic program. This hypothesis received substantial experimental support in respect of hematopoietic (cord blood and bone marrow) tissues by research from the McGuckin and coauthors (2004), Verfaile (Jin et al. 2002) and Ratajczak (Kucia et al. 2006) groups, as well as by our recent data (Habich et al. 2006) concerning the cord blood population.

Neurally-committed cord-blood-derived cell progenitors can attain infinitely-self-renewing NSC-like properties

Neural progenitors derived from cord blood and growing temporarily in monolayer culture can be further selected and expanded *in vitro*. Repeated passages of only floating, low-differentiation, nestin-positive, round-shaped cells appearing in these cultures in the presence of EGF allowed us to establish the first clonogenic, non-immortalized human umbilical cord blood neural stem cell-like line (HUCB-NSC) (Buzanska et al. 2006b).

The phenotypic and functional identity of HUCB-NSCs with classical CNS-derived neural progenitor cells (NPC) were critically evaluated in various *in vitro* analyses. Neuronal differentiation induced by dBcAMP treatment resulted in the expression of several functional genes and proteins, including GluR2, dopamine, serotonin and acetylcholine receptors, transmitters and growth factors, evaluated by microarray technique, RT PCR, immunocytochemistry and electrophysiology. Whole cell-patch clamp recording of two types of voltage-sensitive currents and several ligand-gated currents typical for neuronal cells were found in differentiated HUCB-NSCs (Sun et al. 2005). The firing of action-potential-like spikes has recently been recorded after long-term (terminal) differentiation of this cell culture (unpublished). These results confirm that HUCB-NSCs could conceivably be differentiated, at least *in vitro*, into functional neuronal cells (Bużańska et al. 2006a).

The HUCB-NSC line can be cultured and halted at defined stages of the hierarchical neural stem cell differentiation process, and their developmental decisions can be further manipulated and studied *in vitro*. We would like to implement this culture system to test whether or not defined drugs or toxic substances are harmful to human brain cells, especially during fetal and early childhood development (Buzanska et al. 2005). Well-defined human neural stem cell culture should provide a better model for such observations of the cumulative neural-specific effects of long-term chemical exposure than do animal-based methods, and thus provide an ideal surrogate testing system for screening large numbers of compounds and drug candidates using reproducible, quantitative and cost-effective methods.

Implantation of HUCB-NSCs into rat-brain tissue cultures *in vitro*

We have confirmed intrinsic neurogenic potential of HUCB-NP/NSCs under co-culture with rat-brain primary cultures (Jurga et al. 2006). The close proximity and contacts with rat brain cells promotes the appearance of typical neuron-like phenotypes among implanted HUCB-NSCs. Immunocytochemical examination revealed that 7 days of co-culture decreased the number of Nestin-positive cells, and promoted the cells

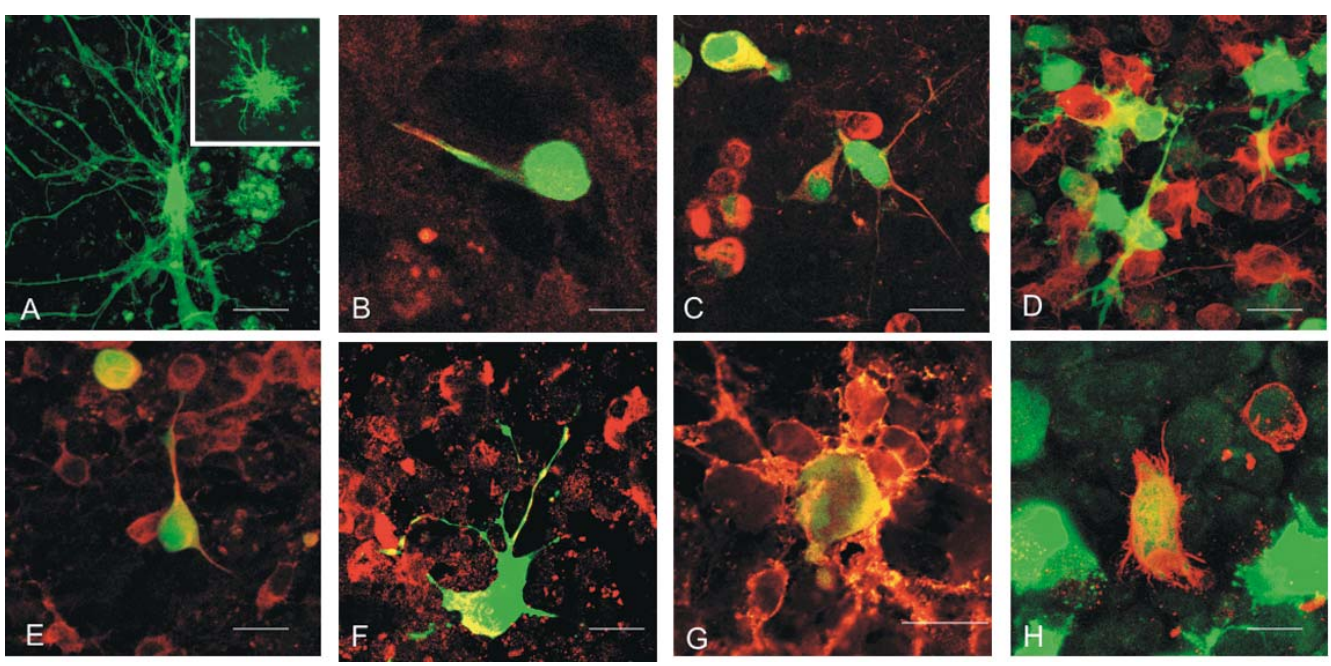

Fig.4. Visualization of HUCB-NSCs 7 days after transplantation into hippocampal organotypic slice culture cultured in serum-free (SF) conditions. HUCB-NSCs were labeled with GFP (green). (A) Morphology of HUCB-NSCs 7 days after transplantation. Neuron-specific markers: nestin (B) NF 200 (C) ß-tubulin III (D) MAP-2 (E) and GluR1 (F), oligodendroglial markers: O4 (G) and GalC (H) expressed by HUCB-NSCs cultured in the presence of rat hippocampal organotypic culture. Co-localization of red (specific for neuronal antigens) and green (pEGFP-N1 transfection) appears yellow in HUCB-NSCs after overlaying of these two images. Scale bars are 20 μm.

expressing mature neuronal markers like type III β-tubulin and MAP-2.

Furthermore, in the rat organotypic hippocampal culture we have observed advanced phenotypic differentiation and directional migration of the grafted HUCB-NSCs, as well as their subsequent histological integration with the recipient cyto-architecture. At 7 days after transplantation, most HUCB-NSCs had developed multipolar or pyramidal neuronlike cell morphology, depending on the histological region of cell transplantation. Most of the HUCB-NSCs differentiated well when seeded on a molecular layer, though did not do so in the DG region of hippocampal slices. Differentiated cells seem to integrate well with the recipient neuronal network, elaborating extensive cell-cell contacts with surrounding rat hippocampal tissue.

Moreover, we have recently established (Fig. 4) that HUCB-NSCs can differentiate well, even in the absence of serum in culture medium. This indicates that rat-brain-tissue-derived signals can efficiently and sufficiently direct transplanted cells into neuronal (Fig. 4B–F) or oligodendroglial (Fig. 4G–H) lineages (unpublished data).

Interestingly, HUCB-NSCs *in vitro* displayed a relatively low drive to differentiate into an astrocytic lineage. After co-culture of HUCB-NSCs with neonatal rat cortex culture a decrease in numbers of positive GFAP cells was observed (Jurga et al. 2006). The same preference toward a neuronal cell fate was noticed after seeding of HUCB-NSCs on hippocampal organotypic culture. During 14 days of common cultivation, about 15% of the transplanted cells expressed neuroblast markers and morphology, whereas none of them displayed GFAP immunoreactivity. Surprisingly, reactive astrogliotic reaction is typical for hippocampal slices cultivated *in vitro* (Bucks et al. 1993). It remains to be determined whether this means that tissue reactive gliosis, dependent on stimulation of transiently-growth-arrested astrocytes, is induced by different or even opposite signals to that responsible for SC-derived astrogenesis.

Transplantation of HUCB-NSCs into adult rat brain *in vivo*

Despite several reports that human cord blood cells can assume neural phenotypes after their infusion/transplantation into animal brain (Kogler et al. 2004, Lu et al. 2002, Zigova et al. 2002) there is a consensus that the number of persisting cells is extremely low in comparison with the number of cells provided, and not fully relevant to the reported clinical benefits of such treatments.

Our novel HUCB-NSC line can be treated as a prototypic transplantation material rich in well defined, neurally-committed cord-blood derivatives for treatment of various models of CNS injury. Recent experiments have been addressed the therapeutic effectiveness of our cell line in stroke-injured rats. However, their initial transplantation into intact rat brain striatum or cerebral cortex revealed that only a small fraction of the cell population can survive the first few days, independently of CsA-induced immunosuppression of the host animals. Moreover, the cell implant evoked acute local tissue reaction with strong infiltration by host inflamatory (ED1-reactive) cells. In the light of this feature typical for primary, non-specific graft rejection, it is important to note here that HUCB-NSCs express human leukocyte antigen – HLA ABC (MHC- class I) molecules, whereas highly immunogenic HLA-DR (MHC- class II) or CD45-antigen-presenting cells are almost completely absent from this cell population (unpublished data).

Surprisingly, the animals compromised by a focal brain lesion were rather more tolerant of HUCB-NSC transplantation than were intact recipients. The migrating donor cells which accumulated in the peri-infarct area were even found over 7 days after transplantation, while it was possible to show the appearance of differentiated phenotypes with expression of neuronal (NF200) but only moderate astrocytic (GFAP) cell markers (unpublished data). However, the paucity of HUCB-NSCs detected within the post-ischemic parenchyma at the end of one month following transplantation combines with the acute rejection of grafted cells by intact (yet immunosuppressed) rat brain tissue, strongly suggests development of severe immunological graft/host conflict. Accordingly, a recent study observed lymphocyte proliferation after xenogenic transplantation of human MSC into rats as an indication of a possible immune response (Grinnemo et al. 2004). Our own data reveal that further scientific work is needed to clarify the immunological behavior of HUCB-NSCs, and their xenogenic availability for experimental animal transplantation purposes (Rogers and Casper 2004).

Toward the use of cord-blood derived neural stem-like cells in brain repair

The beneficial effect of rehabilitation in stroke patients was long explained by augmentation of the two types of inherited endogenous responses: neuroplasticity and neurogenesis (Biernaskie and Corbett 2001, Komitowa et al. 2005). However, little is so far known of the relationship between neurogenesis and observed functional recovery. An interesting recent report held that endogenous neurogenesis is stimulated in response to a brain lesion in both the hippocampus and subventricular zone in rodents (Lichtenwalner and Parent 2006), and also in other, less-defined compartments/niches in the human brain (Jin et al. 2006). Moreover, an enriched environment and spatial learning enhance neurogenesis and salvage ischemic penumbra after focal cerebral ischemia (Matsumori et al. 2006). Newly-generated neurons have been shown capable of directed migration toward a lesion site, surviving there for a limited period of time. However, these processes indicating endogenous repair of damaged nervous tissue are abortive in the long run, probably due to the hostile local environment (Lindvall and Kokaia 2005).

Hypothesizing that neurogenesis is contributing to functional recovery from brain injury through the production of new neurons, we are justified in focusing research on the elements aiming to enhance these mechanisms. One such approach is to introduce through implantation an additional pool of exogenous cells capable of specific migration, differentiation and integration into the neural circuitries (Kelly et al. 2004, see Scheffler et al. 2006 for review, Tabar et al. 2005). However, it must be noted that this neuro-supplementation-based scenario is not the sole approach to the use of stem cells in CNS therapy. Another attractive possibility involves enhancement of endogenous neurogenesis through growth-factor release and/or the positive in this respect graft-host immunomodulation (Chen et al. 2003, English et al. 2006, Newman et al. 2006, Taguchi et al. 2004).

A variety of cell types have been considered an optimal source for neural cell therapy. While it remains difficult to depict detailed features of the ideal cell for this purpose at this stage, there are several general properties defined in order to direct and narrow the search focusing on the most-promising sources. In general, transplantable cells should be available in large, homogenous quantities, sufficient for treatment of an entire population of patients, be resistant to cryopreservation, and express low-immunogenicity and genetic stability with appropriate, fixed differentiation potential. Cell sources based on their origin throughout development can be grouped into: embryonic stem cells, fetal multipotent stem cells and post-developmental/adult stem and progenitor cells. Many investigators favor embryonic stem cells (ES), for their indefinite expandability and impressive, almost unlimited, differentiation repertoire. However, this source of cells is plagued with ethical issues. The other negative side of ES is that proliferation and differentiation are largely uncontrolled, leading to the formation of teratomas (Cao et al. 2006, Fujikawa et al. 2005). While there is a significant effort to decipher and control the mechanisms governing their development, the results at present are far from satisfactory, eliminating the possibility of prompt clinical applications (Bjorklund et al. 2002).

A major advantage of the second of the mentioned groups of cell sources – fetus- or adult-CNS-derived progenitors - is that they are usually more defined in terms of organ-specific phenotypical commitment, thus carrying less risk of uncontrolled division. Unfortunately, this source is very limited in quantity, so has to be considered of experimental value only for as long as the *in vitro* cell expansion problem remains unresolved.

In the third group (of post-developmental/adult sources), we find stem cells derived from bone marrow and peripheral blood (Shen et al. 2006, Shyu et al. 2006). A major advantage of these sources is the prospect of autogenic transplantation with the additional benefit that ethical concerns are lacking. The prospect of autogenic transplantation performed in a large population of patients is laced with logistical difficulties, however, *inter alia* those related to the pace of *in-vitro* cell expansion and manipulation, or the availability of an on-site GLP-level laboratory in every cell-therapy unit. Thus therapy individually tailored for each stroke victim seems impractical, and beyond the financial scope that society is able to handle. A more-realistic strategy is to establish a source of standardized, allogenic material, produced by the industry and available as a cryopreserved, standardized, drug-like product.

CONCLUSION

Human umbilical cord blood has a good chance of becoming the ideal source for standardized, allogenic transplantable material. It is easily obtainable and rich in ontogenically young stem cells already used in

clinics for bone-marrow replacement. Importantly, there are no reports of their tumorogenic or other detrimental activity. Our laboratory has successfully derived a non-immortalized cell-line with properties of neural stem cells. The cell line of HUCB-NSCs serves us as a useful prototype of cord blood derived, human neural-like stem cells for basic research as well as for testing it in animal models of brain pathology. We do believe that upon sufficient characterization, the neuraly committed cord blood-derived progenitors would have the potential for clinical use.

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