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A novel, neural potential of non-hematopoietic human umbilical cord blood stem cells

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ABSTRACT From the time of discovery that among the cord blood mononuclear cell population there are cells capable of changing their fate towards the neural lineage and producing functional neurons and macroglial cells, our attempts have been focused on the understanding of the underlying mechanism of this transition. We have deciphered the first steps of neural stem/progenitor gene induction in aggregating culture of cord blood mononuclear cells, their rapid phenotypic conversion under the influence of neuromorphogenic signals due to mitogen activation and their ability to expand and develop a prototypic, long-living line with neural stem cell properties. Evidence has accumulated that human umbilical cord-derived and neurally committed cells, due to their capacity for self-renewal, multilineage differentiation, plasticity and ability for long-lasting growth *in vitro,* provide unique material for the cell therapy of a wide spectrum of neurological diseases. The putative regenerating potential of these cord blood-derived neural stem/progenitor cells was evaluated after transplantation in experimental models of brain injury. In spite of initial promising data, the results indicate an urgent need to improve available animal model protocols in order to increase immuno-tolerance toward transplanted human cells.

KEY WORDS: *human cord blood, neural stem cell, neurosphere, transplantation, CNS*

Introduction

All cells in the organism are descendants of a single cell – the fertilized egg. The development requires multiplication and differentiation of its descendants into all the different cell types in the body. It is clear that in mammals, various primitive cells – stem cells (the embryo's inner cell mass or cells residing in tissue regenerative niches of organism) may still maintain their expansion and differentiation potentials when propagated *in vitro.* In the presence of differentiation inhibiting signals they are able to multiply for many cells generations as cell lines or "strains" called embryonic (ESC) or adult (ASC) stem cells. Due to their expansion potential and intrinsic ability to create cells of different properties it is believed that stem cells growing *in vitro* would provide an ideal transplantation material for regenerative medicine. Moreover, after genetically-aided transformation they can be used as a semi-pharmaceutical product for different clinical applications.

Until recently, the majority of stem cell research was focused on ES cells as those of having hypothetical potency to create any somatic cell type either *in vitro* or after transplantation in response to local differentiation signals. Apart of practical obstacles to reach these goals, the ethical concern surrounding any technique involving human embryos, even these in pre-implantation stages, indicated the need for finding alternative sources of SC. One appealing option is to bypass creation of ES cells from embryo by "reprogramming" a somatic cell to its undifferentiated state by mimicking events that take place in a somatic nuclear transfer (Wilmut *et al.,* 1997). Moreover, the finding that differentiation associated changes are reversible and controlled by epigenetic status of DNA and chromatin (Tagoh *et al.,* 2004; Ansel *et al.,* 2003; Hsieh and Gage, 2004; Xi and Xie, 2005) can indicate potential ability of any particular somatic cell to change or reverse its already established developmental state through the delivery of appropriate instructive signal(s).

At the beginning of this century it was assumed that the differentiation potential of adult stem cells is already determined and strictly tissue specific. However, at the same time several laboratories documented, especially in the hematopoietic tis-

Abbreviations used in this paper: CMFDA, chloromethyl-fluorescein-diacetate; HUCB, human umbilical cord blood; N, neurosphere; NP, neural progenitor; NSC, neural stem cell.

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sues, several meaningful exceptions from this general rule (Eglitis and Mezey, 1997; Ferrari *et al.*, 1998; Woodbury *et al.,* 2000; Lagasse *et al.,* 2000; Sanchez-Ramos *et al.,* 2000; Orlic *et al.,* 2001; Krause *et al.,* 2001; Jiang *et al.,* 2002). In our group we have shown for the first time that mononuclear fraction from human umbilical cord blood (HUCB), containing ontogenetically young but not hematopoietically determined (CD34-negative) stem cells, can acquire features of neural stem cells under defined *in vitro* conditions (Buzanska *et al.,* 2001; Buzanska *et al.,* 2002), and are able to differentiate in all three types of CNS cell lineages: neurons, astrocytes and oligodendrocytes.

Recent studies have confirmed that adult stem cells extracted *in vitro* even from other than hematopoietic tissues have also a much broader plasticity of differentiation than originally anticipated (Cao *et al.,* 2003 - for skeletal muscle SC; Wurmser *et al.,* 2004 - for neural SC; Amoh *et al.,* 2005 - for hair follicle SC; Guan *et al.,* 2006 - for testis-derived SC; De Coppi *et al.,* 2007 - for amniotic fluid-derived SC). Another distinct subpopulation of bone marrow cells has been shown to differentiate into various cell lineages (Yoon *et al.,* 2005; Kucia *et al.*, 2006). In addition, such multipotent differentiation of adult stem cells can occur without cell fusion (Harris *et al.,* 2004; Jang *et al.,* 2004; Wurmser *et al.,* 2004) and can contribute to regeneration of different tissues. Discovering embryonic-like potential and differentiation plasticity in several adult stem cell population(s) risen again the question, whether the stem cells (ESC and ASC) have similar very high transcriptional potential for all tissue-specific genes or as in case of adult stem cells the transcriptional potential is limited to certain genes and the transcription is directed by tissue-derived or neighboring cell-specific signals. It is worth to mention here that in the case of inner mass cells (ancestors of ESC) we are dealing with pure population of stem cells which, in the presence of differentiation inhibiting signals *in vitro*, can be propagated for many cell generations as ES cell lines (Evans and Kaufman, 1981). In contrast, adult SCs appear exclusively in mixed populations of different partially committed cells and there is no proven method (with exception of hematopoietic SC) for their purification

Why we are interested in Human Umbilical Cord Blood (HUCB) - derived cells?

The beneficial effect of rehabilitation in patients with brain injury was always believed to be based on the augmentation of the two types of inherited endogenous responses: neuroplasticity and neurogenesis (Biernaskie and Corbett, 2001; Komitowa *et al.* 2005). However, these processes, indicating the existence of endogenous mechanism of repair of damaged nervous tissue, are short lived, probably due to the hostile local environment (Lindvall and Kokaia, 2004).

Hypothesis that neurogenesis is contributing to functional recovery from brain injury focused the research on the development of therapeuthic methods allowing for the enhancement of these naturally occurring mechanisms. One such approach is to introduce, through implantation, an additional pool of exogenous stem cells capable of specific migration, differentiation and integration into the neural circuitries (Kelly *et al.* 2004; Tabar *et al.* 2005, for review see Scheffler *et al.* 2006). Another attractive therapeutic possibility involves enhancement of endogenous neurogenesis through the introduction of growth-factors released by naive or genetically manipulated, infused stem cells.

A variety of cell types have been considered an optimal source for neural cell therapy. In general, transplantable cells should represent a homogenous population available in large quantities, be resistant to cryopreservation, and express low-immunogenicity and genetic stability with appropriate, established differentiation potential. Many investigators favor embryonic stem cells (ES), for the reasons already mentioned above (indefinite expandability and impressive, almost unlimited, differentiation repertoire) (Kelly *et al.*, 2004; Hayashi *et al.*, 2006). However, this source of cells is plagued with severe 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.,* 2000; Fujikawa *et al.,* 2005; Erdo *et al.*, 2003). While there is a significant effort to decipher and control the mechanisms governing the behavior and differentiation of ESC (Baharvand *et al.,* 2007), the results are far from satisfactory, eliminating the possibility of prompt clinical applications of ESC.

A major advantage of the second type of stem cell – derived from fetus or adult tissue-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 and not easy to expand, so has to be considered as having experimental value only.

The third group of non-invasively obtainable stem cells contains cells derived from bone marrow, peripheral blood, cord blood, and, more recently, amniotic fluid (Hermann *et al., 2004*; Zhao Y *et al.*, 2003; Buzanska *et al., 2002;* De Coppi *et al.,* 2007). A major advantage of these sources is the prospect of auto/allogenic transplantation with the additional benefit of lack of ethical issues A logistically and economically realistic strategy for such transplantations is to establish a source of standardized, genotypically-defined material available as a cryopreserved, standardized combination of transplant and quasi-pharmaceutical product. HUCB has a good chance of becoming the ideal source for such standardized transplantable material. Our laboratory has successfully derived a non-immortalized cell-line with properties of neural stem cells (Buzanska *et al.,* 2003; Buzanska *et al.,* 2006b). This cell line of HUCB-NSCs serves as a useful prototype for the ideal transplantable material depicted above, and, upon sufficient characterization in animal models, it has a strong potential for clinical use. However the unique properties of HUCB-NS cells must be fully understood before any clinical application.

Historical note

The function of human stem cells in adult central nervous system have been of interest to our group since 1996 - long before the first evidence was provided that human stem cells can be maintained in culture *in vitro* (Thomsom *et al.*, 1998). The cellular model for this initial research concerning neural stem cells was the cell line derived from human medulloblastoma - named DEV (Dufay *et al.,* 1994). It was characterized by indefinite growth and ability to differentiate into three neuronal cell types, thus showing neural stem cell line character (Derrington *et al.*, 1998). Our special interest focused on transcriptional regulation of glial or neuronal fate decision undertaken by non-differentiated human neural stem-like cells. For that purpose the cells were transfected

with evolutionary conserved murine or invertebrate genes encoding transcription factors known to be involved in the neuronal or glial differentiation cascade (Buzanska *et al.*, 2001b). Our results obtained in cooperation with the group of Prof. B. Zalc (INSERM) not only confirmed functional conservation of basic Helix-Loop-Helix (bHLH) transcriptional factors involved in human neurogenesis, but also pointed out that cellular isolates from human brain tumors contain cells with neural stem potential, which under appropriate conditions, can be multi-directionally differentiated into mature neurons, astrocytes and oligodendrocytes. This is consistent with the recent theory of the cancer stem cells role in oncogenesis and as such it is citied in the relevant literature (Singh *et al.*, Oncogene, 2004).

Evidence for surprising presence of stem cells in the adult human central nervous system together with the proofs for ongoing neurogenesis during adulthood (Eriksson *et al.*, 1998) provoked us to look for the source of nontransformed neural stem cells suitable for further experimental approaches as well as for putative therapy. However, neural stem cells, which are present in adult or developing human brain, are not easily harvestable. What's more, somatic stem cells, unlike embryonic ones, in general have limited life span as they spontaneously differentiate in laboratory culture and thus enter an irreversible growth arrest after a finite number of cell divisions. This phenomenon (except of the latest research data, Conti *et al.*, 2005; De Coppi *et al.,* 2007) was believed as typical for progenitor cells isolated from any fetal or adult human tissue. Thus our challenge was to isolate ethically neutral and non-transformed SC population of neural character, suitable for long-lasting *in vitro* expansion.

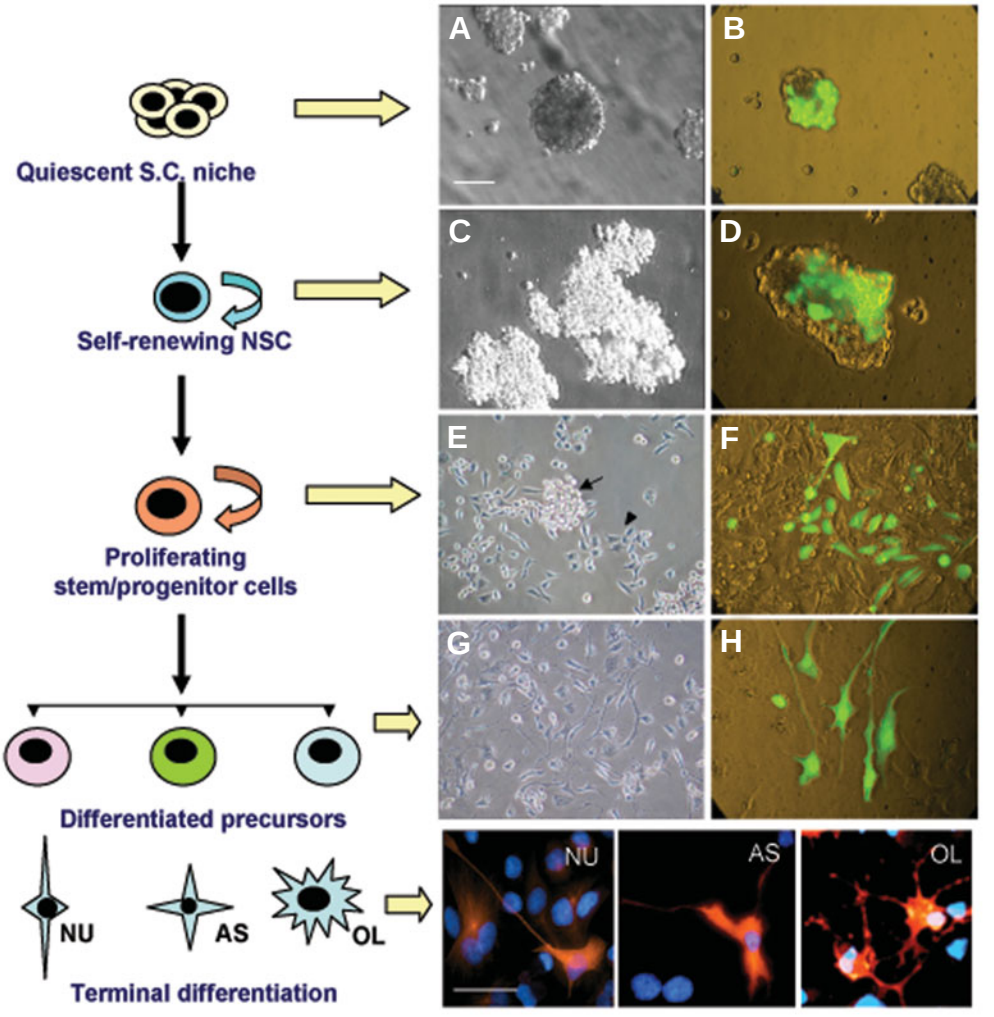

Fig. 1. Developmental steps of human umbilical cord blood-neural stem cells (HUCB-NSC) toward neural lineages. *Phase contrast microscopy* **(A–H)** *with additional fluorescence* **(B, D, F, H)** *for cultures transfected with green fluorescent protein.* **(A,B)** *HUCB-NSC derived neurospheres - quiescent stem cell niche;* **(C,D)** *self-renewing, floating HUCB-NSC in defined serum-free medium;* **(E,F)** *intermediate, neural committed HUCB-NSC grown in low serum medium at floating (arrow) and adherent (arrowhead) fraction;* **(G,H)** *HUCB-NSC derived neuronal, astrocytic and oligodendroglial precursors differentiated in media supplemented with neuromorphogens. Immunocytochemistry: (NU) β-Tubulin III, (AS) S100 β, (OL) GalC for neuronal, astrocytic and oligodendrocytic cells respectively. Scale bars, 50 μm.*

Human umbilical cord blood stem cells (HUCB-SC), in contrast to adult bone marrow stem cells (BM-SC), are less mature and therefore less immunogenic and have a higher proliferation potential associated with extended life span and longer telomeres. We assumed that due to their immaturity and specific postnatal ontogenic position the population of HUCB-SC would retain enhanced capacity to proliferate and expand also *in vitro* and studies that it may contain a subpopulation of undefined stem cells with flexible phenotype or embryonic-like properties which would allow them to differentiate into neural cell types. The first positive results confirming these assumptions were obtained in 2001 (Buzanska *et al.,* 2001a) and then published in 2002 (Buzanska *et al.,* 2002). Thereafter, through repeated passages of floating, round-shaped, nestin-positive and clonogenic cells from these neural progenitors population we have established the first, expanding, indefinitely growing HUCB-NSC cell line (Buzanska *et al.,* 2003; Buzanska *et al.*, 2006b).

Neurally-committed cord-blood-derived stem/progenitor cells can attain infinitely-self-renewing NSC-like properties

Phenotypic and functional identity of HUCB-NSC line, their stability, clonogenity, ability to form neurospheres and to differentiate into all neural cell types (Buzanska *et al.*, 2006a; Buzanska

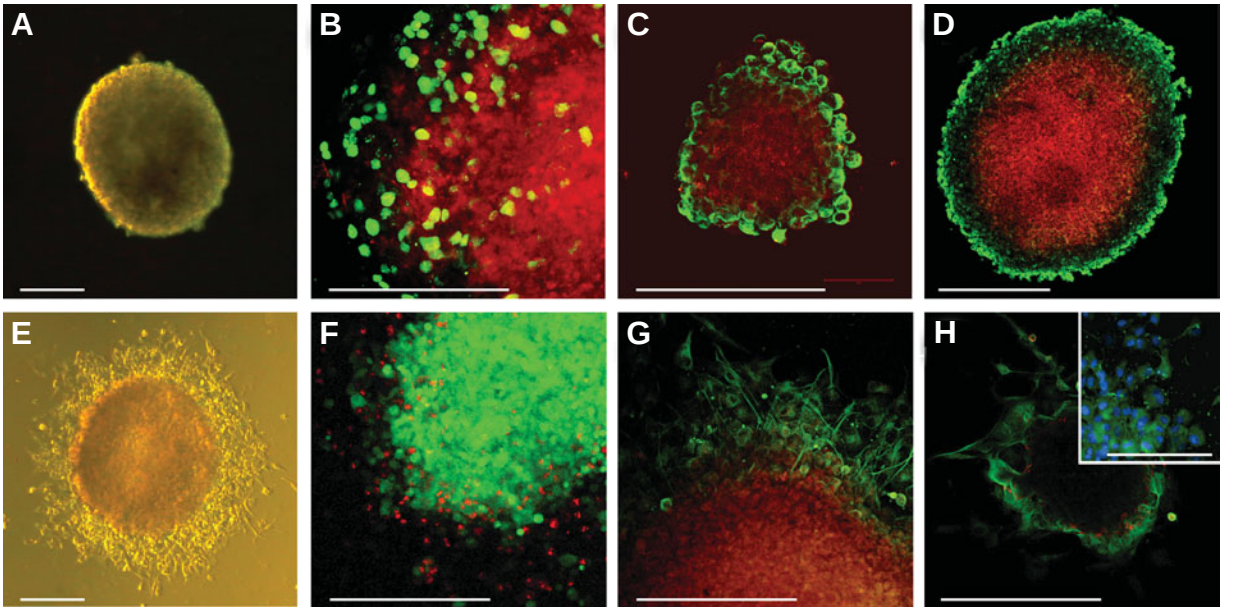

Fig. 2. Immunocytochemical detection of cell markers in neurospheres (N-HUCB) formed from HUCB-NSC. *Resting stage of N-HUCB (upper panel); activated stage of N-HUCB (lower panel).* **(A)** *Phase contrast microscopy image of floating N-HUCB in serum-free medium; confocal microscopy image of cells expressing:* **(B)** *Ki-67 (green) at the surface of N-HUCB;* **(C)** *connexin 43 (red) and GFAP (green);* **(D)** *nestin (red) and GFAP (green) markers in the different regions of N-HUCB.* **(E)** *Phase contrast microscopy image of activated (attached) N-HUCB. Confocal microscopy image of cells expressing:* **(F)** *Ki-67 (red) present in migrating from attached neurosphere composed of GFP positive cells;* **(G)** *connexin (red) in the centre of N-HUCB and β-Tubulin III (green) neuroblasts visible at the surface of neuroshere;* **(H)** *the majority of cells surrounding activated N-HUCB display β-Tubulin III (green) and MAP-2 (inset: green). Few cells are GFAP-positive (red), while the cells in the core region of N-HUCB remain negative for these markers. Scale bars, 100 μm.*

et al., 2006b) were confirmed in further studies. Critical *in vitro* analysis involved contrast-phase microscopic observations, gene expression studies using cDNA microarray and quantitative RT-PCR followed by immunocytochemical evaluation of the protein expression. Non-transformed character of the HUCB-NSC cell line was proved by the karyotypic analysis and transplantations into immunodeficient non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. It was shown, that HUCB-NSC grown in continuous culture (for more than 65 passages) displayed normal human karyotype without identifiable chromosomal aberrations in early (9^{th}-13^{th}) and late (22^{nd}-45^{th}) passages. After transplantation into NOD/SCID mice tumor formation was not observed. In addition we did not find enhanced expression of the classical protooncogenes in HUCB-NSCs as compared to the starting population of HUCB mononuclear cells. Stability of the HUCB-NSC cell line was further confirmed by the lack of significant differences in the growth rates, clonogenicity, and differentiation potency between the early and late passages. Single cells isolated from the long-term expanded HUCB-NSC produced clones (cloning efficiency approximately 10%) of multipotent neural progenitors differentiating into neuronal, astrocytic and oligodendroglial lineages.

Depending upon culture conditions HUCB-NSCs can be maintained at different developmental stages, which reproduce neural stem cell differentiation process (Buzanska *et al.,* 2005), and they can display following phenotypes: 1) non-differentiated "dormant" stem cells residing in neurosphere-like structures (serum-free conditions) (Fig. 1 A, B); 2) proliferating, but still non-differentiated neural progenitors in free-floating aggregates (serum-free conditions but under growth factor stimulation) (Fig. 1C, D); 3) proliferating at a high rate cultures of mixed, nondifferentiated (floating) and already committed (adherent cells) neural progenitors when grown in low serum (2%) conditions (Fig. 1 E, F); 4) the adherent cells differentiating into neuronal, astrocytic and oligodendroglial lineages when cultured in high serum or low serum media supplemented with neuromorphogens (Fig.1 G, H).

HUCB-NSC line can be easily halted and harvested at these different stages of neural commitment, thus provides a suitable model for the study of the mechanisms governing neural stem cells developmental fate decisions. Such system can be experimentally manipulated *in vitro* by the presence of trophic factors, mitogenes, neuromorphogenes and transduced genes. These cells can be also used for large-scale screening of compounds for their neurotoxic effect or for new drug discovery. Currently HUCB-NSC line is under implementation for developmental neurotoxicity testing in collaborative effort with ECVAM/JRC.

The ability of HUCB-NSCs to form neurospheres, a hallmark of genuine neural stem cells, further confirmed stem character and neural commitment of cord blood-derived line. Neurosphere, a well characterized, multicellular structure (Doetsch *et al.,* 2003) is assumed to follow typical NSC behavior in the CNS-specific niches. Nonadherent HUCB-NSC spheres (Jurga and Domanska-Janik, 2004) are linage negative for advanced neuronal, astroglial or oligodendroglial markers, but contain cells expressing markers of uncommitted human neural stem cells, like Nestin and GFAP (Fig. 2). Upon attachment, in the presence of serum, surface cells of neurosphere start to migrate away from the center then differentiate into neurons (β-tubulin III – red), astrocytes (GFAP – green) and oligodendrocytes (data not shown). These results are consistent with the pattern of differentiation in neurospheres

derived from the human CNS (Caldwell *et al*., 2001) or human embryonic stem cells (Benzing *et al.,* 2006).

Neural character of HUCB-NSC line was further evaluated by comparative analysis of the transcriptional profile (Affymetrix DNA microarray probes HG-U133, covering the expression of about 40.000 genes), between the free-floating, non-differentiated HUCB-NSC and its starting, nonselected cell population – mononuclear cell (MNC) fraction of human cord blood. It was shown that more than 90 % of stem and neural related genes are up-regulated in HUCB-NSC as compared to its reference population of MNC. "Stemness" up-regulated in HUCB-NSC genes encode proteins involved in Wnt, Lif and Notch signaling pathways, that are known to regulate stem cell self-renewal and proliferation (Sato *et al.,* 2004; Melton and Cowan, 2004). They are represented mainly by frizzled, wingless, Lif and notch gene families (*Fzd8*, *Fzd7*, *Wnt5a*, *Ctnmb1*, *Lif*, *LifR*, *Erbb2, Jak3, Notch2*, *Notch3*, *Notch4, Jag2, Dll3, Hey1*, *Pnn1* and *2, Adam15*, *17*, *23*). In addition to activation of pro-neural genes like: *NeuroD1, Otx1,* and *Msi,* studies reveled expression of *Oct4*, *Sox2, Mdr1, Rex1* - the genes characteristic for pluripotent embryonic stem cells. Thus HUCB-NSC can retain their potential pluripotency, while executing exclusively neural-restricted gene expression (Jurga *et al*., 2006b).

Of special interest was the high activity of FGF signaling pathway in HUCB-NSC. Up-regulation of *FgfR1* was shown to be a hallmark for neural commitment of clonally related stem cells (Hsieh and Gage, 2004). In non-differentiated HUCB-NSC expression of *FgfR1* gene was increased up to 600-fold. Since FGF signaling pathway was also shown to be involved in epigenetic control of self renewal activity of stem cells (Hsieh and Gage, 2004; Rao, 2004), significantly increased expression of *FgfR1* may be important not only for neural commitment, but also for the self-renewal capacity of HUCB-NSC. Microarray data and the parallel study by quantitative RT-PCR revealed activation in HUCB-NSCs of several pro-neural genes (*Sox2, NeuroD1, Otx1,* and *Msi1*), but also the lack of expression genes typical for mesodermal (T gene) and endodermal differentiation (*Foxa1*) (Jurga *et al*., 2006b; Buzanska *et al*., 2006a), further indicating neural commitment of the cell line.

Nestin-positive HUCB-NSC may undergo spontaneous differentiation into cells expressing markers typical for all main neural-type cells, but extend of this differentiation highly depends upon plating density, attachment conditions and presence of serum in the medium. In standardized conditions: on poly-l-lysine coated surface and in low serum medium (2%) HUCB-NSC cells cultured for two weeks expressed neuronal (β-tubulin III), astrocytic (S-100β) and oligodendroglial markers up to 28%, 10% and 1% of total cell number, respectively (Buzanska *et al*., 2005). Application of neuromorphogenes (RA, BDNF and di-Butyryl cyclic AMP (dBcAMP)) to HUCB-NSC or co-culture in conditions reassembling natural niche: with rat cortical brain cells or on hippocampal slices (Fig. 3), induced further differentiation and expression of more advanced neuronal markers like NF-200 or MAP-2 (Jurga *et al*., 2006b; Buzanska *et al*., 2006a). We have also shown, that lineage specific, directed differentiation of HUCB-NSC is also possible. Different combinations of several growth factors and neuromorphogens, such as PDGF-AA, PDGF-BB, Ciliary Neurotrophic Factor (CNTF), GDNF, T3 and dBcAMP, applied to standardized, plating and culture conditions (2% serum, no mitogens, for 2 weeks in culture), allowed us to establish controlled conditions for the directed commitment into neuronal, astrocytic and oligodendroglial lineages (unpublished).

Nevertheless, nearly exclusive (more than 80%) commitment into neuronal phenotype was achieved upon prolonged (2 to 4 weeks) differentiation in the presence of dBcAMP. The gene expression profile of HUCB-NSCs changed substantially in these conditions. Activated genes (as compared to non-differentiated HUCB-NSCs) were mainly related to the signaling pathways, neurotransmitters, receptors, or channels involved in neuronal functions (Buzanska *et al.,* 2006a; Buzanska *et al.,* 2006b; Sun *et al*., 2005). Up-regulation of genes was followed by the expression of corresponding proteins, which included: GluR2, dopamine, serotonin and acetylcholine receptors, Gad 65, GABARAPL1and 3; TAU, SV2, TH and many more involved in synaptic transmission and function of gabaergic, glutamatergic and serotoninergic neurons (Fig. 3).

Mechanisms underlying neuronal differentiation of HUCB-

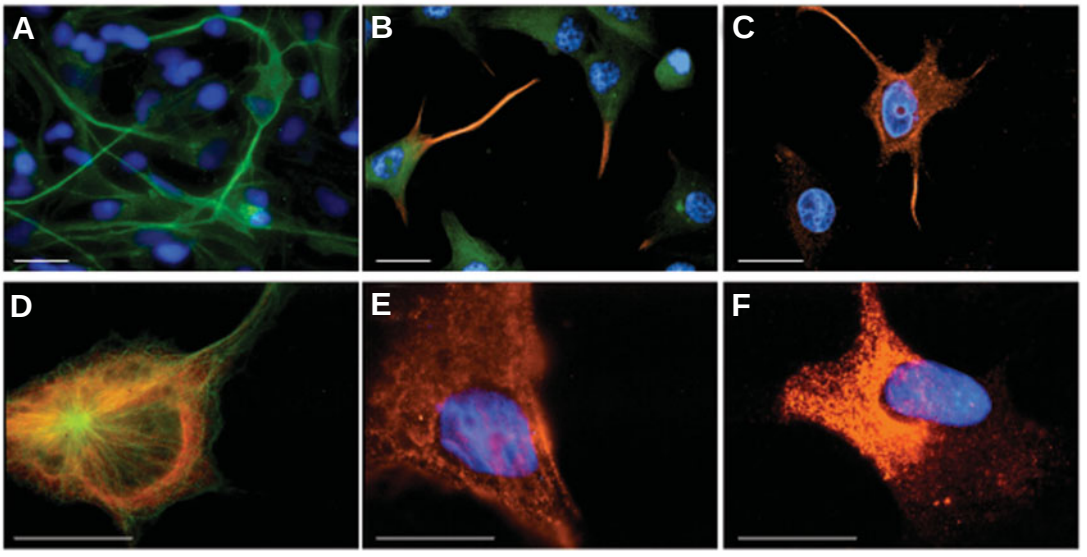

Fig. 3. Immunocytochemical detection of neuronal markers in dBcAMP-differentiated HUCB-NSC. (A) *Expression of β-Tubulin III (green);* **(B)** *merger of β-Tubulin III (green) and microtubule associated protein 2 (MAP2, red) immunostaining. Note that MAP2 is localized to neuronal extensions.* **(C)** *Expression of GABA receptor 1-associated protein (GABAR1AP, red);* **(D)** *β-Tubulin III (green) and neurofilament 200 (NF200, red) co-expression. Note filamentous organization of immunostained proteins;* **(E)** *glutamate receptor 2 (GluR2, red) and* **(F)** *dopamine receptor 2a (DR2a, red) immunodetection. Cell nuclei were stained blue with Hoechst 33258. Scale bars, (A,B,C) 50 μm; (D,E,F) 20 μm.*

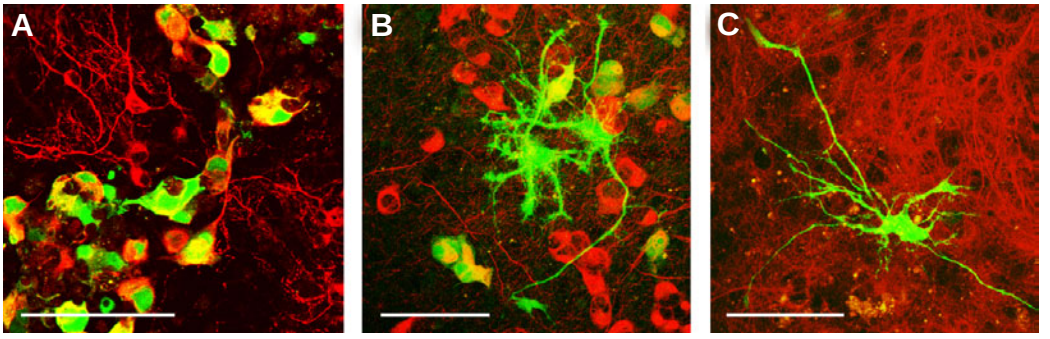

Fig. 4. Confocal microscopy image of HUCB-NSC transplanted onto rat hippocampal organotypic slices and cultured for 2 weeks. *HUCB-NSC expressing:* **(A)** *GFP (green) and β - Tubulin III (red);* **(B)** *GFP (green) and NF-200 (red);* **(C)** *GFP (green) and TUJ1 (red) are seen on the slices with co-expression of these markers (yellow). Scale bars, 100 μm.*

NSC are thought to resemble the mechanisms operating in developing mammalian brain, thus involve bHLH cascade of transcriptional regulation. In gain-of function experiments we have shown that ID1 (endogenous inhibitor of bHLH transcriptional factors) inhibits neurogenesis. Negative regulation of HUCB-NSC neuronal differentiation is coupled with translocation of ID1 protein from cytoplasm to the nucleus suggesting its possible usage as a stage-specific marker of neurogenesis (Jurga *et al.,* 2006a).

However, assuming that HUCB-NSC line may be a source of cells for possible therapeutic application or reliable model for *in vitro* tests of terminal, neuronal differentiation, we needed to provide the strong physiological evidence for its functional differentiation. For such purpose HUCB-NSC must not only develop morphological characteristics of neurons, but also voltage- and ligand- gated ion channels, that would allow them to function within a neural network and respond to neurotransmitters released from neighboring neurons. The whole-cell patch clamp technique for recording electrophysiological properties of HUCB-NSCs was applied to address this issue (Sun *et al.,* 2005). Two types of voltage-sensitive currents: (1) inward rectifying current (Kir) and (2) outward rectifying potassium current (IK+) together with several ligand-gated currents typical for neuronal cells, were recorded from differentiated HUCB-NSC. All this currents could be blocked by specific antagonists and restored after their removal.

Further functional evidence is provided by the ability of HUCB-NSC to grow and terminally differentiate directly on silicon or glass multi-electrode neurosensor (Bionas Neurosensorix Measurement System, Rostock and Central Network for Neurons, Texas respectively). Chip-cultured HUCB-NSCs formed a neuronal-like network capable to generate spontaneous field potential activity (unpublished data). These results show that HUCB-NSC could conceivably be differentiated in spontaneously firing, functional neuron-like cells.

Possible mechanisms of transition of cord blood mononuclear cells toward neural phenotypes

It is still not clear whether exposure to specific factors *in vitro* makes HUCB cells disregard their hematopoietic identity due to epigenetic reprogramming in the mechanism called trans- or de-differentiation (Abkowitz, 2002; Jiang *et al.,* 2002; Korbling *et al.,* 2002; Poulsom *et al.,* 2002; Terskikh *et al.,* 2001), or whether they acquire new features by directed expansion of a preexisting, embryonic-like, pluripotent stem cell subpopulation positively selected during employed experimental procedures (McGuckin *et al.,* 2004; Habich *et al.,* 2006).

According to our recent data (Habich *et al.,* 2006) freshly-isolated HUCB mononuclear fraction does not express or has very low level of expression of gene or protein markers characteristic for lineage-specific progenitors. Instead, genes believed to be master regulators of the pluripotent stem state, i.e. Oct3/4, Sox2, Mdr1 and Rex1, have been found to be expressed in the native, hematopoietic progenitors-depleted ($CD34^-$) cord blood isolates. During 24-48 hr cell culture in strictly specified conditions, the frequency of Nestin and CD133 immunopositive cells (lineage-specific NSC markers) increased rapidly in parallel with cell proliferation rate (Ki67 labeling), and a near 10-fold increase of the "side population" (as measured by high Hoechst 33342 efflux, an approved test for the presence of stem/progenitor cells). Concomitantly, cultured cells start to form aggregates and successively express other pro-neural genes, i.e. enhanced Sox2, OTX1, Nestin, GFAP and NF-200. During the next few days immunoreaction for the wide panel of neural markers: β-tubulin III, MAP2, GFAP, S100β, Doublecortin, NG2 and GalC, increased in the majority of cultured cells with reciprocal lowering of all of the "stemness" markers. At this stage, cells adhere to the bottom, disperse to form a monolayer and decrease their proliferation rate (Ki67 expression). Additional treatments with neuromorphogenes or co-culturing with rat-brain primary culture induced further differentiation of these early neural precursors toward more advanced neuronal phenotypes. However, gradual depletion of Oct3/4, Rex-1, Sox2 and Ki67 clearly indicates the onset of predominance of non-dividing, differentiated cells over the uncommitted, self-renewing and proliferating stem/progenitors (HUCB-NS/P), leading to their successive depletion and dilution *in vitro* and finally to senescent of the culture.

However, repeated passages of floating, nestin-positive, round-shaped cells appearing occasionally in the supernatant of these primary monolayer cultures treated with Epithelial Growth Factor (EGF) or EGF/FGF, allowed us to prolong culture expansion and, for the first time, to establish described above non-immortalized human umbilical cord blood neural stem cell line (HUCB-NSC) (Buzanska *et al.,* 2003; Buzanska *et al.,* 2006b). The exact mechanisms responsible for such substantial change in HUCB cells behavior *in vitro* are unknown at present. The asymmetric type of the progenitor proliferation kinetics leading to culture senescence (Sherley, 2002) is assumed as typical for all stem cells residing in the specialized niches of adult organism whereas the symmetric balance between maintenance of undifferentiated stem cells and lineage committed progeny would provide a force

to enormous tissue expansion observed mainly during development. Thus, symmetric type of cell expansion kinetic, responsible for indefinite growth *in vitro* has been attributed to embryonic SC whereas asymmetric division is treated as evolutionary conserved feature of somatic stem cells involved in space- and time-limited organogenesis and tissue maintenance. Until now, all non-transformed stem cell lines developed from non-embryonic tissues such as CB-derived (Kogler *et al*., 2004; Xiao *et al.,* 2005), amniotic fluid AFC (De Coppi *et al.,* 2007) and our HUCB-NSC line (Buzanska *et al.,* 2006b) contain the cells expressing stage specific embryonic markers (Oct3/4, Seea-4, Rex1, Mdr1, ect.), that may suggest maintenance of the stem cell population with the ability to perform embryonic-like, symmetric type of stem cell division.

These primitive, pluripotent stem cells *in vitro* are particularly sensitive to environmental influence provided by direct contacts with other cell types or tissue-specific instructive signals, among them these promoting "spontaneous" differentiation. Thus, effective inhibition of cell differentiation signals and promotion of symmetric type of stem cell division would be primary requirement for long-lasting culture expansion (Sherley, 2002). The precise definition of these events and establishment of standard procedure for prolonged HUCB-NSC expansion *in vitro* is the main goal of our study.

The cord blood challenge for brain repair

Cord blood cells have been a part of clinical practice for over 15 years and offer several advantages in terms of clinical transplantation (Broxmeyer *et al.,* 1989; Gluckman *et al.,* 1989; Laughlin *et al*., 2001). Apart from hematological disorders CNS diseases represent especially daunting challenges for cell-based strategies of repair. Studies by a number of groups support the idea that infusion of cells obtained from human cord blood lessen neural damage resulting from stroke, neurodegenerative diseases and spinal cord injury (Nan *et al*., 2005; Ende *et al*., 2000; Chen *et al*., 2006).

Cord blood has been shown to contain hematopoietic stem cells, mesenchymal stem cells and endothelial precursor cells. At the present time it is not known whether any of these stem cells can ameliorate deficits seen in neurologically compromised animals treated with cord blood but it is possible that a combination of all these cells may contribute to observed functional restitution. The results from our study addressed this problem and demonstrated that intra-carotid artery infusion of human umbilical cord blood cells depleted of hematopoietic precursors and directed *in vitro* toward neural phenotype (NP) (Habich *et al.,* 2006), ameliorate neurological deficits associated with stroke-like, ouabain-induced brain injury. Rats transplanted with HUCB-NP showed significant behavioral improvement in walking beam and rotarod performance after one week, with further progress in recovery of locomotor's deficits observed after 4 weeks (Janowski *et al*., 2004; Gornicka *et al*., 2005; Gornicka *et al*., 2006). Functional therapeutic benefit was correlated with obvious reduction in lesion volume in transplanted rats in comparison to control, non-transplanted counterparts. Our results are consistent with other experiments which, in contrast to ours, have utilized non-selected cord blood to treat CNS injury. In all these reports HUCB cell administrated intravenously or intraperitonealy modified positively the severity of neurological deficits caused by middle cerebral artery occlusion (MCAO) (Willing *et al.,* 2003; Vendrame *et al*., 2004; Xiao *et al*., 2005; Newcomb *et al.,* 2006; Meier *et al*., 2006). Also intravenous administration of umbilical cord blood improved functional state of the brain and behavioral deficits in rats suffering from hemorrhagic or traumatic brain and spinal cord injury (Nan *et al*., 2005; Lu *et al*., 2002; Ereniev *et al*., 2005; Saporta *et al*., 2003). All these data suggest that cells with neural progenitor properties are contributing substantially to all these CB therapeutic effects.

Furthermore, therapeutic benefits of CB infusion have been reported in chronic neurodegenerative diseases. It has been shown that infusion of HUCB cells delayed the progression of amyotrophic lateral sclerosis (ALS) and increased lifespan of the diseased, transgenic mice (Ende *et al.,* 2000; Garbuzova-Davies *et al*., 2003). Systemic delivery of HUCB cells has an ability to prevent circulatory shock and resulting brain damage in the heatstroke animal model (Chen *et al*., 2006). Transplantation of umbilical cord blood in babies with infantile Krabbe's disease that favorably altered their natural history could be the first, promising example from human clinic. Infants who underwent HUCB cell graft before the development of symptoms showed progressively increasing central myelination and continued gains in developmental skills and cognitive function (Escolar *et al.,* 2005; Escolar *et al.,* 2007). These therapeutic benefits of cord blood infusion may hold hope for slowing the progression of other chronic neural diseases.

Beside of all these promising data it is still not clear how transplanted stem cells can participate in the CNS repair process. Besides our studies there are many reports from other laboratories of cord blood cells turning into brain cells i.e. neurons, astrocytes, oligodendrocytes, endothelial cells and microglia both *in vitro* and *in vivo* (Buzanska *et al*., 2006; Borlongan and Hess, 2006; English *et al*., 2006; Zandonella, 2005; Tagushi *et al.,* 2004; Asheuer *et al*., 2004). Thus, the most straightforward idea is that cord blood stem cells infused systemically reach brain, differentiate into mature cell types and simply replace the lost cells. However, until now this mechanistic view cannot reconcile the experimental findings. In our studies only a few transplanted HUCB-NP cells were detected in the ipsilateral hemisphere of ischemic brain of injured rats (unpublished). This is consistent with majority of other studies where only low number of human cells was detected in the injured tissue of grafted rats (Vendrame *et al*., 2004; Nan *et al*., 2005; Nystedt *et al*., 2006). The limitation of the presence of HUCB cells after systemic transplantation might be the effect of their rejection in discordant xenogeneic recipients. It is well known that immunosuppressive treatment is a key determinant of grafted cells survival in a manner of xenograft paradigms (Brevig *et al.,* 2000; Vendrame *et al*., 2004). Thus, a majority of experimental xenografting protocols included the use of high doses of cyclosporin A. However, although this classical immunosuppressant can effectively inhibits T cell activity it is inadequate at protecting xenografts from direct immune attack that involves the innate responses of the recipients such as complement system, natural antibodies, the coagulation cascade and natural killer cells (Cascalho and Platt, 2001).

The next concern is related to brain accessibility for systemically transplanted cells. We have detected intra-arterially infused cells only at the border of lesion at 24 h after delivery (Janowski

et al., 2004). Borlongan *et al.* (2004) have shown that HUCB cells do not enter the brain parenchyma even when co-infused with blood-brain barrier permeabilizer. Apart of these confusing results numerous studies steadily revealed that HUCB graft can reduce cerebral infarct volume and improve behavioral recovery. Therefore, rapid improvement within few days as seen in HUCB transplanted animal models may indicate that mechanisms other than cell replacement are of primary importance in all these cases. It has been suggested that transplanted HUCB stem cells may repair brain damage acting as bystander regulators of neuron and glia rescue *via* the release of neurotrophic factors. Human umbilical cord blood cells as well as their neural derivatives produce different neurotrophic factors i.e. brain-derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4-5 (NT4-5) (Fan *et al.*, 2005). The results of Borlongan *et al.* (2004) demonstrated that intravenous HUCB infusion significantly increase GDNF, BDNF and NGF brain levels in stroke animals as compared with nontransplanted counterparts. It is also possible that in addition to the role of already known or unknown factors secreted by transplanted cord the blood cells themselves are responsible for functional improvement following CNS lesion.

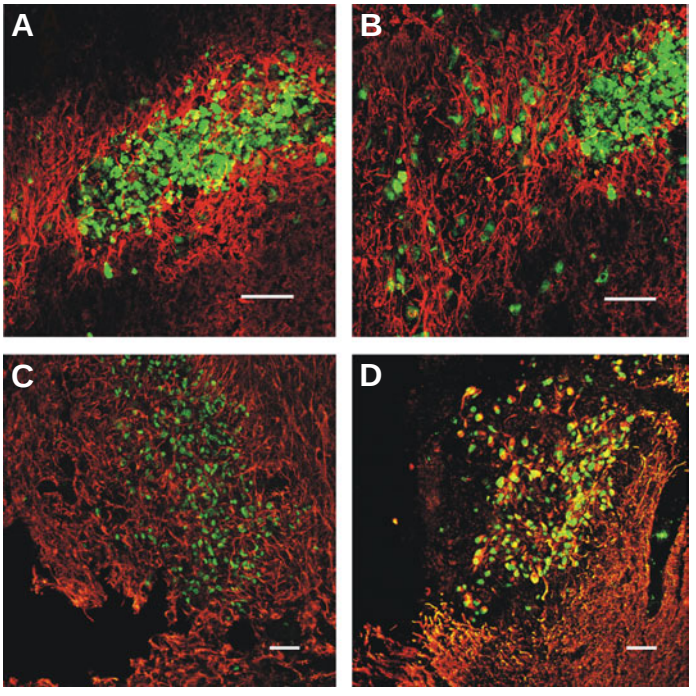

Fig. 5. Immunohistochemical staining of HUCB-NSCs transplanted into lesioned rat brain. (A) *3 days after transplantation of CMFDA-labeled donor cells (green) in transplantation side. Co-staining of these cells with neuron specific anti-NF-200 antibody (yellow) can be found in a minimal number of cells.* **(B)** *Migrating CMFDA-labeled HUCB-NSCs (green) surrounding NF-200 reactive host neuron (red) 3 days after cells injection.* **(C)** *7 days after transplantation, HUCB-NSCs stained with the human nuclei marker NuMA (green) were seen in close vicinity of GFAP reactive astrocytes (red).* **(D)** *7 days after transplantation, HUCB-NSCs stained with human nuclei marker NuMA (green) co-expressed the neuronal marker NF-200 (red cytoplasm and protrusions) in the infarct area. Scale bar, 50 μm.*

Another mode of delivery of stem cells, proposed in various experimental and clinical settings, is their direct brain implantation. In our own studies of the methods of selection of HUCB-NSCs we have tested behavior of these cells after intraparenchymal brain grafting. We showed that shortly after transplantation into intact brain of adult rats the cells migrate from the injection site and then disperse through the host brain tissue during the first few days. However, their survival is seriously compromised by local inflammation and then acute rejection (Janowski *et al.*, 2006a; Janowski *et al.,* 2006b). Our results are in agreement with studies from other laboratories where relatively low cell survival was noticed after intraparenchymal xenograft despite of the usage of classical Cyclosporin A (CsA)-induced immunosuppression of the host immune response (Walczak *et al.*, 2004; Kogler *et al.*, 2004; Pan *et al.*, 2005). To explain this rapid adverse reaction Newman *et al.* (2006) have shown that HUCB cells produced a variety of cytokines i.e. IL-8, IL-1α and chemokines i.e. MCP-1. These factors, being chemoattractants for the cells mobilized as a first line defense system after xenotransplantation, must be considered as one of the critical factors. The previously held view that the brain was an absolute immunologically neutral site allowing indefinite survival without rejection of cell grafts has proven to be wrong. Thus, the brain should be regarded rather as a site where immune responses occur albeit in a tissue-specific, modified form, which directly limits graft survival.

To shed a light on this problem we have studied HUCB-NSCs behavior after their transplantation into organotypic rat hippocampal cultures, the model where immunological host reactions are lacking (Jurga *et al.,* 2006b). In this condition we have observed advanced phenotypic differentiation of the transplanted cells mainly toward neuronal lineage. Transplanted cells seemed to integrate well with the rat neuronal network elaborating extensive cell-cell contacts with surrounding rat hippocampal tissue (Fig. 4).

Also neonatal rats with incompletely developed immunological system provide more permissive model to intracranial HUCB cells transplantation (Zigova *et al.*, 2000; Zigova *et al.,* 2002). In our experiments we have shown that HUCB-NSC transplanted into subventricular zone of neonatal 1-day old rats survive significantly better than in adults, migrate along the rostral migratory pathway and express lineage-specific markers (TUJ-1, NF-200, GFAP, S-100β) (Kozlowska *et al.*, 2005).

Surprisingly, CNS injury, when compared with intact tissue, apparently improved viability of transplanted stem cells and directed their migration toward injured area. In our ouabain model of brain injury there is a high activation of macrophage/microglia and astrocytes, induced by the infarct itself (Kozlowska *et al.*, 2006). It seems plausible to postulate that dynamic interplay between various cytokines and trophic factors released by injured tissue, inflammatory cells and factors provided by grafted HUCB-NSCs, may create favorable microenvironment for longer cell survival. Indeed, the substantial number of transplanted HUCB-NSC cells migrated and accumulated around the injured area (Fig.5). Seven days after transplantation high number of these cells exhibited neuronal phenotypes (NF-200) and, to lower extend, glial (GFAP, S-100β) cell markers. Some of these cells survived for 4 weeks; however most of them disappeared during this time, despite of employed CsA immunosuppression (Kozlowska *et al.*, 2007). These results agree with other reports where only a few transplanted HUCB cells were found in injured

CNS at 4-6 weeks after transplantation (Liu *et al.*, 2006; Kuh *et al.*, 2005). Thus, it seems evident that HUCB cells grafted to the brain of adult rats are the subject of considerable immune surveillance. The transplantation of any type of alien cells invariably initiates mechanisms that coordinate innate and adaptive immune responses. As a consequence the majority of HUCB cells transplanted to the adult brain of rodents exhibit poor long-term survival and meager differentiation.

The past two decades have shown significant progress in basic understanding of adult stem cells biology. HUCB-derived and neurally committed cells, due to their capacity for self renewal, multilineage differentiation, plasticity and ability for long-lasting culturing *in vitro*, make them unique as a powerful tool for the cell therapy of a wide spectrum of neurological diseases. However, in spite of the first promising data it would be much premature at this step of our knowledge to launch any relevant clinical trials. Instead, there is an urgent need for deepening of our understanding of adult stem cell biology and to improve available animal models to induce tolerance toward alien human cells. This would be critical for exploring the real power of human stem cells in any preclinical experimental settings. In addition, the immunology of grafted neural stem/progenitor cells of hematopoietic-tissue origin has not been extensively studied. Overall, only after solving all these problems we would be able to determine whether, to what extend, and by which mechanism(s), the transplantation of defined CB-derived neural progenitor/stem cell populations can be used to cure human brain.

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