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The influence of BDNF on human umbilical cord blood stem/progenitor cells: Implications for stem cell-based therapy of neurodegenerative disorders

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Umbilical cord blood (UCB)-derived stem/progenitor cells (SPCs) have demonstrated the potential to improve neurologic function in different experimental models. SPCs can survive after transplantation in the neural microenvironment and induce neuroprotection, endogenous neurogenesis by secreting a broad repertoire of trophic and immunomodulatory cytokines. In this study, the influence of brain-derived neurotrophic factor (BDNF) pre-treatment was comprehensively evaluated in a UCB-derived lineage-negative (Lin^-) SPC population. UCB-derived Lin^- cells were evaluated with respect to the expression of i) neuronal markers using immunofluorescence staining and ii) specific (TrkB) receptors for BDNF using flow cytometry. Next, after BDNF pre-treatment, Lin^- cells were extensively assessed with respect to apoptosis using Western blotting and proliferation *via* BrdU incorporation. Furthermore, NT-3 expression levels in Lin^- cells using RQ PCR and antioxidative enzyme activities were assessed. We demonstrated neuronal markers as well as TrkB expression in Lin^- cells and the activation of the TrkB receptor by BDNF. BDNF pre-treatment diminished apoptosis in Lin^- cells and influenced the proliferation of these cells. We observed significant changes in antioxidants as well as in the increased expression of NT-3 in Lin^- cells following BDNF exposure. Complex global miRNA and mRNA profiling analyses using microarray technology and GSEA revealed the differential regulation of genes involved in the proliferation, gene expression, biosynthetic processes, translation, and protein targeting. Our results support the hypothesis that pre-treatment of stem/progenitor cells could be beneficial and may be used as an auxiliary strategy for improving the properties of SPCs.

Key words*:* gene expression, BDNF, lineage-negative cells, stem/progenitor cells, umbilical cord blood

INTRODUCTION

Human umbilical cord blood (UCB) is an attractive source of unique stem/progenitor cells (SPCs) with immature characteristics and abundant proliferative potential related to an elongated life span and longer telomeres compared with SPCs derived from other sources, such as bone marrow or peripheral blood (Kögler et al. 2004). An immunomagnetic cell separation based on the presence of surface antigen is an efficient method for the rapid and gentle sorting of SPCs to prepare selected cell types for use in research and clinical applications. Efficacy and safety of intravenous administration of immunoselected autologous $CD34^+$ cells in hematopoietic reconstitution following high dose chemotherapy in patients with cancer has been proved (Chou et al. 2005). $CD34^+$ stem cells isolated by magnetic sorting are also chosen for SPC transplantation in tissue regeneration in clinical trials (Fujita et al. 2014, Tsuji et al. 2014). However, a growing body of evidence indicates that cells lacking the CD34 antigen also contain valuable SPCs. An alternative stem cell marker prominin-1 (CD133) was found to be applicable for positive selection methods targeting

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more primitive hematopoietic SPCs enriched in $CD34^+$ cells (Passino et al. 2000). A methodology based on immunomagnetic negative selection has been developed to deplete mononuclear cells (MNCs) of hematopoietic lineage marker-expressing mature cells. This strategy for isolating the lineage-negative (Lin^-) cell population was developed to confirm the presence of multipotent cells in various sources, e.g., human UCB. Unfortunately, Lin^- cells are a poorly characterized heterogeneous SPC population that contains a small percentage of stem cells. Most of the cells in this population are progenitors. UCB-derived Lin^- cells contain both primitive $CD34^+$ and $CD133^+$ cells as well as a proportion of $CD34^-$ cells that only intracellularly express the CD34 protein; these cells also represent a promising population of immature cells (Forraz et al. 2004).

Neurodegenerative diseases affect millions of people worldwide. UCB contains a population of cells capable of differentiating into neural cells, and this ability holds special promise for the treatment of neural diseases currently lacking effective therapy (Buzanska et al. 2002). The therapeutic potential of UCB SPCs may be attributed to the assumed replacement of damaged cells in injured tissues, or alternatively, and more likely, to their ability to augment repair processes in damaged tissues through neural protection and the secretion of neurotrophic factors (Sanberg et al. 2005). In a previous paper, we provided evidence for the presence of neurotrophins (NTs) and their receptors in distinct UCB-derived SPC populations, showing that these cells express NTs and NT receptors at both the mRNA and protein levels (Paczkowska et al. 2013). Moreover, the humoral activity of UCB-derived SPCs has been described in *in vitro* experiments as well as *in vivo* experiments involving animal models of cell transplantation (McGuckin et al. 2004, Sanberg et al. 2005).

The aim of this study was to elucidate the effects of pre-treatment with a potent neurotrophic growth factor (brain-derived neurotrophic factor, BDNF) on unique UCB-derived Lin^- SPCs. BDNF plays a critical role in the CNS and is involved in the development of the nervous system and neuronal differentiation, survival, plasticity, and function (Huang and Reichard 2001). In addition to these important functions in neurons, BDNF also promotes angiogenesis, exerts proangiogenic properties, and modulates inflammatory processes (Jiang et al. 2010, Blais et al. 2013, Paczkowska et al. 2014). BDNF activates two classes of receptors: neurotrophin receptor (p75NTR), which is a member of the tumor necrosis factor receptor family, and the more specific high-affinity binding TrkB receptor, which possesses internal tyrosine kinase activity (Bartkowska et al. 2010). The binding of BDNF with its relevant receptors mediates specific effects by activating downstream signaling pathways. As a result, TrkB receptors mediate the proliferation, survival or differentiation of neuronal cells (Huang and Reichardt 2003).

In our previous study, we demonstrated that a UCB-derived Lin^- cells spontaneously express NTs and their receptors at the mRNA and protein levels and that after a short-term incubation under serum-free conditions, Lin^- cells express significantly higher mRNA and protein levels of BDNF and NT-3 than under steady-state conditions (Paczkowska et al. 2013). However, although Lin^- cells possess receptors for most NTs, little is known regarding the response of Lin^- cells to BDNF stimulation. The pre-treatment of transplanted cells with NTs may play a role in improving the efficiency of stem cell-based therapies by influencing the survival of transplanted stem cells and facilitating neurotrophic effects. Therefore, in this study, our purpose was to examine the presence of BDNF receptors and their activation during the BDNF stimulation of Lin^- cells. Next, we assessed the influence of BDNF on the apoptosis, proliferation, oxidative stress, and expression of neurotrophin-3 (NT-3) as well as the overall mRNA and miRNA expression patterns. Our hypothesis was that BDNF pre-treatment is associated with beneficial changes in the activity of UCB-derived Lin^- cells.

METHODS

Sample collection

Human UCB samples were obtained from the Department of Feto-Maternal Medicine and Gynecology of the Pomeranian Medical University in Szczecin, Poland. UCB specimens were collected from the placentas and umbilical cords of healthy full-term deliveries. This study adhered to the tenets of the Declaration of Helsinki, and approval was obtained from the Local Research Ethics Committee. Moreover, the women involved gave written informed consent prior to involvement.

Isolation of human umbilical cord blood-derived Lin⁻ cells

The full population of UCB nucleated cells (NCs) was obtained after erythrocyte lysis using BD PharmLyse Lysing Solution (BD Biosciences Pharmingen, San Jose, CA, USA) for 15 min at room temperature in the dark. The cells were then washed twice in phosphate-buffered saline (PBS). The obtained suspension of NCs was subjected to immunomagnetic separation procedures. Lin⁻ cells were isolated using a Lineage Cell Depletion Kit (Miltenyi Biotec, Auburn, CA, USA). Isolation procedures were performed according to the manufacturer's instructions, as previously described (Paczkowska et al. 2013). The total number of isolated Lin⁻ cells was determined using a TC Automated Cell Counter (Bio-Rad, Philadelphia, PA, USA).

Flow cytometry

Freshly immunomagnetically isolated UCB-derived Lin⁻ cells were subjected to flow cytometry to determine the presence of specific TrkB receptors for BDNF. The Lin⁻ cells were resuspended in 100 µL PBS and stained for TrkB receptor using mouse anti-human monoclonal anti-TrkB antibody (R&D Systems, Minneapolis, MN, USA) for 15 min at room temperature in the dark. The cells were washed twice with PBS and incubated with secondary goat anti-mouse monoclonal antibody conjugated with FITC (BD Biosciences) for 15 min at room temperature in the dark. Subsequently, the cells were washed twice with PBS and resuspended in 1% paraformaldehyde.

Fluorescence was measured and the data were analyzed using a fluorescence-activated cell analyzer (LSRII, BD Biosciences) and the BD FACSDiva software. Typically, 10 000 events were acquired to determine the percentage of cells expressing surface receptors.

Immunofluorescence analysis

Freshly immunomagnetically isolated UCB-derived Lin⁻ cells were subjected to immunofluorescence staining for neuronal markers. First, the cells were fixed with 3.7% paraformaldehyde and then smeared on polylysine-coated slides. After permeabilization in 0.5% Tween 20 (Bio-Rad) and blocking with 10% normal goat serum, the smears were incubated at 4°C overnight with one of the following primary antibodies: rabbit anti-Doublecortin (1:100) (Novus Biologicals, Littleton, CO, USA), rabbit anti-β-III-tubulin (1:50) (OriGene Technologies, Rockville, MD, USA), chicken anti-GFAP (1:50) (LifeSpan Biosciences, Seattle, WA, USA) or mouse anti-NeuN (1:100) (GenTex, Zeeland, MI, USA). Subsequently, the cells were incubated in the dark with the relevant secondary antibodies: goat anti-rabbit-TR (1:100) (Vector Laboratories, Burlingame, CA, USA), goat anti-chicken Alexa Fluor 488 (1:100) (Life Technologies, Paisley, UK), or goat anti-mouse Alexa Fluor 488 F(ab')2 (1:100) (Life Technologies). Upon termination, all of the sections were counterstained with DAPI solution (Thermo Fisher Scientific, Waltham, MA, USA), mounted, and examined using an LSM700 confocal system (Carl Zeiss, Jena, Germany). For quantification of the percentage of cells expressing a specific marker the number of positive cells was determined in relation to the total number of DAPI labeled nuclei. Counts of immunoreactive cells were made in 10 random fields in the slides for each with a 20 objective.

Incubation of Lin⁻ cells with BDNF

Lin⁻ SPCs were incubated at a density of 1.5 to 2.5×10^6 cells/well in 24-well culture plates on non-treated plastic in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich, St. Louis, MO, USA) containing bovine calf serum (BCS) (5%), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) at 37°C under a humidified atmosphere containing 5% CO_2. The cells were treated or not with recombinant human BDNF 50 ng/mL (Sigma-Aldrich) and collected after 2 or 24 h of incubation. The incubated cells were then subjected to mRNA, microRNA, and protein isolation, antioxidant activity measurements, and proliferation assays.

Lin⁻ cell proliferation

Cell proliferation was assessed by measuring BrdU incorporation into newly synthesized DNA strands of actively proliferating cells using an Apoptosis, DNA damage, and Cell Proliferation Kit according to the manufacturer's protocol (BD Biosciences). The Lin⁻ cells, some of which had been treated with BDNF for 24 h, were incubated with a 10 µM BrdU labeling solu-

tion at 37°C for 2 h and then collected. BrdU incorporation was measured *via* flow cytometry using a FITC-conjugated mouse monoclonal anti-BrdU antibody. The results are expressed as the percentage of cells in S phase.

Assays of Lin⁻ cell antioxidants

To quantitatively assess changes in the Lin⁻ cells' antioxidative response, Lin⁻ cells treated or untreated with BDNF were collected after 2 and 24 h of incubation, washed twice in PBS and then resuspended in 0.5 mL of PBS. Afterwards, cells were frozen and stored at −80°C. Lin⁻ cell lysates were obtained by adding Triton X-100 (0.1%) to defrosted Lin⁻ cell suspensions. SOD, catalase and glutathione transferase (GST) activity were measured in cell lysates *via* a kinetic method using a UV/VIS Lambda 650 (Perkin-Elmer, USA) spectrophotometer according to previously described methods (Dołęgowska et al. 2010). The SOD sensitivity was 0.1 U/mL and specificity was 97%; the coefficient of variation was lower than 5%. The enzyme activity was calculated per 1 mg of cellular protein. All reagents were purchased from Sigma-Aldrich.

Real time QRT-PCR

Total mRNA was isolated from Lin⁻ cells using the mirVana™ miRNA Isolation Kit (Life Technologies). Subsequently, the mRNA was reverse-transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). A quantitative assessment of gene expression was performed using real time QRT-PCR carried out on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Inc.). The 15 µL reaction mixture contained 7.5 µL of SYBR Green PCR Master Mix, 10 ng of cDNA template, and one pair of primers 5'- GGT ACG CGG AGC ATA AGA GTC-3' (forward) and 5'-GAG AGT TGC CCG TTT TGA TCT-3' (reverse) for NT-3; 5'-AAT GCG GCA TCT TCA AAC CT-3' (forward) and 5'-TGA CTT TGT CAC AGC CCA AGA TA-3' (reverse) for beta-2 microglobulin (BMG). The threshold cycle (Ct), i.e. the cycle number at which the amount of the amplified gene of interest reached a fixed threshold, was subsequently determined. The relative target gene mRNA expression was quantified using the comparative Ct method. The relative quantification value of the target was normalized to the endogenous control BMG gene and expressed as $2\Delta Ct$, where ΔCt = [Ct of target genes] − [Ct of endogenous control gene (BMG)]. The expressions of the genes in Lin⁻ cells after exposure to BDNF were expressed as percentages of the relative expressions in untreated cells.

Western blot analysis

Western blot analysis was performed to evaluate the expression of the unphosphorylated and phosphorylated kinase Akt, procaspase 3, and active caspase 3. The UCB-derived Lin⁻ cells (3×10^6) were lysed for 10 min on ice in M-Per lysing buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma-Aldrich) (10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 mM sodium fluoride, and 2 mM Na_3VO_4). Three cell lysates were pooled and equal amounts of protein (20 µg/well) were loaded and separated on a 4–20% sodium dodecyl sulfate polyacrylamide gel *via* electrophoresis (SDS-PAGE, mini-PROTEAN II electrophoresis system, Bio-Rad) and then transferred to a 0.2 µm polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Kaleidoscope polypeptide standard wide range (10–250 kD) protein markers (Bio-Rad) were used to determine the molecular weights of the analyzed proteins. After blocking non-specific binding for 2 h at room temperature with a 3% BSA, Tris-HCl and NaCl solution with 0.05% Tween 20, the membrane was probed with a specific monoclonal/polyclonal IgG antibody directed against amino acid sequences of the selected proteins (unphosphorylated and phosphorylated kinase Akt, procaspase 3, active caspase 3): rabbit anti-Akt monoclonal antibody (at 1:1 000 dilution, Cell Signaling), rabbit anti-phospho-Akt (Ser473) monoclonal antibody (at 1:500 dilution, Cell Signaling), rabbit anti-proCASP-3 polyclonal antibody (at 1:750 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-activeCASP-3 polyclonal antibody (at 1:750 dilution, Santa Cruz Biotechnology,) and incubated overnight at 4°C. Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary Ab (Santa Cruz Biotechnology) specific to the primary antibody used in the previous step. Chemiluminescence detection was performed using the ECL Advance Detection Kit (Amersham Life Sciences, Buckinghamshire, UK), and the bands were subsequently visualized with a UVP camera (Gel DOC-It Imaging system, Bio-Rad).

RNA Isolation and Affymetrix GeneChip Microarray and data analysis

We analyzed samples of UCB Lin⁻ cells after 2 and 24 h of incubation with or without BDNF. Total RNA was isolated using the mirVana™ miRNA Isolation Kit (Life Technologies). RNA isolates from three separate cell incubations were pooled to generate one sample for subsequent experimental procedures. Sense-strand cDNA generated from total RNA using an Ambion WT Expression Kit (Life Technologies) was fragmented and labeled using the GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) and hybridized onto an Affymetrix WT Array Strip. The hybridization and subsequent fluidics and scanning steps were performed using an Affymetrix GeneAtlas™ system (Affymetrix). The microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2743. All microarray data analysis were done in R statistical environment. miRNA data were preprocessed using ExiMiR Bioconductor package and NormiR function (with default settings). mRNA data were analyzed using oligo Bioconductor package with default options. In our analysis, groups of over- and under-expressed genes were verified for over-representation of genes from Gene Ontology terms (biological processes ontology) using Fisher-exact test. Computed *P*-values were adjusted using Bonferroni correction.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was used for the investigation of global dysregulations of the biological pathways between the Lin⁻ cells after 2 and 24 h of incubation with or without BDNF. For the pathway investigation, the pre-defined gene sets are from Molecular Signatures Database (MsigDB) (Subramanian et al. 2005). For comparison of BDNF treated cells to not treated, GSEA was performed using the normalized data of entire 48 803 transcripts (GSEA version 2.07, Broad Institute http://www.broad.mit.edu/gsea). Permutation was conducted 1 000 times according to default-weighted enrichment statistics and using a signal-to-noise metric to rank genes according to their differential expression levels across the BDNF treated and untreated Lin⁻ cells. To adjust for multiple hypothesis testing, the maximum enrichment score (ES) was normalized to account for the gene set size (NES) and the false discovery rate (FDR) corresponding to each NES was calculated. Significant gene sets were defined as those with a nominal *P*-value <0.05.

miRNA Isolation and Affymetrix GeneChip Microarray and data analysis

We prepared and analyzed samples of UCB Lin⁻ cells after 2 and 24 h of incubation with or without BDNF. The total RNA was isolated from the incubated cells using the mirVana™ miRNA Isolation Kit (Life Technologies) following the manufacturer's protocol. RNA isolates from three separate cell incubations were pooled to generate one sample for the subsequent experimental procedure. A total of 130 ng of the RNA was used. The process was started with a poly (A) tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA. The samples were then hybridized onto an Affymetrix miRNA 4.1 Array Strip (Affymetrix) at 48°C for 20 h. After washing and labeling with streptavidin-PE, the array was scanned with the use of an Affymetrix GeneAtlas system (Affymetrix). The microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2794. All microarray data analysis were done in R statistical environment. miRNA data were preprocessed using ExiMiR Bioconductor package and NormiR function (with default settings). mRNA data were analyzed using oligo Bioconductor package with default options. In our analysis, groups of over- and under-expressed genes were verified for over-representation of genes from gene Ontology terms (biological processes ontology) using Fisher-exact test. Computed *P*-values were adjusted using Bonferroni correction.

miRNA-mRNA Correlation Analysis

We used six databases to determine which miRNA targets were annotated (Targetscan database http://www.targetscan.org/, TarBase http://diana.cslab.ece.ntua.gr/tarbase/, Miranda http://www.ncrna.org/, miRBase http://www.mirbase.org/, mi RNA Target database http://www.ncrna.org/, and PicTar http://pictar.

mdc-berlin.de/). We assumed that the miRNA targets the mRNA when putative mRNA targets were annotated in at least four databases. Then, we analyzed the mRNAs and miRNAs by selecting a group of down-regulated miRNAs to predict the target upregulated mRNAs (included in the differential expression genes obtained in the mRNA microarray analysis). Next, all of the differentially expressed genes with corresponding miRNA changes were classified (using Bonferroni's correction) according to the Gene Ontology (GO) Classification of Biological Processes.

Statistics

The arithmetical means and standard deviations were calculated using MS Excel. Comparisons of parameters between two groups were made using unpaired Student's *t*-test. Because of abnormal distributions of analyzed data the Mann-Whitney test was used to compare the expression levels of NT-3 in Lin^- cells after 2 h or 24 h of exposure to BDNF compared with control cells. $P<0.05$ was considered statistically significant.

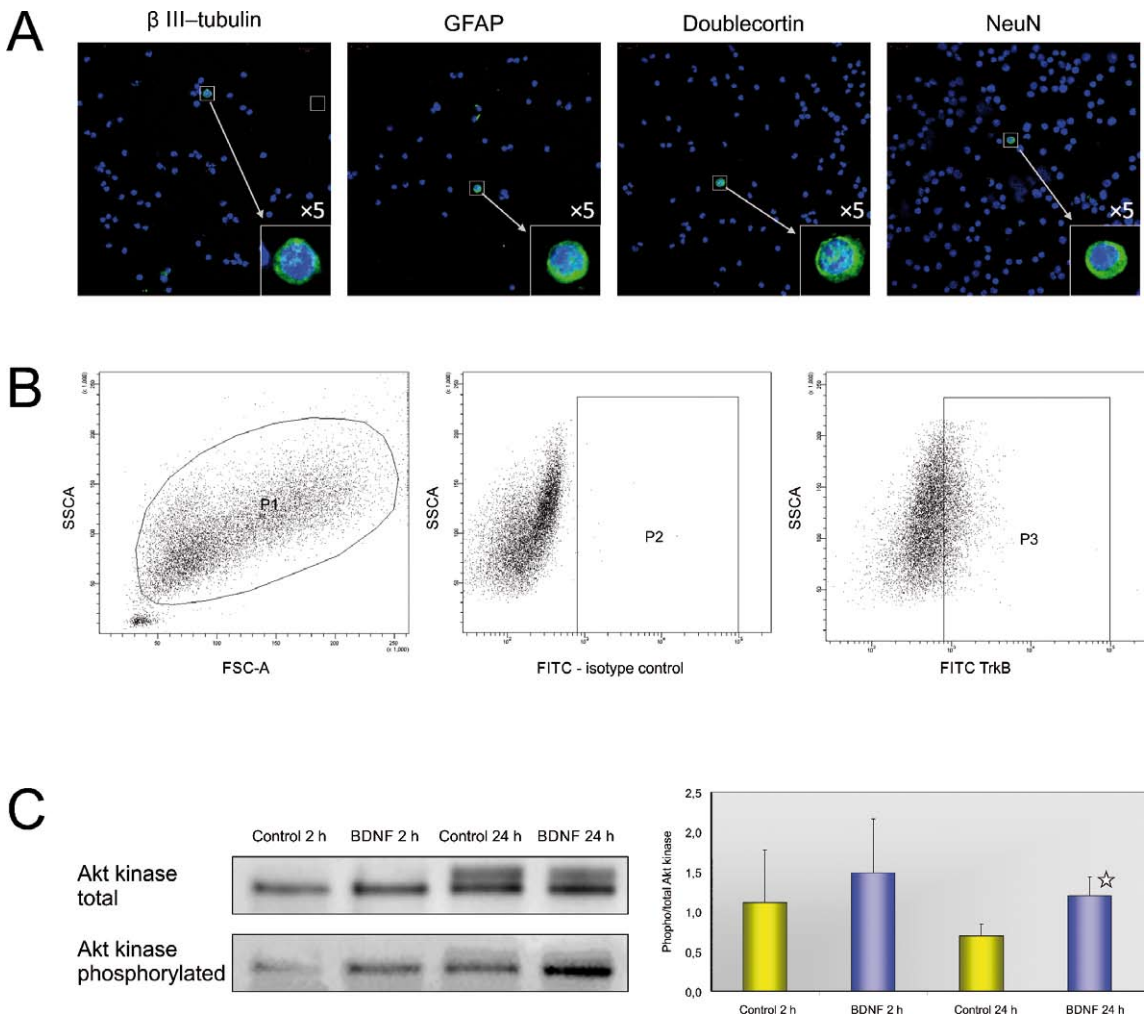

Fig. 1. (A) Immunofluorescence analysis of the spontaneous expression of neural markers by UCB-derived Lin^- cells. Unique cells were β III-tubulin, GFAP, Doublecortin, and NeuN positive. Nuclei were visualized *via* DAPI staining. All images were captured using a LSM700 confocal system (Carl Zeiss, Jena, Germany). Representative and selected data are shown. (B) UCB-derived Lin^- cells express BDNF TrkB receptors. Representative dot-plots showing Lin^- cells gating on the side scatter (SSC) and forward scatter (FSC) (gate P1). Events contained by gate P3 were characterized by the presence of TrkB receptor. (C) BDNF activates TrkB receptors. Lin^- cells were cultured and treated with BDNF (50 ng/mL) for 2 and 24 h. Western blotting analysis showed increased activation (phosphorylation) of Akt with BDNF stimulation in Lin^- cells compared with cells without BDNF stimulation after 24 h of incubation. Total Akt were used to control the amount of protein loaded and the ratio of phosphorylated/total protein was quantified (n=4).

RESULTS

Human UCB-derived Lin⁻ cells

The mean volume of the collected UCB units was 60.76±23.7 mL and ranged from 47 to 117 mL. The total average number of NCs recovered from a single unit was 613.7±305.4×10⁶ cells. We successfully isolated the Lin⁻ cells from human UCB using immunomagnetic separation. The number of Lin⁻ cells recovered from 100×10⁶ NCs was 1.41±1.0×10⁶.

Lin⁻ cells contain cells expressing specific neuronal markers

Accumulating data indicate that Lin⁻ cells are a very heterogeneous population and are therefore not well characterized. By employing immunofluorescence staining, we here showed the expression of specific neuronal markers in a small portion of Lin⁻ cells. We found that 6.43±5.13% of freshly isolated Lin⁻ cells expressed β-III-tubulin, 3.01±2.3% expressed GFAP, 2.17±1.46% expressed Doublecortin, and similarly 1.66±2.75% expressed NeuN (Fig. 1A). These results support the hypothesis that Lin⁻ cells are heterogeneous population and contain cells with different characteristic.

UCB Lin⁻ cells express neurotrophin receptors

Before BDNF stimulation of Lin⁻ cells we evaluated the expression levels of the specyfic BDNF receptors TrkB on the Lin⁻ cells by flow cytometry. TrkB receptors were expressed by Lin⁻ cells, as demonstrated in Figure 1B. Cytofluorometric analysis revealed that 44.3±23.19% of Lin⁻ cells expressed TrkB receptors.

Priming UCB-derived Lin⁻ cells with BDNF activates the relevant receptors

BDNF binds TrkB with high affinity to trigger its dimerization as well as to trigger the autophosphorylation of tyrosine residues in the cytoplasmic kinase domain, which act as docking sites for effector molecules and induce the activation of three main signaling pathways (the PLCγ, PI3K and ERK cascades) (Huang and Reichardt 2003). We focused on the PI3K signaling pathway because it has already been described as playing a pivotal role in NT receptor signaling. The expression levels of phosphorylated Akt were analyzed after 2 h and 24 h incubation with BDNF using Western blotting. We observed the phosphorylation of Akt after Lin⁻ cells were exposed to 50 ng/ml of BDNF for 24 h (Fig. 1C). Taken together, our analysis using the Western blotting technique demonstrated the phosphorylation of Akt, which indicated that BDNF exposure leads to the activation of TrkB receptors present on UCB-derived Lin⁻ cells. The efficient activation and signaling of relevant receptors could stimulate Lin⁻ cells and be responsible for the effects of BDNF in these cells.

Apoptosis in cultured Lin⁻ cells is noticeably reduced by BDNF

UCB cells were subjected to red blood cell lysis and immunomagnetic isolation. Both of these processes could be stress stimuli for these cells and could involve apoptotic mechanisms; thus, we examined this phenomenon in Lin⁻ BDNF-treated cells. After 24 h of incubation, we observed that the level of activated caspase 3 was lower in the BDNF-treated cells compared with the untreated cells (Fig. 2A). We observed that BDNF effectively suppressed the cleavage of procaspase 3 in UCB-derived Lin⁻ cells suggesting the anti-apoptotic action of BDNF on Lin⁻ cells.

The Lin⁻ cell antioxidative system is modified during incubation with BDNF

An analysis of the mean activity of Lin⁻ antioxidants measured after 2 h of incubation revealed no significant differences between the BDNF-treated and untreated Lin⁻ cells. In contrast with these observations, we found the lower activities of SOD and tendency toward decreased GST activity in Lin⁻ cells following 24 h BDNF pre-treatment than in control Lin⁻ cells (Fig. 2B, C, D). We observed increased SOD activity in untreated cells after 24 h incubation compared with 2 h and a tendency toward increased GST activity in these cells. Pre-treatment with BDNF decreased antioxidant activity levels in UCB-derived Lin⁻ cell after 24 h of incubation.

BDNF pre-treatment does not influence proliferation of the UCB-derived Lin⁻ cells in culture after 24 h

We did not observe significant differences in percent of BrdU positive cells after 24 h incubation of

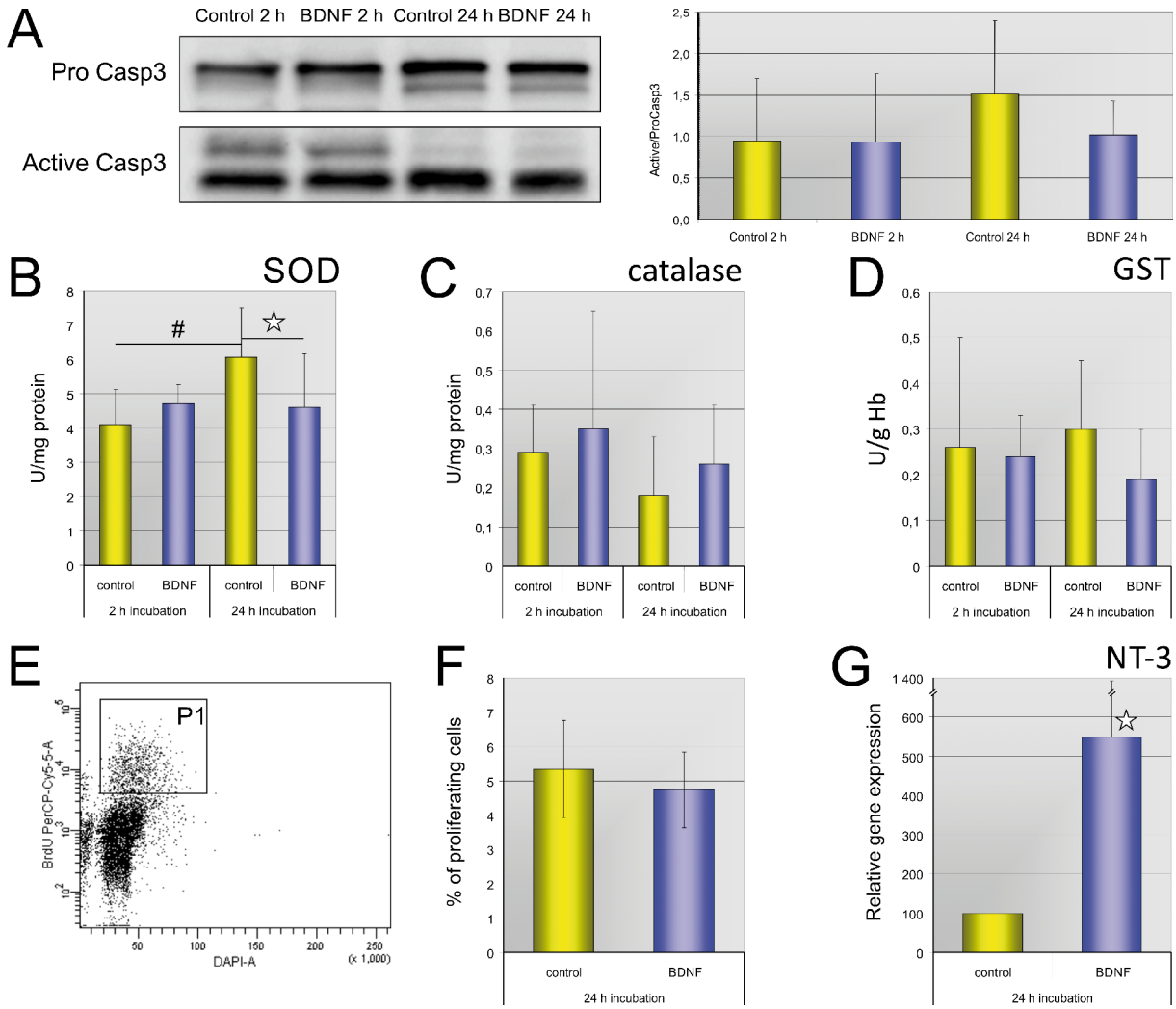

Fig. 2. (A) Effect of BDNF on apoptosis in UCB-derived Lin⁻ cells. Lin⁻ cells were cultured and treated with BDNF (50 ng/mL) for 2 and 24 h. Protein lysates were stained with antibodies against cleaved caspase 3. Procaspase 3 was used to control the amount of protein loaded and the ratio of active/procaspase was quantified. Western blotting analysis showed decreased activation (cleavage) of caspase 3 in cells with BDNF stimulation compared with the cells without BDNF stimulation after 24 h of incubation (n=5). (B) Effects of BDNF stimulation on superoxide dismutase (SOD) activity in Lin⁻ cells after 2 (n=5) and 24 h (n=9). Data are presented as the means ± SD. *P<0.05 *versus* control without BDNF stimulation. #P<0.05 *versus* control cells without BDNF stimulation after 2 h. (C) Effects of BDNF stimulation on catalase activity in Lin⁻ cells after 2 (n=5) and 24 h (n=9). Data are presented as the means ± SD. (D) Effects of BDNF stimulation on glutathione S-transferase (GST) activity in Lin⁻ cells after 2 (n=5) and 24 h (n=9). Data are presented as the means ± SD. (E) Effects of BDNF 24 h stimulation on BrdU incorporation by UCB Lin⁻ cells measured using flow cytometry (representative flow cytometry plot). Gate 1 contained proliferating population indicated *via* BrdU and DAPI staining. (F) Bar chart presents the proliferation of Lin⁻ cells measured using flow cytometry with data expressed as the percentage of proliferating cells. Data are presented as the means ± SD (n=5). (G) QRT-PCR analysis showed an increased expression of NT-3 in Lin⁻ cells stimulated with BDNF after 24 h of incubation. Relative mRNA expression values were normalized against BMG expression values. Data are expressed as the percentage of expression compared with the cells without BDNF stimulation. Data are presented as the means ± SD. *P<0.05 *versus* control without BDNF stimulation (n=11).

Table I

Top ten genes with the highest up- or downregulation in UCB-derived lineage-negative cells after 2 h of BDNF treatment

Symbol	Gene title	Fold change
CANX	calnexin	2.12
PARK7	parkinson protein 7	2.052
KAT7	K(lysine) acetyltransferase 7	2.05
USP22	ubiquitin specific peptidase 22	1.928
SEPT2	septin 2	1.911
EIF3D	eukaryotic translation initiation factor 3, subunit D	1.847
ANAPC5	anaphase promoting complex subunit 5	1.793
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.672
RPS3AP5	ribosomal protein S3A pseudogene 5	1.641
SOWAHC	GDP dissociation inhibitor 2	1.612
RNA5SP74	RNA, 5S ribosomal pseudogene 74	−1.497
CAMTA2	calmodulin binding transcription activator 2	−1.53
CBX3	chromobox homolog 3	−1.532
RPL14P1	ribosomal protein L14 pseudogene 1	−1.582
RNA5SP403	RNA, 5S ribosomal pseudogene 403	−1.776
RNA5SP191	RNA, 5S ribosomal pseudogene 191	−1.823
TMED2	transmembrane emp24 domain trafficking protein 2	−1.836
RNA5SP443	RNA, 5S ribosomal pseudogene 443	−1.938
SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	−2.005
Y_RNA	Y RNA	−2.544

UCB-derived Lin$^-$ cells treated or not with BDNF (Fig. 2E, F). It suggests that BDNF does not influence the proliferation of Lin$^-$ cells after 24 h.

BDNF pre-treatment increases NT-3 expression in the UCB Lin$^-$ cells

To verify whether BDNF stimulation altered the expression of NT-3 in Lin$^-$ cells, we performed real time QRT-PCR and determined the expression levels of NT-3 in BDNF-treated Lin$^-$ cells and controls after 2 and 24 h of incubation. We found that NT-3 expression was significantly higher in Lin$^-$ cells following BDNF exposure compared with unstimulated cells after 24 h (Figure 2G). However, NT-3 expression levels were not elevated after 2 h of incubation (data not shown). These data suggest that BDNF priming has a favorable influence on the trophic activity of Lin$^-$ cells, increasing the expression of neuroprotective factor, NT-3, in Lin$^-$ SPCs.

Whole-genome microarray analysis of gene expression in Lin$^-$ cells treated with BDNF shows an increase in genes associated with the translational processes and protein production

To further characterize the influence of BDNF priming on UCB Lin$^-$ cells, we analyzed the global gene expression pattern of the BDNF-treated Lin$^-$ cells

Table II

Top ten genes with the highest up- or downregulation in UCB-derived lineage-negative cells after 24 h of BDNF treatment

Symbol	Gene title	Fold change
LAMP3	lysosomal-associated membrane protein 3	3.893
TCEB2	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)	3.416
U6	U6 spliceosomal RNA	3.124
ANAPC5	anaphase promoting complex subunit 5	3.021
Y_RNA	Y RNA	3.011
HNRNPA1	heterogeneous nuclear ribonucleoprotein A1	2.499
VTRNA1-1	vault RNA 1-1	2.267
STARD7	StAR-related lipid transfer (START) domain containing 7	2.263
SRSF9	serine/arginine-rich splicing factor 9	2.249
SOWAHC	sosondowah ankyrin repeat domain family member C	2.201
TARDBP	TAR DNA binding protein	−2.141
SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	−2.257
KARS	lysyl-tRNA synthetase	−2.266
CANX	calnexin	−2.304
ILF2	interleukin enhancer binding factor 2, 45kDa	−2.333
SEPT2	septin 2	−2.438
NARS	asparaginyl-tRNA synthetase	−2.542
SART3	squamous cell carcinoma antigen recognized by T cells 3	−2.592
SART1	squamous cell carcinoma antigen recognized by T cells	−2.66
CAPNS1	calpain, small subunit 1	−3.458

versus the controls. Microarray analysis revealed that 56 genes were at least 2-fold upregulated and that 43 genes were at least 2-fold downregulated in the Lin^- cells treated with BDNF for 2 h compared with the cells cultured without BDNF (control). Moreover, the microarray analysis revealed that 94 genes were at least 2-fold upregulated in the Lin^- cells treated with BDNF for 24 h compared with the untreated control cells; 74 genes were 2-fold (or greater) downregulated in the Lin^- cells treated for 24 h with BDNF compared with the control cells. The genes with the largest change in expression are presented in Table I for the Lin^- cells treated with BDNF after 2 h compared with the untreated control cells. The most strongly up-regulated gene after 2 h treatment of BDNF was the calnexin (CANX). Calnexin is a chaperone, characterized by assisting protein folding and quality control, ensuring that only properly folded and assembled proteins proceed further along the secretory pathway. The other most strongly up-regulated genes included the parkinson protein 7 (PARK7) which product acts as a positive regulator of androgen receptor-dependent transcription and protects neurons against oxidative stress and cell death; K(lysine) acetyltransferase 7 (KAT7), which has been implicated in the pre-replication complex assembly, transcriptional regulation, and organ regeneration. The genes with the largest change in expression are presented in Table II for the Lin^- cells

treated with BDNF after 24 h compared with the untreated control cells. The most strongly up-regulated gene after 24 h treatment of BDNF was the lysosomal-associated membrane protein 3 (LAMP3). The product of this gene mediates signal transduction events that plays a role in the regulation of cell development, activation, growth and motility. The other most strongly up-regulated genes included the transcription elongation factor B (TCEB2), which activates elongation by RNA polymerase II and U6 which participate in several RNA-RNA and RNA-protein interactions.

Next, all of the differentially expressed genes were classified according to the Gene Ontology (GO) Classification of Biological Processes. Functional analysis using GO revealed that a number of pathways were specifically and diversely represented in the analyzed UCB-derived SPCs. Given the ability of SPCs to exert a neuroprotective effect *via* trophic action, we were interested in biological processes related to biosynthetic processes and cytokine production. Comparing the bioinformatic analysis of the complex gene dataset in Lin^- cells treated with BDNF with that of untreated cells indicated that the genes involved in the translational processes were among the most upregulated; these genes included the specific genes RPL18, RPL27, RPL14, RPS7, SRP14, FAU, RPL30. A summary of the selected distribution of genes of interest according to the Gene Ontology Classification of Biological Processes is shown in Figure 3A. Overall, our analysis of the global gene expression changes revealed that a number of genes are expressed in different patterns within Lin^- cells after BDNF treatment. An increased expression was observed in the genes that are associated with translational processes and protein production.

Gene enrichment analysis

To further consider the biological implication of changes in gene expression after BDNF stimulation, we used GSEA in the classical manner, to identify pathways that are altered under BDNF stimulation after 2 h and 24 h (Subramanian et al. 2005). GSEA evaluates how genes in queried pathways are distributed in the fold change (lesional *versus* non-lesional) ordered list generated by our data. This was quantified by using the Enrichment Score (ES), a weighted Kolmogorov-Smirnov-like statistic that evaluates if the members of the pathway are randomly distributed or found at the extremes (top or bottom) of the list. A normalized enrichment score (NES) evaluates the number of genes in the pathway. A positive NES denotes that the list of genes is enriched at the beginning of the ordered fold change list, and a negative NES denotes that the list in question is enriched at the end of the list.

GSEA revealed altered regulation of the biological pathways in Lin^- cells treated by BDNF for 2 h compared to untreated cells. For the proliferation and apoptosis, the cell cycle related pathways including DNA replication, cell cycle check points, G1/S transition, and mitotic M-M/G1 phases, were significantly enriched in BDNF treated cells after 2 h compared to control cells. Further, among the pathways enriched in BDNF treated cells were pathways involved in the gene expression including reactome mRNA processing, reactome mRNA splicing, reactome processing of capped intron-containing pre mRNA (Table IV, Fig. 4). In contrast, GSEA analysis revealed only two biological pathways with over-representation in Lin^- cells under BDNF stimulation after 24 h with FDR <25% (pyeon cancer head and neck vs. cervical dn, reactome beta defensins).

BDNF pre-treatment modulates miRNA Expression Profiling

miRNAs are small, noncoding RNA molecules that regulate gene expression by binding the 3′-untranslated region (3′-UTR) of the mRNAs of their target genes, resulting in mRNA cleavage or translation repression. miRNAs act on approximately 60% of all protein-coding genes (Friedman et al. 2009). Emerging evidence supports the important functions of miRNAs in coordinating many fundamental cellular processes, including development, proliferation, differentiation, death, and metabolism. In the current study, we used miRNA microarrays to identify the miRNAs that Lin^- cells express when treated with BDNF. The result of miRNA expression microarray analysis showed that 9 miRNAs were upregulated and 28 were downregulated after Lin^- cells were treated with BDNF for 2 h (fold change ≥2). A total of 58 upregulated miRNAs and 26 downregulated miRNAs (test *versus* control, fold change ≥2) were obtained after the Lin^- cells were treated with BDNF for 24 h. Table III presents a list of the miRNAs that exhibited the greatest level of differential expression after 2 and 24 h of BDNF treatment.

Furthermore, an miRNA/mRNA correlation analysis was carried out in our study to further charac-

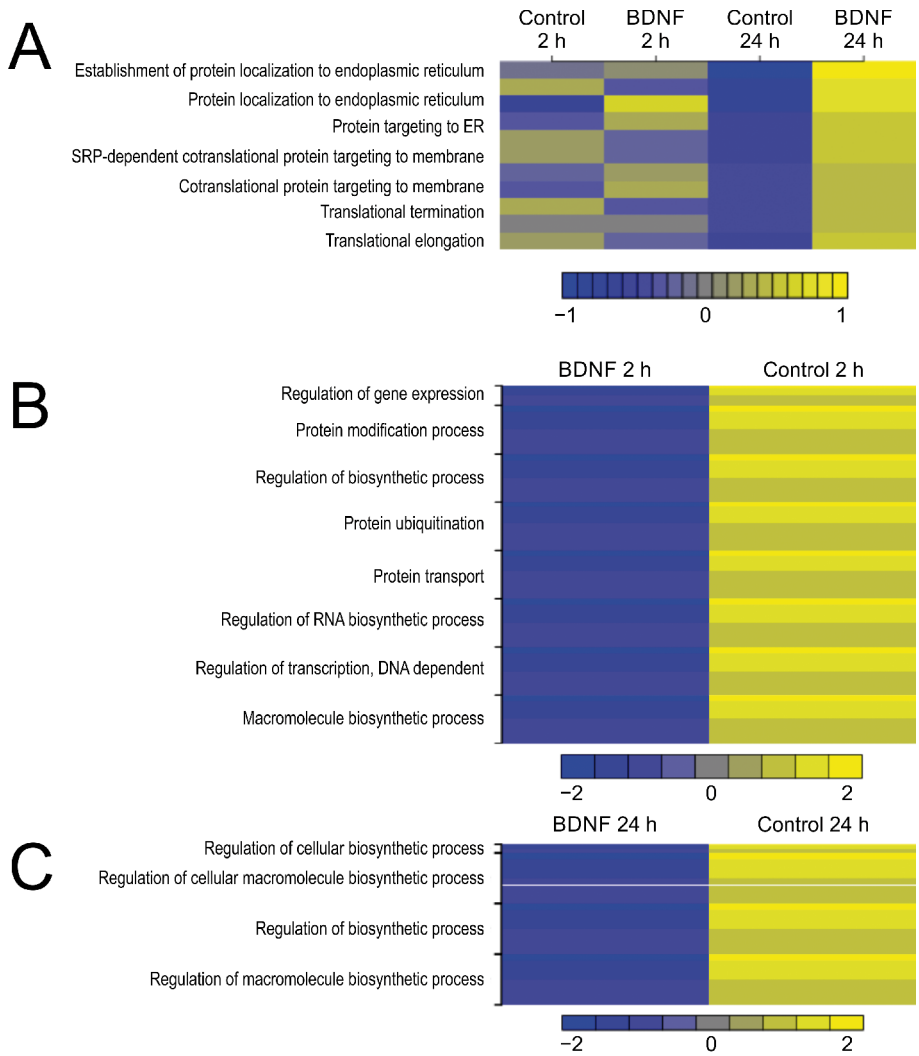

Fig. 3. (A) Global gene expression changes in human UCB-derived Lin⁻ cells stimulated with BDNF compared with untreated cells. The heatmap represents the expression levels of highly overexpressed genes. Individual genes are assigned according to the GO classification of specific biological processes listed on the left side of the graph. Each column comprises a set of horizontal lines, each representing a single gene. Levels of gene expression are indicated on a color scale with yellow corresponding to the highest level of expression and blue corresponding to the lowest level. The range of expression rates of the analyzed genes is shown below the graph. (B) Global miRNA expression changes in human UCB-derived Lin⁻ cells stimulated with BDNF after 2 h. The heatmap represents the expression levels of highly differentially expressed miRNAs associated with genes that are assigned according to the GO classification of specific biological processes listed on the left side of the graph. (C) Global miRNA expression changes in human UCB Lin⁻ cells stimulated with BDNF after 24 h compared to untreated control cells. The heatmap represents the expression levels of highly differentially expressed miRNAs associated with genes that are assigned according to the GO classification of specific biological processes listed on the left side of the graph. Each column comprises a set of horizontal lines, each representing a single miRNA. Levels of miRNA expression are indicated on a color scale with yellow corresponding to the highest level of expression and blue corresponding to the lowest level.

terize the effect of BDNF on regulating Lin⁻ cell genes. We analyzed miRNA as well as mRNA expression changes (obtained using the mRNA microarray analysis) by selecting a group of down-regulated miRNAs with concomitant upregulated target mRNAs. Next, all of the differentially expressed mRNAs were classified according to the GO Classification of Biological Processes. A functional analysis using GO revealed that a number of pathways were specifically represented in the analyzed Lin⁻ cells treated with BDNF after 2 h. Among these pathways were gene expression and its positive regulation, the regulation of the macromolecule biosynthetic process, the regulation of the macromolecule modification biosynthetic process, the regulation of cellular biosynthetic processes, the establishment of protein localization, protein transport, the regulation of RNA synthesis, the regulation of transcription, and the positive regulation of the macromolecule metabolic process (Fig. 3B).

Similarly, GO analysis revealed that a number of pathways were specifically represented in the analyzed Lin⁻ cells treated with BDNF after 24 h. Among these pathways were the regulation of the cellular biosynthetic process, the regulation of the cellular macromolecule biosynthetic process, and the regulation of the macromolecule biosynthetic process (Fig. 3C).

DISCUSSION

In recent decades, various stem cells have been verified and tested for neuroregeneration, including hematopoietic, embryonic, and mesenchymal stem cells, among others (Abraham and Verfaillie 2012, Drela et al. 2013). Both *in vitro* and animal experiments have demonstrated positive effects of these cells in improving neural tissue function (Abraham and Verfaillie 2012). Lin⁻ cells are described as not committed to any lineage and promising for assessment in transplantation purposes. Consequently, the population of Lin⁻ cells is considered as an attractive candidate for examining its ability in neural tissue repair due to their neurogenic potential (McGuckin et al. 2004). It has been shown that Lin⁻ cells are capable of generating a high number of neuroblasts after *in vitro* induction in defined serum-free media (Jurga et al. 2012). Nevertheless, only a few groups have demonstrated that human UCB cells give rise to neural progenitors *in vitro* (Sanchez-Ramos et al. 2001, Buzanska et al. 2002, Habich and Domanska-Janik 2011, Jurga et al. 2012). In addition to such *in vitro* studies, Schwarting and colleagues (2008) using MCAO mouse model has demonstrated significant decrease in infarct volume and apoptosis after about 72 hours of intravenous transplantation of Lin⁻ cells. However, in another study, transplanting UCB Lin⁻ cells into the brain parenchyma did not result in their differentiation into neuronal phenotypes (Coenen et al. 2005). Lin⁻ cells have been demonstrated to get incorporated into laser-injured retina in a dose-dependent manner when transplanted through either the intravitreal or intravenous route (Singh et al. 2012). One study by Koike-Kiryama and coworkers (2007) has shown that Lin⁻ cells express markers specific for retinal nerve cells two weeks after transplantation into subretinal space. Recently, we have clearly demonstrated that transplantation of Lin⁻ cells exerts a potent neuroprotective function after acute chemical injury in murine retinas (Machalinska

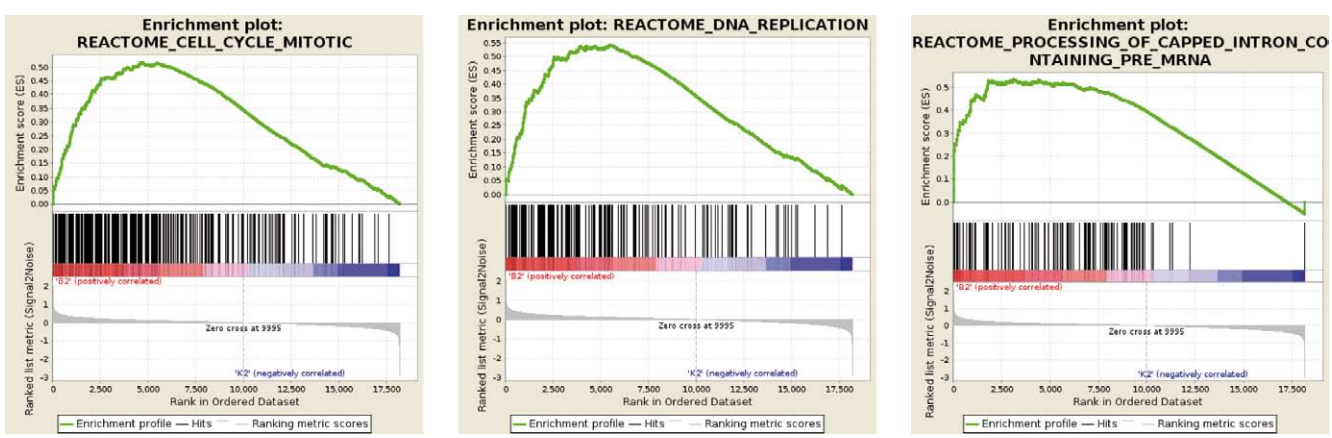

Fig. 4. Examples of enriched GSEA terms for the BDNF treated Lin⁻ cells after 2 h are shown.

Table III

Top ten miRNAs with the highest up- or downregulation in lineage-negative cells after 2 or 24 h of BDNF treatment

2 h incubation				24 h incubation			
miRNA up	Fold change	miRNA down	Fold change	miRNA up	Fold change	miRNA down	Fold change
miR-510	1.333	miR-1200	−1.738	miR-744	2.316	miR-1827	−2.077
miR-1201	1.325	miR-429	−1.629	miR-769-5p	2.293	miR-194	−1.774
miR-1226	1.247	miR-16-1	−1.570	miR-548d-3p	2.179	miR-513b	−1.666
miR-548c-3p	1.199	let-7f	−1.513	miR-198	2.174	miR-106b	−1.577
miR-516b	1.134	miR-493	−1.320	miR-514	2.006	miR-519b-3p	−1.557
miR-513c	1.073	miR-1274b	−1.270	miR-520e	1.986	miR-874	−1.418
miR-1323	1.071	miR-135b	−1.268	miR-660	1.972	miR-148b	−1.391
miR-720	1.036	miR-30c-2	−1.241	miR-99a	1.930	miR-490-5p	−1.350
miR-19b-1	1.032	miR-514	−1.228	miR-524-3p	1.905	miR-181a-2	−1.314
		miR-211	−1.222	miR-1226	1.868	miR-1208	−1.305
		miR-491-3p	−1.221	miR-146a	1.727	miR-21	−1.296
		miR-875-3p	−1.217	miR-500	1.684	miR-219-1-3p	−1.273
		miR-320c	−1.216	miR-548c-3p	1.683	miR-105	−1.250
		miR-369-3p	−1.211	miR-518e	1.683	miR-320c	−1.243
		miR-518b	−1.206	miR-196a	1.680	miR-493	−1.230

et al. 2015). The precise therapeutic mechanisms by which UCB-derived cells affect neural tissue regeneration in animal models remain unclear. The beneficial neurotrophic influence of transplanted cells on CNS cells is widely accepted. Neurotrophic factors secreted by transplanted UCB-derived cells may be partially responsible for the amelioration of neurological deficits in animal models after transplantation (Paul and Anisimov 2013). NTs could provide neuroprotection by supporting the growth stimulation of neural progenitor cells or by attenuating the apoptotic signaling of chronic inflammation that characterizes neurodegenerative disease.

In contrast to HSCs, which are well defined by the presence of the CD34 marker, the Lin^- cells are variously and individually established by researchers who choose the markers used for the depletion of lineage-committed cells. The Lin^- population is heterogeneous and contains various early stem/progenitor cell types. To date, Lin^- cells have not been comprehensively characterized. In this study, we used a commercially available MACS kit to detect many lineage antigens (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a). The isolation of Lin^- cells enabled us to obtain cells free from magnetic beads and antibody attachments; these states are beneficial for their clinical use in cell transplantation.

The efficacy of stem cell-based therapies largely depends on the fate and function of the engrafted cells. Therefore, strategies for enhancing SPCs survival, proliferation, and humoral activities have become important issues. The pre-treatment of transplanted cells using NTs, such as BDNF, could play a role in improving the efficiency of stem cell-based therapy by enhancing the survival of transplanted stem cells, inhibiting apoptosis and facilitating neurotrophic

Table IV

GSEA terms that are enriched in lineage-negative cells after BDNF incubation for 2 h

GSEA term	ES	NES	FDR *q*-val	FWER *P*-value
Cell Cycle				
Reactome Cell Cycle Mitotic	0.52	2.58	0.0	0.0
Reactome Cell Cycle	0.50	2.55	0.0	0.0
Reactome DNA Replication	0.54	2.55	0.0	0.0
Reactome G1/S Transition	0.58	2.52	0.0	0.0
Reactome Autodegradation of Cdh1 by Cdh1:APC/C	0.66	2.51	0.0	0.0
Reactome Mitotic M-M/G1 Phases	0.54	2.47	0.0	0.0
Reactome Mitotic G-G1/S Phases	0.56	2.46	0.0	0.0
Reactome SCF(Skp2)-mediated Degradation of p27/p21	0.64	2.43	0.0	0.0
Reactome Cell Cycle Checkpoints	0.55	2.41	0.0	0.0
Reactome S Phase	0.56	2.40	0.0	0.0
Reactome Synthesis of DNA	0.58	2.40	0.0	0.0
Reactome Regulation of Mitotic Cell Cycle	0.59	2.39	0.0	0.0
Reactome APC/C:Cdc20 Mediated Degradation of Mitotic Proteins	0.61	2.38	0.0	0.0
Reactome CDT1 Association with the CDC6:ORC:origin Complex	0.61	2.36	0.0	0.0
Gene Expression				
Reactome mRNA Splicing	0.58	2.54	0.0	0.0
Reactome Processing of Capped Intron-containing Pre-mRNA	0.54	2.40	0.0	0.0
Reactome mRNA Processing	0.50	2.27	1.75E−4	0.005
Varia				
Shen SMARCA2 Targets Up	0.52	2.66	0.0	0.0
Zhang Tlx Targets 36hr Dn	0.55	2.55	0.0	0.0
Wong Embryonic Stem Cell Core	0.46	2.30	8.29E−5	0.002

(FDR) False-discovery rate calculated based on the normalized statistics of the permutation data to account for the variable sizes of genes and pathways

effects (Nowakowski et al. 2013). Although the conceptual basis for pre-conditioning cells for transplantation is widely accepted, the subsequent priming mechanisms involved remain to be fully elucidated. A large array of studies have demonstrated that cell-cell interactions are an important mechanism in neural tissue, suggesting that transplanted SPCs can protect neurons from oxidative stress and metabolic insults and augment endogenous regeneration processes by secreting protective molecules and growth factors (Ou et al. 2010, Shang et al. 2011). We previously demonstrated that UCB-derived Lin^- cells spontaneously express NTs and their receptors at a higher level than unseparated nucleated cell populations of UCB (Paczkowska et al. 2013). In various experimental studies, SPCs are administered into cerebrospinal fluid or directly into

the neurogenic niche; at this site, these cells could interact with the neural microenvironment through the paracrine and/or autocrine axis. Here, we assessed the influence of pre-treatment with BDNF on the properties of UCB-derived Lin^- cells. Thus, our experiments highlight that BDNF pre-treatment noticeably modifies the metabolic activity of Lin^- cells.

First, we assessed BDNF receptor TrkB activation in Lin^- cells followed by BDNF stimulation in culture. BDNF binds TrkB with high affinity to trigger its dimerization as well as to trigger the autophosphorylation of tyrosine residues in the cytoplasmic kinase domain, which act as docking sites for effector molecules and induce the activation of three main signaling pathways (the PLCγ, PI3K and ERK cascades). We demonstrated that BDNF pre-treatment triggers the phosphorylation of Akt in Lin^- cells, a downstream target of phosphatidylinositol 3-kinase (PI3K). The phosphorylation of Akt is involved in a number of important cell processes. Furthermore, this signaling protein is a pivotal switch that mediates anti-apoptotic processes. The phosphorylation of the Bcl-2 family member BAD and the protease caspase 9 by Akt suppresses their proapoptotic function, thereby accounting for the anti-apoptotic effects of Akt in a variety of situations, including oxidative and osmotic stress, irradiation, and ischemic shock (Datta et al. 1997). In this study, the apoptosis of Lin^- cells was inhibited following BDNF stimulation; however, the effect was more pronounced after 24 h *versus* 2 h of incubation. This observation is in line with a number of reports supporting the survival-promoting effects of BDNF through the activation of the PI3K pathway (Numakawa et al. 2010). Moreover, we hypothesize that BDNF could exert a protective effect by activating the PI3K cascade during transplantation procedures, which are thought to stress the transplanted cells.

A large number of studies have demonstrated that oxidative stress plays a crucial role in a number of neurodegenerative diseases (Gatta et al. 2009). The BDNF activation of TrkB, leading to the initiation of signaling pathways, is implicated in influencing the actions of the transcription factor CREB, which is involved in neuroprotection. Transgenic mice expressing A-CREB, a dominant negative form of CREB, showed a significant increase in vulnerability to seizure activity. The A-CREB mice presented increased ROS levels and decreased neuroprotection *via* BDNF application (Lee et al. 2009). These experimental data suggest that CREB is a vital upstream effector of neuroprotection against oxidative toxicity. Because cell culturing could cause oxidative stress, we compared antioxidant system enzymes (SOD, catalase, and GST) in Lin^- cells incubated in the presence or absence of BDNF. We found lower cellular SOD and tendency to decreased GST activity in Lin^- cells treated with BDNF compared with cells untreated with BDNF after 24 h in culture. The other antioxidant enzyme, catalase, did not differ after BDNF pre-treatment. Superoxide dismutase belongs to the first line of defense enzymes and specifically acts against the formation of hydroxyl radicals (Clarkson and Thompson 2000). Hydrogen peroxide (H_2O_2) is produced following the two-step dismutation of the superoxide anion (O_2^-) with SOD. Thereafter, the product of this process may be neutralized in the disproportionation reaction with CAT and reduction reaction with GPx (Dolegowska et al. 2010). SOD and GST activity could be an index for superoxide production (Rukmini et al. 2004, Giebutowicz et al. 2014). Similarly, the SHH pathway is activated in primary cultured cortical neurons after exposure to hydrogen peroxide and protects neurons from apoptosis by increasing the activities of SOD and glutathione peroxidase (Dai et al. 2011). We suggest that *in vitro* incubation stimulates modest levels of oxidative stress (Halliwell 2003), resulting in a significant increase in SOD activity and no significant change in GST observed in Lin^- cells after 24 h of incubation compared with 2 h. Interestingly, BDNF treatment diminished this effect, leading to lower SOD and tendency to decreased GST activity, which is likely an adaptive response of this enzyme to decrease oxygen production. Notably, the expression of GST is regulated *via* the Nrf2 and NF-κB signaling pathways, which are activated in oxidative stress (Morceau et al. 2004). Given the notion that higher antioxidant system activity is a biomarker of oxidative stress, the decrease in antioxidant enzyme activity in cells treated with BDNF could be interpreted as indicating anti-oxidative properties of BDNF.

To further characterize the influence of BDNF pre-treatment on UCB-derived Lin^- cells, we studied the effect of BDNF on the expression of NT-3 in these cells. NT-3 is one of the most promising growth factors for neuronal regenerative therapy and regulates a number of neuronal functions, including survival, neurogenesis

(Shang et al. 2011). The transcription factors that may play a role in the expression of neurotrophic proteins include CREB, NF-κB, CCAAT/enhancer-binding protein beta (C/EBPβ), and activator protein 1 (AP-1). The phosphatidylinositol 3-kinase (PI3K)/AKT pathway induces NFkB activation (Meffert et al. 2003). NFkB plays a role in cell survival and also has diverse functions in the nervous system, including roles in plasticity, learning, and memory. Neurotrophic growth factors (e.g., BDNF, NGF) are counted among many NF-κB target genes that may be important for plasticity and learning (Zaheer et al. 2001). Here, we investigated the influence of BDNF pre-treatment on NT-3 expression in UCB-derived Lin^- cells. It has been previously showed by Leingartner and co-workers that BDNF acting *via* TrkB increases NT-3 mRNA levels in cerebellar granule neurons (Leingärtner et al. 1994). It has been suggested that transplanted SPCs could protect neurons from oxidative stress and various metabolic insults by secreting growth factors such as BDNF (Zhang et al. 2011). Our results suggest that short-term priming by BDNF may enhance the trophic activity of Lin^- cells. There is a number of factors that could influence the cells transplanted into neural tissue. Here we assessed only the influence of mature form of BDNF on Lin^- cells, whereas BDNF is present in two forms in the brain. It is well known that BDNF is synthesized as a pre-proBDNF protein, which has its pre-sequence cleaved off in the endoplasmic reticulum. ProBDNF is either proteolytically cleaved and secreted as mature BDNF or secreted as proBDNF and cleaved by extracellular proteases. Once released, proBDNF binds preferentially to the pan-neurotrophin receptor p75NTR, while mature BDNF can also bind p75NTR with low affinity; however, mature BDNF binds preferentially with high affinity to TrkB receptors. These two types of receptors activate different intracellular secondary messenger cascades and trigger distinct cellular responses. The binding of p75NTR by proBDNF initiates both prosurvival NF-kB and pro-apoptotic Jun kinase signaling cascades. However, we examined only the action of mature BDNF on Lin^- cells in this study.

Next, to further characterize the response of Lin^- cells to BDNF pre-treatment, we compared the global gene expression patterns of Lin^- cells treated with BDNF with those of the controls. A study comparing the genome-wide gene expression profiles of UCB-derived Lin^- cells treated with BDNF indicated enhanced translation processes and protein targeting. Regarding protein synthesis, BDNF facilitates the local translation of proteins in dendrites *via* the activation of mammalian target of rapamycin (mTOR) through the PI3K signaling pathway (Schratt et al. 2004). There are two important signaling pathways (mTOR and ERK) that regulate the compilation of the eIF4e complex and the activation of S6K1, both contributing to enhanced mRNA translation initiation at active synapses (Klann and Dever 2004). The differential activation and role of these cascades in neuronal survival may depend on both the cell type and the involvement of specific physiological or pathological stimuli. To further consider the biological implication of changes in gene expression after BDNF stimulation, we used GSEA which revealed that gene sets related to the cell cycle and mitosis as well as the gene expression were significantly enriched in BDNF treated cells after 2 h compared to control cells. The identification of the over representation of the pathway connected with mitosis in Lin^- cells after 2 h in this study by GSEA could be considered a good indication for the proliferation induced by BDNF. It could indicate on the very early stimulation of proliferation in the group of cells by BDNF which couldn't be observed after 24 h as revealed by GSEA of cells at this time as well as by BrdU incorporation observed by flow cytometry.

Moreover, we investigated the expression patterns of miRNAs in Lin^- cells treated with BDNF after 2 and 24 h and identified several miRNAs with significantly changed expression. Recent studies have uncovered a large role of miRNAs in the regulation of NT signaling; these studies suggested that the aberrant expression of one or more NT-regulated miRNAs may be involved in the pathogenetic hallmark of neurodegenerative diseases. Here, we simultaneously analyzed miRNAs and corresponding target genes following BDNF treatment in Lin^- cells. We revealed that target upregulated genes of the downregulated miRNAs in Lin^- cells following 2 h BDNF treatment were involved in gene expression, transcription, RNA biosynthesis, protein transport, and the regulation of nucleobase-containing compound metabolic processes. Genes that were upregulated with a corresponding downregulation of miRNAs in Lin^- cells after 24 h of BDNF treatment were involved in cellular biosynthetic processes. Together, the changes in global miRNAs and gene expression patterns suggest an anabolic effect of BDNF in Lin^- cells. Interestingly, BDNF exerts anabolic effects on dendritic development (Burkhalter et al. 2007). Further knowledge of the global miRNA alterations and transcriptional program involved in the BDNF treatment process may lead to a more explicit understanding of stem/progenitor cells' molecular mechanism of response to neurotrophic factors.

CONCLUSIONS

Beneficial effects of UCB-derived cell transplantation in experimental models of nervous tissue injuries has been demonstrated in numerous studies on neural tissue regeneration (Schwarting et al. 2008, Arien-Zakay et al. 2009). In our study we observed that BDNF exerts an influence on Lin^- cells acting through its TrkB receptors. The influence consists of diminishing of apoptosis in these cells, modulation of antioxidants and an increasing of NT-3 expression. Microarray gene expression profiling, GSEA and global miRNAs analysis revealed that BDNF influenced the gene expression, proliferation, and the regulation of metabolism by exerting anabolic effects on Lin^- cells. Our results support the hypothesis that pre-treatment of stem/progenitor cells could be beneficial and may be used as an auxiliary strategy for improving the properties of SPCs. The usefulness of the strategy involving pre-treatment of stem/progenitor cells with neurotrophins before transplantation in experimental studies could be a subject of further research.

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REFERENCES

Abraham R, Verfaillie CM (2012) Neural differentiation and support of neuroregeneration of non-neural adult stem cells. Prog Brain Res 201: 17–34.

Arien-Zakay H, Lecht S, Bercu MM, Tabakman R, Kohen R, Galski H, Nagler A, Lazarovici P (2009) Neuroprotection by cord blood neural progenitors involves antioxidants, neurotrophic and angiogenic factors. Exp Neurol 216: 83–94.

Bartkowska K, Turlejski K, Djavadian RL (2010) Neurotrophins and their receptors in early development of the mammalian nervous system. Acta Neurobiol Exp (Wars) 70: 454–467.

Blais M, Lévesque P, Bellenfant S, Berthod F (2013) Nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and glial-derived neurotrophic factor enhance angiogenesis n a tissue-engineered in vitro model. Tissue Eng Part A 19: 1655–1664.

Burkhalter J, Fiumelli H, Erickson JD, Martin JL (2007) A critical role for system A amino acid transport in the regulation of dendritic development by brain-derived neurotrophic factor (BDNF). J Biol Chem 282: 5152–5159.

Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features in vitro. J Cell Sci 115: 2131–2138.

Chou T, Sano M, Ogura M, Morishima Y, Itagaki H, Tokuda Y (2005) Isolation and transplantation of highly purified autologous peripheral CD34+progenitor cells: purging efficacy, hematopoietic reconstitution following high dose chemotherapy in patients with breast cancer: results of a feasibility study in Japan. Breast Cancer 12: 178–188.

Clarkson PM, Thompson HS (2000) Antioxidants: what role do they play in physical activity and health? Am J Clin Nut 72 Suppl: 637S–646S.

Coenen M, Kögler G, Wernet P, Brüstle O (2005) Transplantation of human umbilical cord blood-derived adherent progenitors into the developing rodent brain. J Neuropathol Exp Neurol 64: 681–688.

Dai RL, Zhu SY, Xia YP, Mao L, Mei YW, Yao YF, Xue YM, Hu B (2011) Sonic hedgehog protects cortical neurons against oxidative stress. Neurochem Res 36: 67–75.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231–241.

Dołęgowska B, Błogowski W, Domański L (2010) Clinical evidence of the association between serum perioperative changes in xanthine metabolizing enzymes activity and early post-transplant kidney allograft function. J Am Coll Surg 211: 587–595.

Drela K, Siedlecka P, Sarnowska A, Domanska-Janik K (2013) Human mesenchymal stem cells in the treatment of neurological diseases. Acta Neurobiol Exp (Wars) 73: 38–56.

Forraz N, Pettengell R, McGuckin CP (2004) Characterization of a lineage-negative stem-progenitor cell population optimized for ex vivo expansion and enriched for LTC-IC. Stem Cells 22: 100–108.

Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92–105.

Fujita Y1, Kinoshita M, Furukawa Y, Nagano T, Hashimoto H, Hirami Y, Kurimoto Y, Arakawa K, Yamazaki K, Okada Y, Katakami N, Uno E, Matsubara Y, Fukushima M, Nada A, Losordo DW, Asahara T, Okita Y, Kawamoto

A (2014) Phase II clinical trial of CD34+ cell therapy to explore endpoint selection and timing in patients with critical limb ischemia. Circ J 78: 490–501.

Gatta L, Cardinale A, Wannenes F, Consoli C, Armani A, Molinari F, Mammi C, Stocchi F, Torti M, Rosano GM, Fini M (2009) Peripheral blood mononuclear cells from mild cognitive impairment patients show deregulation of Bax and Sod1 mRNAs. Neurosci Lett 453: 36–40.

Giebułtowicz J, Sołobodowska S, Bobilewicz D, Wroczyński P (2014) Blood ALDH1 and GST activity in diabetes type 2 and its correlation with glycated hemoglobin. Exp Clin Endocrinol Diabetes 122: 55–59.

Habich A, Domanska-Janik K (2011) Aggregation-promoted expansion of neuraly committed human umbilical cord blood progenitors in vitro. Acta Neurobiol Exp (Wars) 71: 1–11.

Halliwell B (2003) Oxidative stress in cell culture: an underappreciated problem? FEBS Lett 540: 3–6.

Huang EJ, Reichardt L (2001) Neurotrophins: Roles in neuronal development and function. Annu Rev Neurosci 24: 677–736.

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72: 609–642.

Jiang Y, Wei N, Zhu J, Lu T, Chen Z, Xu G, Liu X (2010) Effects of brain-derived neurotrophic factor on local inflammation in experimental stroke of rat. Mediators Inflamm 2010: 372423.

Jurga M, Forraz N, Basford C, Atzeni G, Trevelyan AJ, Habibollah S, Ali H, Zwolinski SA, McGuckin CP (2012) Neurogenic properties and a clinical relevance of multipotent stem cells derived from cord blood samples stored in the biobanks. Stem Cells Dev 21: 923–936.

Klann E, Dever TE (2004) Biochemical mechanisms for translational regulation in synaptic plasticity. Nat Rev Neurosci 5: 931–942.

Kögler G, Sensken S, Airey JA, Trapp T, Müschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Müller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200:123–135.

Koike-Kiriyama N, Adachi Y, Minamino K, Iwasaki M, Nakano K, Koike Y, Yamada H, Mukaide H, Shigematsu A, Mizokami T, Matsumura M, Ikehara S (2007) Human cord blood cells can differentiate into retinal nerve cells. Acta Neurobiol Exp (Wars) 67: 359–365.

Lee B, Cao R, Choi YS, Cho HY, Rhee AD, Hah CK, Hoyt KR, Obrietan K (2009) The CREB/CRE transcriptional pathway: protection against oxidative stress-mediated neuronal cell death J Neurochem 108: 1251–1265.

Leingärtner A, Heisenberg CP, Kolbeck R, Thoenen H, Lindholm D (1994) Brain-derived neurotrophic factor increases neurotrophin-3 expression in cerebellar granule neurons. J Biol Chem 269: 828–830.

Machalińska M, Rogińska D, Pius-Sadowska E, Kawa MP, Paczkowska E, Rudnicki M, Lejkowska R, Baumert B, Wiszniewska B, Machaliński B (2015) Neuroprotective and antiapoptotic activity of lineage-negative bone marrow cells after intravitreal injection in a mouse model of acute retinal injury. Stem Cells Int 2015:620364. doi:10.1155/2015/620364.

McGuckin CP, Forraz N, Allouard Q, Pettengell R (2004) Umbilical cord blood stem cells can expand hematopoietic and neuroglial progenitors in vitro. Exp Cell Res 295: 350–359.

Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D (2003) NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 6: 1072–1078.

Morceau F, Duvoix A, Delhalle S, Schnekenburger M, Dicato M, Diederich M (2004) Regulation of glutathione S-transferase P1-1 gene expression by NF-kappaB in tumor necrosis factor alpha-treated K562 leukemia cells. Biochem Pharmacol 67: 1227–1238.

Nowakowski A, Andrzejewska A, Janowski M, Walczak P, Lukomska B (2013) Genetic engineering of stem cells for enhanced therapy. Acta Neurobiol Exp (Wars) 73: 1–18.

Numakawa T, Suzuki S, Kumamaru E, Adachi N, Richards M, Kunugi H (2010) BDNF function and intracellular signaling in neurons. Histol Histopathol 25: 237–258.

Ou Y, Yu S, Kaneko Y, Tajiri N, Bae EC, Chheda SH, Stahl CE, Yang T, Fang L, Hu K, Borlongan CV, Yu G (2010) Intravenous infusion of GDNF gene-modified human umbilical cord blood CD34+ cells protects against cerebral ischemic injury in spontaneously hypertensive rats. Brain Res 1366: 217–225.

Paczkowska E, Kaczyńska K, Pius-Sadowska E, Rogińska D, Kawa M, Ustianowski P, Safranow K, Celewicz Z, Machaliński B (2013) Humoral activity of cord blood-derived stem/progenitor cells: implications for stem cell-based adjuvant therapy of neurodegenerative disorders. PLoS One 8: e83833.

Paczkowska E, Rogińska D, Pius-Sadowska E, Jurewicz A, Piecyk K, Safranow K, Dziedziejko V, Grzegrzółka R, Bohatyrewicz A, Machaliński B (2014) Evidence for proangiogenic cellular and humoral systemic response in

patients with acute onset of spinal cord injury. J Spinal Cord Med doi: 10.1179/2045772314Y.0000000227.

Pasino M, Lanza T, Marotta F, Scarso L, De Biasio P, Amato S, Corcione A, Pistoia V, Mori PG (2000) Flow cytometric and functional characterization of AC133(+) cells from human umbilical cord blood. Br J Haematol 108: 793–800.

Paul G, Anisimov SV (2013) The secretome of mesenchymal stem cells: potential implications for neuroregeneration. Biochimie 95: 2246–2256.

Rukmini MS, D'Souza B, D'Souza V (2004) Superoxide dismutase and catalase activities and their correlation with malondialdehyde in schizophrenic patients. Indian J Clin Biochem 19: 114–118.

Sanberg PR, Willing AE, Garbuzova-Davis S, Saporta S, Liu G, Sanberg CD, Bickford PC, Klasko SK, El-Badri NS (2005) Umbilical cord blood-derived stem cells and brain repair. Ann N Y Acad Sci 1049: 67–83.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. Exp Neurol 171: 109–115.

Schwarting S, Litwak S, Hao W, Bähr M, Weise J, Neumann H (2008) Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. Stroke 39: 2867–2875.

Schratt GM, Nigh EA, Chen WG, Hu L, Greenberg ME (2004) BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. J Neurosci 24: 7366–7377.

Shang AJ, Hong SQ, Xu Q, Wang HY, Yang Y, Wang ZF, Xu BN, Jiang XD, Xu RX (2011) NT-3-secreting human umbilical cord mesenchymal stromal cell transplantation for the treatment of acute spinal cord injury in rats. Brain Res 1391: 102–113.

Singh T, Prabhakar S, Gupta A, Anand A. (2012) Recruitment of stem cells into the injured retina after laser injury. Stem Cells Dev 21: 448–454.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545–1550.

Tsuji M, Taguchi A, Ohshima M, Kasahara Y, Sato Y, Tsuda H, Otani K, Yamahara K, Ihara M, Harada-Shiba M, Ikeda T, Matsuyama T (2014) Effects of intravenous administration of umbilical cord blood CD34(+) cells in a mouse model of neonatal stroke. Neuroscience 263: 148–158.

Zaheer A, Yorek MA, Lim R (2001) Effects of glia maturation factor overexpression in primary astrocytes on MAP kinase activation, transcription factor activation, and neurotrophin secretion. Neurochem. Res 26: 1293–1299.

Zhang MJ, Sun JJ, Qian L, Liu Z, Zhang Z, Cao W, Li W, Xu Y (2011) Human umbilical mesenchymal stem cells enhance the expression of neurotrophic factors and protect ataxic mice. Brain Res 1402: 122–131.