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Endothelial Progenitor Cell-Derived Factors Exert Neuroprotection in Cultured Cortical Neuronal Progenitor Cells

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Abstract

There is substantial evidence that stem and progenitor cells secrete trophic factors that have potential for repairing injured tissues. We have previously reported that the conditioned medium (CM) obtained from endothelial progenitor cells (EPC) cultures protects striatal neurons against 3-nitropropionic acid-induced toxicity. In the present study we tested the hypothesis that EPC-CM may support cortical neuronal cell function and/or survival. EPC were isolated from the peripheral blood of healthy human donors and cultured in hypoxic conditions (1.5% O_2) to stimulate the secretion of growth factors. The supernatant or conditioned medium (EPC-CM) was then collected and used for the various experiments. Primary cultures of cerebral cortex from fetal rat embryonic day 14 were treated with EPC-CM and challenged by glucose and serum deprivation. We observed that EPC-CM treatment significantly increased total cell number and cell viability in the cultures. Similarly, the number of Iba1-expressing cells was significantly upregulated by EPC-CM, while western blot analyses for the astroglial marker glial fibrillary acidic protein did not show a marked difference. Importantly, the number of beta-III-tubulin-positive neurons in the cultures was significantly augmented after EPC-CM treatment. Similarly, western blot analyses for beta-III-tubulin showed significant higher signal intensities. Furthermore, EPC-CM administration protected neurons against glucose- and serum deprivation-induced cell loss.

In sum, our findings identified EPC-CM as a means to promote viability and/or differentiation of cortical neurons and suggest that EPC-CM might be useful for neurorestorative approaches.

Keywords

endothelial progenitor cells, paracrine factors, cortical cultures, beta-III-tubulin, neurons, neuroprotection

Introduction

There is growing evidence that stem and progenitor cells mediate their effects predominantly by secreting trophic factors. The repertoire of bioactive molecules released in the extracellular environment is referred as secretome[1]. The beneficial effect of secretome has been described for several pathological conditions including neurodegenerative diseases[2]. In addition, endothelial progenitor cells (EPC) and their soluble factors have also been successfully used in traumatic brain injury, ischemic stroke, and white matter damage models[3–6]. We have recently demonstrated that the conditioned medium of peripheral blood- derived EPC (EPC-CM) promotes viability of neurons in primary cultures from the ventral mesencephalic as well as the ganglionic eminence and importantly, in these cultures EPC-CM elicited neuroprotective responses against neurotoxic insults induced by 1-methyl-4-phenyl pyridinium and 3-nitropropionic acid treatment, respectively[7,8].

In the framework of therapeutic applications, secretome-based applications have the advantage of evoking low immunogenic reactions thus allowing an allogenic use. On the other hand, in addition to the various beneficial effects reported, the high variety of active factors present in

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different secretomes may also elicit unwanted side effects, e.g., cell senescence[9]. Hence, detailed studies are needed to understand the current advantages and limitations of secretome-based treatments for tissue regeneration[1]. To explore whether EPC-CM has a wide neuroprotective activity spectrum, in the present study we investigated the effect of EPC-CM on differentiating primary cortical progenitor cells from rat fetuses. In particular, we analyzed whether EPC-CM might influence the yield and survival of neurons and attempted to analyze the neuroprotective potential of EPC-CM against a metabolic insult induced by glucose and serum deprivation (GSD).

Materials and Methods

Animals

Time pregnant Wistar rats were purchased from Janvier Labs (Le Genest-Saint-Isle, France) and housed at 12-h light–dark cycle with food and water ad libitum. All experiments were carried out in the light phase and in accordance with the guidelines of the Animal Research Ethics Committee of the Canton Bern, Switzerland, and the University of Bern Animal Care and Use Committee, Switzerland (Study No. 6/12, 10/15, and 119/16).

Preparation and Culture of Rat Dissociated Fetal Cortical Cultures

Cultures of cerebral cortex (CTX) were prepared from embryonic day 14 (E14) old rat fetuses as described previously with slight modifications[10]. In brief, pregnant Wistar rats were anesthetized using gas inhalation (5% isoflurane, 75% N_2O, 20% O_2; Peachtree Corners, GA, USA) followed by an intraperitoneal injection of a mix of ketamine (120 mg/kg; Vetoquinol AG, Ittigen, Switzerland) and Xylaxine (20 mg/kg; Vetoquinol AG). The fetuses were separated by cesarean section and the dams immediately thereafter decapitated and exsanguinated. Then fetuses were euthanized by decapitation and the cortical areas were dissected bilaterally in cold Dulbecco's modified Eagle's medium (DMEM; Gibco, Reinach, Switzerland) according to standard procedures[11]. The CTX explants were mechanically dissociated and plated at a density of 0.3 and 2.0 embryos/well (seeding density of 700 to 800 viable cells per mm^2) into the 24-well and 6-well plates (Falcon), respectively. Cell viability, as assessed using the Trypan Blue dye exclusion method in a Neubauer chamber, was always above 65%. The 24 wells contained glass inserts (glass coverslips of 12 mm diameter, Assistent, Sandheim, Germany). Culture wells were precoated with poly-L-lysine (0.1 mg/ml; Sigma, Buchs, Switzerland). The neurons were grown in 0.5 and 2.5 ml, for 24- and 6-well plates, respectively, of standard culture medium consisting of 55% DMEM, 32.5% Hank's balanced salt solution (Gibco), 0.3% glucose, 10% fetal calf serum (FCS; Gibco), and 1% 0.01 M HEPES (Merck, Darmstadt, Germany) as well as antibiotics/antimycotics (061-05240 D; Gibco). The medium was refreshed after 2 days *in vitro* (DIV) and exchanged at DIV5 to the different treatment groups, as described below, for another 2 days. At the end of the experimental period, at DIV7, the cultures were washed with PBS and processed for the different analyses as described below.

EPC Culture and CM Preparations

EPC were cultivated from peripheral blood mononuclear cells of healthy human anonymous donors as previously described[12]. The buffy coats utilized were purchased from the Interregional Transfusion Centre of the Swiss Red Cross (Bern, Switzerland) and no experimental approval or informed consent was needed. All samples were handled according to the regulations and guidelines of the University Hospital Bern, Switzerland. Mononuclear cells were isolated and plated on fibronectin (Sigma)-coated six-well plastic dishes (Falcon) in endothelial cell basal medium-2 (EBM-2; Lonza, Basel, Switzerland) containing endothelial growth medium BulletKit and 5% FCS (Lonza). To manufacture EPC-CM, EPC were incubated for 48 h under hypoxic conditions (1.5% O_2, 5% CO_2, 93.5% N_2) using a humidified gas-sorted anoxic incubator-gloved box (InVivo2 400, Ruskinn Technologies, Bridgend, UK) in growth factor-free EBM-2 containing 1% FCS as previously described[13]. After incubation, EPC-CM was collected, sterile filtered, and snap-frozen until further use[13]. The EBM-2 was treated in parallel to the cell cultures and served as control medium (unconditioned medium).

Treatments

For the immunohistochemical and Western blot analyses the culture wells were randomly assigned to the different treatment groups and incubated from DIV5 until the end of the experimental period at DIV7 in EPC-CM or with EBM-2.

We have recently shown that EPC-CM exerted neuroprotection for cultured striatal progenitor cells[7]. Hence, we assessed whether EPC-CM administration offers a survival-promoting potential also for cerebral cortical cultures. For that purpose we tested two regimens, i.e., pre- or posttreatment with EPC-CM. In the pretreatment experiments cultures were either grown in maintenance culture or EPC-CM supplemented medium up to DIV2 in culture when they were exposed to a GSD for 2 days as described previously by Kaneko and co-workers with slight modification (solution consisting of 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 mM Na_2HPO_4, 0.01 mM glycine, 26.2 mM $NaHCO_3$, and 10 mM HEPES, pH 7.2)[14]. In the posttreatment experiments cells were after the GSD consequently grown for additional 2 DIV in the absence or presence of EPC-CM. Controls were kept in regular maintenance medium for the whole experimental period.

Cell Viability Assay

Cell viability was assessed as previously described with minor modifications[15]. Briefly, cultures were treated as described above. At DIV7 the experimental media were replaced with incubation medium and the number of viable cells was assessed by the Presto Blue assay (Invitrogen, Reinach, Switzerland) using a microplate reader (Ex. 560 nm, Em. 590 nm; VarioSkan, ThermoFisher Scientific, Reinach, Switzerland).

Western Blotting and Protein Measurements

The cells were lysed in RIPA buffer (ThermoFisher Scientific, Reinach, Switzerland) containing protease inhibitor cocktail set V (Sigma), 10 mM phenylmethylsulfonyl fluoride (Sigma), 1 mM leupeptin (Sigma), and 1 μM sodium orthovanadate (Sigma). The protein concentration was determined in two aliquots per lysate using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). The Western blotting was carried out according to the method of Laemmli[16] with some modifications. Equal amounts of the proteins were run on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories AG, Cressier, Switzerland). The transfer efficiency was checked by Coomassie Blue staining of the gels. Blots were blocked for 45 min in 5% fat-free milk powder in TBS-Tween 0.01% and incubated with the same solution overnight with primary antibodies [mouse anti-glial fibrillary acidic protein (GFAP), 1:500, Millipore, Zug, Switzerland; mouse anti-beta-III-tubulin, 1:1,000, Promega, Dübendorf, Switzerland; mouse anti-microtubule-associated protein 2 (MAP2), 1: 500, Sigma; rabbit anti-α-tubulin, 1:2,000, Sigma]. After washing in TBS-Tween 0.01% the blots were incubated for 1 h with antibody-peroxidase conjugates (anti-mouse or anti-rabbit, 1:10,000; Jackson ImmunoResearch, Ely, UK) in 5% fat-free milk powder in TBS-Tween 0.01%. Subsequent to washing the blots were visualized with the enhanced chemiluminescence substrate kit (ThermoFisher Scientific) using the Fusion Pulse TS System (Vilber Lourmat, Collégien, France). Bands were quantified on 8-bit converted images using the Fiji software and normalized against α-tubulin. Six cultures from four independent experiments were included for the analyses.

Immunocytochemistry

At the end of the experimental period cell cultures were fixed for 30 min in 4% paraformaldehyde, washed, and blocked with 10% horse serum in 0.1% Triton-X-100/PBS. Thereafter, the cells were incubated overnight with the primary antibodies rabbit anti-GABA (1:5,000; Sigma) in 0.1% Triton-X-100/PBS with 2.5% horse serum at 4°C. Subsequent to washes in PBS, the cultures were incubated for 2 h with biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Servion, Switzerland). Endogenous peroxidase was blocked for 10 min in 3% hydrogen peroxide and 10% methanol in PBS. Bound antibodies were visualized after washes in PBS by the avidin–biotin complex (VECTASTAIN® ABC-Peroxidase Kit; 1:250, PK-4000; Vector Laboratories) in combination with the DAB Substrate Kit (34002, ThermoFischer Scientific). The cultures were mounted with Aquatex (Millipore, Darmstadt, Germany).

For the analysis of microglial cells and neurons the cultures were treated as described above and incubated overnight at 4°C with the primary antibodies rabbit polyclonal anti-ionized calcium-binding adapter molecule 1 (Iba-1, 1:1,000, WAKO, Richmond, VA, USA); mouse anti-beta-III-tubulin (1:1,000, Sigma); Subsequent to washes in PBS, the cultures were incubated for 2 h with the fluorescently labeled antibodies Alexa® Fluor donkey anti-rabbit 594 nm and donkey anti-mouse 488 nm 1:250, Molecular Probes, Carlsbad, CA, USA). Cultures were then washed for 4 × 10 min in PBS and mounted in 0.1 M PBS containing 50% glycerol.

Histological Analyses

All cultures were analyzed under bright field illumination by a researcher blinded to the treatment groups as described by our group before[17–20]. In brief, only cells with distinct immunoreactivity, clear neuronal shape, and visible neurites were counted as GABA-positive neurons. To obtain cell counts, 5.2% of the glass slide surface (113 mm^2) in the culture dish was analyzed in six areas [in the upper, right, lower, left corners (distance 2.5 mm from edge) and twice in the center of the culture; each sized 0.97 mm^2] using a ×10 objective in combination with a ×10 ocular with counting grid (100× magnification). When doubts about the specification of GABA-ir neurons existed then the cell cultures were examined at a higher magnification (×400). Fluorescence pictures stained for beta-lll-tubulin and lba1 were recorded using an Olympus epifluorescence microscope (BX51, Olympus, Tokyo, Japan) equipped with a digital camera (Olympus DP72, Olympus). NeuN-ir cell counts were analyzed in six areas [in the upper, right, lower, left corners (distance 2.5 mm from edge) and twice in the center of the culture; each sized 0.15 mm^2] using a ×20 objective (200× magnification).

Statistical Evaluation

For statistical analysis a commercially available software package was used (Prism 7.04, GraphPad Software, La Jolla, CA, USA). To compare group means of several groups, one-way analysis of variance followed by Tukey's multiple comparison test was used. Statistical significance of two groups only was assessed by two-tailed unpaired *t*-test or by the nonparametric Mann–Whitney test based on the outcome of the D'Agostino and Pearson normality test. Differences were considered statistically significant at $P < 0.05$. Data are presented as mean + SEM.

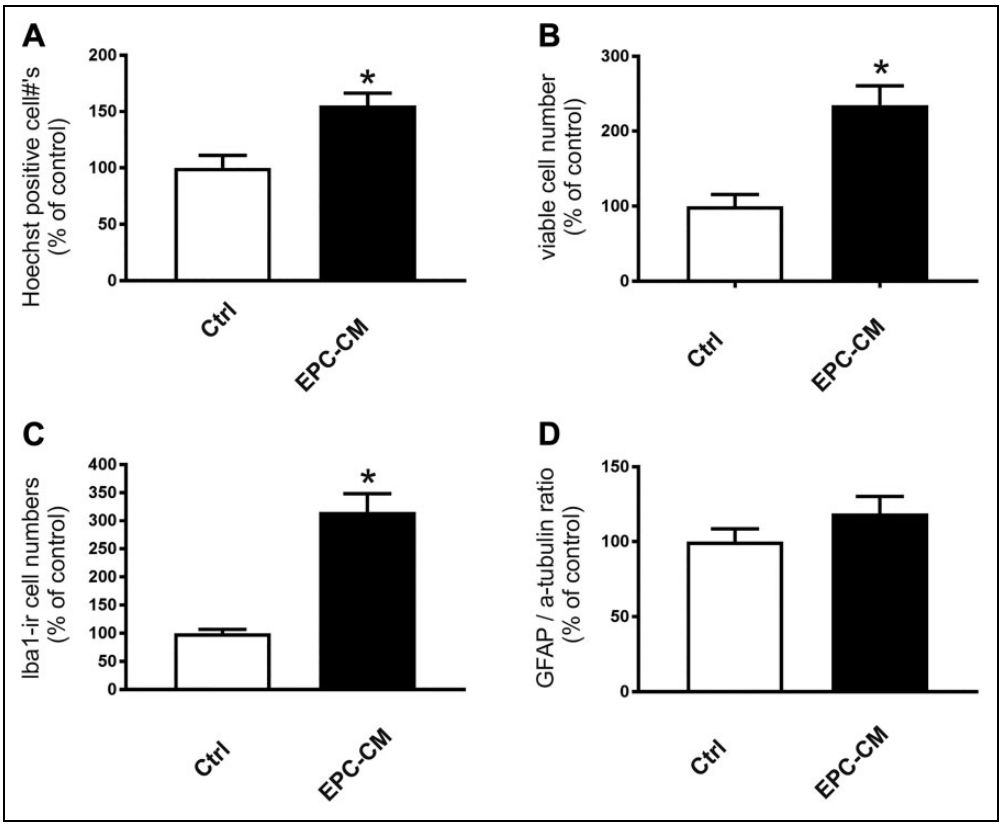

Fig. 1. Quantitative analysis of number of total cell nuclei (Hoechst, A), viable cell number (B), Iba-1-positive cell numbers (C), and Western blot quantification of GFAP (D) in cerebral cortical cultures. Cultures were grown for 7 DIV and treated without (Ctrl) or with endothelial progenitor cells secretome (EPC-CM) from DIV5 to DIV7. Data are presented as a percentage of controls and values given as mean + SEM. *$P < 0.05$ versus corresponding control.
DIV: days *in vitro*; EPC-CM: conditioned medium of peripheral blood-derived endothelial progenitor cells; GFAP: glial fibrillary acidic protein.

Results

Effects of EPC Conditioned Medium Treatment on Total Cell Numbers, Viable Cell Numbers, Number of Microglial Cells, and Levels of GFAP in Dissociated Cortical Cultures

We first observed that the total cell numbers were significantly higher in EPC-CM-treated cultures as compared to controls ($t_{3.6/12}$, $P < 0.05$) (Figure 1A). In line with these results the cortical cultures treated with EPC-CM disclosed a significant increase in number of viable cells ($t_{4.4/17}$, $P < 0.05$) (Figure 1B). Notably, EPC-CM treatment resulted in a pronounced and significant increase in number of lba1-ir cells ($t_{6.5/18}$, $P < 0.05$) (Figure 1C) while only a slight increase in GFAP levels (by 1.2-fold) was found as compared to controls (Figure 1D).

EPC Conditioned Medium Treatment on Number of Beta-lll-Tubulin-Expressing Neurons and of Beta-III-Tubulin Levels

The number of beta-III-tubulin-expressing neurons was significantly augmented after EPC-CM exposure ($t_{6.5/12}$, $P < 0.05$) (Figure 2A). The percentage of beta-III-tubulin-positive neurons of total cell numbers as assessed by the Hoechst staining was significantly higher in the cultures treated with EPC-CM as compared to controls (by 1.37-fold, $t_{2.8/12}$, $P < 0.05$). In line with this observation, EPC-CM treatment resulted in a pronounced and significant increase of beta-III-tubulin levels ($t_{2,7/10}$, $P < 0.05$) (Figure 2B). Similarly, we detected significantly higher MAP-2 levels after EPC-CM treatment as compared to controls (by 1.48-fold, $t_{4,7/10}$, $P < 0.05$).

EPC-CM Exerts Neuroprotection Against Glucose and Oxygen Deprivation

We finally investigated whether administration of EPC-CM prior to or after an insult induced by GSD may have the potential to rescue GABA-ir neurons from cell loss. The presence of EPC-CM prior to the insult resulted in a tendency for higher numbers of surviving GABA-ir cells [F (2,63) = 28.81; $P < 0.05$]; however, this difference did not reach significance (Figure 3A). Notably, administration of EPC-CM after the insult resulted in significantly higher numbers of surviving GABA-ir cells [F (2,28) =

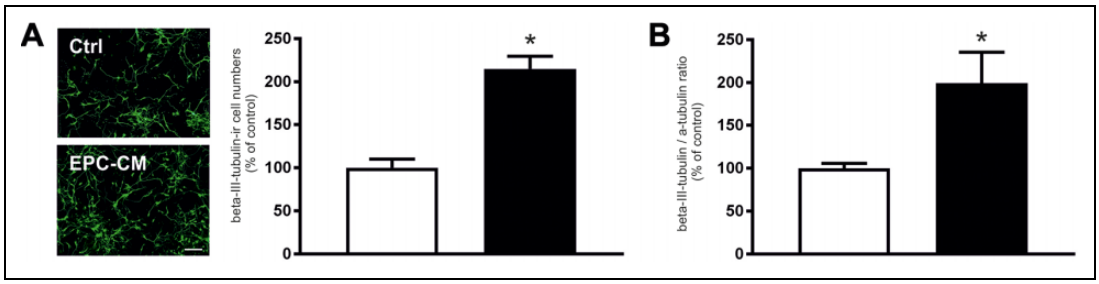

Fig. 2. Representative photomicrographs, quantitative analysis of beta-III-tubulin-positive neurons, and Western blot quantification of beta-III-tubulin in fetal rat cerebral cortical cultures grown for 7 DIV treated without (Ctrl, open bar) or with endothelial progenitor cells secretome (EPC-CM, filled bar) from DIV5 to DIV7. Scale bar: 100 μm. Data are presented as a percentage of controls and values given as mean + SEM. *$P < 0.05$.
DIV: days *in vitro*; EPC-CM: conditioned medium of peripheral blood-derived endothelial progenitor cells.

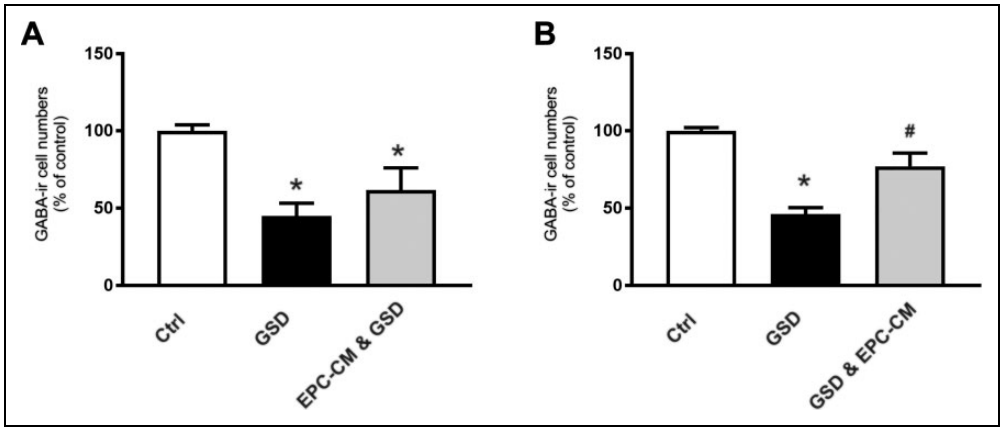

Fig. 3. Quantitative analysis of GABA-ir cell densities in cerebral cortical cultures fetal cultures in a pretreatment and posttreatment regimen. Cultures were grown for 7 DIV. GSD was performed from DIV2 to DIV4. Cultures were exposed to EPC-CM either from DIV0 to DIV2 (EPC-CM and GSD) or from DIV4 to DIV6 (GSD and EPC-CM). Untreated cultures served as controls (Ctrl). Data are presented as a percentage of controls and values are given as mean + SEM. *$P < 0.05$ versus control; #$P < 0.05$ versus GSD-treated cultures.
DIV: days *in vitro*; EPC-CM: conditioned medium of peripheral blood-derived endothelial progenitor cells; GSD: glucose and serum deprivation.

12.17; $P < 0.05$] even though they did not reach control levels (Figure 3B).

Discussion

There is mounting evidence that EPC release a wide array of factors including growth factors, cytokines, lipids, and extracellular matrix, which play a crucial role in supporting tissue viability and trigger a regenerative response[21–23]. In fact, the paracrine actions are believed to be the main mediators for the therapeutic effect of EPC transplanted in host tissues[24,25]. The present work showed that EPC-CM treatment increased total cell numbers as well as overall cell viability hinting to the idea that EPC-CM treatment targeted a variety of neuronal and non-neuronal cell populations. Importantly to note, we demonstrate that EPC-CM exerts important supporting cues on neuronal cells. In particular, we report that EPC-CM substantially increases the density of mature neurons as found by immunocytochemical and immunoblot detection of the specific marker beta-III-tubulin. This notion is further supported by the higher signal intensity of MAP2, which is exclusively expressed in perikarya and dendrites of neurons[26]. Notably and to our knowledge for the first time, we could demonstrate that EPC-CM disclosed the capacity to confer neuroprotection from a metabolic insult. These results extend our previous observations regarding survival-promoting capacity of paracrine factors released by peripheral blood-derived EPC on striatal and ventral mesencephalic neuronal cells[78]. The mechanisms and pathways involved in higher GABA-ir cell densities found with EPC-CM supplementation in cultures challenged by GSD, however, were not addressed in the present study. It is known that insufficient supply of glucose induces neuronal cell death as a result of a series of events including activation of poly(ADP-ribose) polymerase-1, and mitochondrial permeability transition, activation of neuronal glutamate

receptors, and production of reactive oxygen species[27]. Findings from our earlier reports suggest that cell viability supported by EPC-CM may be due to a raise in the antioxidant capacity of the cell[28]. Moreover, it can be hypothesized that EPC-CM supports the bioenergetic homeostasis as it is known that developing cortical neurons' functionality in *in vitro* systems is deeply influenced by the metabolic microenvironment[29].

The identification of the factors for the survival improvement of neuronal progenitors was not specifically addressed in our paper. However, our earlier study identified lipidic factors as important mediators of the actions of EPC-CM[7]. Moreover, we have previously shown that EPC-CM contains a number of neurotrophic factors including brain-derived neurotrophic factor glial cell line-derived neurotrophic factor, neuritin, and vascular endothelial growth factor, which are critical for the developing cerebral cortex[8,30]. In addition, it has been reported that EPC secretome has the capacity to promote oligodendrocyte expansion and maturation in mice in a model of cerebral hypoperfusion[6]. Similar to our earlier observations, in the present study EPC-CM did not display significant effects on astrocytes hinting to the idea that this cell type is not a major target of the EPC-CM-mediated actions. Although it is difficult to compare findings gathered from different culture systems, it is interesting to note that we did not find any newly generated neurons in ventral mesencephalic cultures following EPC-CM treatment[8]. Further experiments will be needed to verify whether EPC-CM promotes the proliferation of immature neurons in cortical cultures and to evaluate the possible role of lipids and other factors. On the other hand, the finding that EPC-CM induced a prominent increase of microglia cell numbers was not surprising and in line with our past report[7]. In fact, we have previously reported that several immunomodulatory cytokines are secreted from EPC[7,8]. It is thus reasonable to speculate that the effects of EPC-CM on cortical neurons are the result of the direct action of neurotrophic factors found in EPC secretome but also influenced by the complex network of signaling cross-talk between the different cell types present in the cortical cultures. In this context, the definition of the actual impact of microglia activation would improve the understanding of the mechanisms of action of EPC-CM and eventually further improve the tissue regenerative capacity of EPC-CM.

In sum, the present study broadens the current knowledge of the tissue-protective and -regenerative potential of EPC-CM by demonstrating the supportive activity of EPC secretome against GSD-induced neuronal degeneration *in vitro*. These novel findings thus highlight the potential of EPC-CM to conceive strategies to promote neuronal viability. It can be prospected that EPC-CM might contribute to the future development of new approaches for neurorestorative therapeutic paradigms alternative or complementary to cell transplantation.

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Ethical Approval

Ethical Approval is not applicable for this article.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Animal Research Ethics Committee of the Canton Bern, Switzerland, and with approval of the University of Bern Animal Care and Use Committee, Switzerland (authorizations numbers BE6/12 and BE10/15 and BE119/16).

Statement of Informed Consent

Informed consent for patient information to be published in this article was not obtained because the buffy coats employed for EPC isolation were purchased from the Interregional Transfusion Centre of the Swiss Red Cross (Bern, Switzerland) and no experimental approval or informed consent was needed.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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