

## Human Mesenchymal Stromal Cells and Their Derivative, SB623 Cells, Rescue Neural Cells via Trophic Support Following In Vitro Ischemia

Ciara C. Tate, Carlos Fonck, Michael McGrogan, and Casey C. Case

SanBio, Inc., Mountain View, CA, USA

Cell transplantation is a promising treatment strategy for many neurological disorders, including stroke, which can target multiple therapeutic mechanisms in a sustained fashion. We investigated the ability of human mesenchymal stromal cells (MSCs) and MSC-derived SB623 cells to rescue neural cells via trophic support following an in vitro stroke model. Following oxygen glucose deprivation, cortical neurons or hippocampal slices were cocultured with either MSCs or SB623 cells separated by a semiporous membrane (prohibits cell–cell contact) or with MSC- or SB623 cell-conditioned medium. MSCs, SB623 cells, MSC-conditioned media, and SB623 cell-conditioned media all significantly reduced neural cell damage/death compared to untreated conditions, and the rescue effect of the conditioned media was dose dependent. We identified 11 neurotrophic factors secreted by MSCs and/or SB623 cells. This study emphasizes the importance of trophic support provided by marrow-derived cells, which likely contributes to the efficacy of cell therapy for brain injury.

Key words: Cerebral ischemia; Mesenchymal stromal cells; Oxygen glucose deprivation; SB623 cells; Trophic support

### INTRODUCTION

Stroke is the leading cause of long-term disability and the third leading cause of death in the US (29), prompting the demand for more effective clinical treatments. Cell transplantation is a promising treatment strategy, which compared to other therapies has the advantage of targeting a variety of mechanisms in a sustained manner. Adult bone marrow-derived mesenchymal stromal cells (MSCs) are a particularly attractive cell candidate for a number of reasons, including the relative ease with which they can be obtained and expanded in vitro. Transplantation of these cells has been shown to improve functional recovery following experimental focal ischemia in rodents [for review, see (26, 36)]. MSCs can also be manipulated in vitro prior to transplantation into assuming specific phenotypes and properties. We have developed a novel human MSC-derived cell known as SB623 by transiently transfecting MSCs with an expression vector encoding human *Notch1* intracellular domain (NICD; regulates stem cell fate) (7). SB623 cells have been shown to reduce the lesion volume and promote functional recovery when delivered to rodent brains following experimental focal ischemia (43). Furthermore, postischemic transplantation of simi-

larly generated SB623-like cells led to enhanced functional recovery compared to transplantation of untransfected MSCs (10,22).

Although cell transplantation has been shown to be beneficial for the treatment of stroke and other neurological disorders, the mechanisms of action are not well understood. Donor cells potentially help injured tissue through cell replacement and/or by providing trophic support. Some studies show that MSCs can transdifferentiate into neural cells, including neurons, astrocytes, and neural stem/progenitor cells, in specific in vitro (7, 31) or in vivo (14,25) environments. Trophic support is a more likely therapeutic mechanism because the effects of transplanted cells occur relatively quickly and persist even as donor cell survival decreases (26). MSCs are known to produce a variety of trophic factors, which may be beneficial to the injured and regenerating brain (3,28). Trophic factors in turn could promote cell survival, blood–brain barrier repair, angiogenesis, regeneration/plasticity (e.g., neurogenesis, neurite outgrowth, synapse formation), and remyelination. To address the complex pathology of the injured brain, it is likely that a combination of these roles occurs and is dependent on cytokines secreted by the donor cells. In this study we utilize in vitro models of cerebral ischemia to investigate

the ability of SB623 cells, as well as their parent MSCs, to rescue injured neural cells via trophic support. In addition, we identify specific cytokines secreted by these cells, which may be beneficial.

## MATERIALS AND METHODS

### *Cell and Tissue Culture*

Two potential human donor cell types were examined in this study: mesenchymal stromal cells (MSCs) and MSC-derived SB623 cells. The effects of these donor cells were examined in an in vitro model of ischemia applied to either primary rat neurons or cultured rat hippocampal brain slices. All reagents were from Invitrogen (Carlsbad, CA) unless indicated otherwise.

### *Donor Cells (MSCs and SB623 Cells)*

Bone marrow aspirates from healthy human adults were obtained from Lonza (Walkersville, MD), rinsed, and plated in tissue culture flasks. Culture medium for the generation and maintenance of donor cells was  $\alpha$ -minimum essential medium ( $\alpha$ MEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin (referred to throughout the text as "growth medium"). Nonadherent cells were discarded, and the remaining cells were passed two times using trypsin (0.25% + 1 mM EDTA). MSCs were then either frozen for later use or plated for SB623 cell preparation. For SB623 preparation, MSCs were transfected with pCI plasmid containing human *Notch1* intracellular domain (NICD; *Notch1* truncated at the transmembrane domain) and neomycin-resistance gene using Fugene6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. The next day, medium was replaced with growth medium containing 100  $\mu$ g/ml G418 and selection continued for 7 days. Selection medium was then replaced with growth medium. After removal of G418, cells were passed two additional times. SB623 cells were harvested using trypsin-EDTA and cryopreserved for later use. For experiments, frozen MSCs and SB623 cells from the same human donor were thawed, replated, and allowed to recover for approximately 1 week. Cells from a total of 11 different human donors were used for this study, typically testing one to three matched sets of donor cells (MSCs and SB623 cells derived from the same donor) per experiment. Both MSCs and SB623 cells were routinely characterized by flow cytometry analysis and were found to be positive (>95%) for CD29, CD90, and CD105, and negative (<5%) for CD31, CD34, and CD45, indicating their mesenchymal nature.

### *Neurons*

Primary rat neurons were used in an in vitro model of cerebral ischemia. Rat cortical tissue pieces from em-

bryonic day 18 were obtained from BrainBits (Springfield, IL) and dissociated. Tissue pieces were placed in Hank's balanced salt solution (HBSS) with trypsin (0.25% + 1 mM EDTA) at 37°C for 5 min. The trypsin-EDTA was inactivated with serum-containing medium, removed, and then rinsed with HBSS. Tissue was then triturated in HBSS containing DNase I (0.15 mg/ml; MP Biomedicals, Solon, OH). Cells were then centrifuged at  $210 \times g$  for 5 min. Following resuspension, cells were plated on poly-*d*-lysine-coated plates at  $\sim 7,000$  cells/cm<sup>2</sup> and cultured in Neurobasal™ medium supplemented with 2% B-27 and 0.5 mM GlutaMAX™ to minimize glial cell contamination (referred to throughout the text as "neuronal medium"). Neuronal medium was changed 50% every 3–4 days, and neurons were used for experiments at 14 days in vitro to give them time to mature and form networks. Cultures were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub> and atmospheric oxygen.

### *Organotypic Hippocampal Slices*

All procedures involving animals conformed to guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals. For an additional and more in vivo-like model of cerebral ischemia, hippocampal brain slices were utilized. Hippocampal slices, which contain multiple cell types normally found in the brain and retain neuronal connectivity ex vivo, were prepared using standard methods. Briefly, 9-day-old Sprague-Dawley rat pups (Charles River, Wilmington, MA) were euthanized by CO<sub>2</sub> inhalation followed by rapid decapitation. Hippocampi were dissected and cut (400  $\mu$ m thick) using a McIlwain tissue chopper. Tissue slices were placed on a 30-mm insert (1 slice/insert; Millipore, Billerica, MA), the base of which was a polytetrafluoroethylene (PTFE) membrane with a pore size of 0.4  $\mu$ m. The insert was in a well containing 50% MEM plus Earl's salts, 25% HBSS, 25% horse serum, and supplemented with 1% penicillin/streptomycin, 8.5 mM HEPES (Sigma-Aldrich, St. Louis, MO), 5.5 mM glucose (Sigma-Aldrich), and 0.5 mM GlutaMAX™. The liquid in the well reached the bottom of the insert and kept the insert wet. After 1 day, the medium was slowly changed over to Neurobasal™ medium supplemented with 2% B-27 and 1.0 mM GlutaMAX™ (referred to throughout the text as "slice medium"). Slices were used at 4–6 days in vitro. Cultures were maintained at 35°C in a humidified incubator containing 5% CO<sub>2</sub> and atmospheric oxygen.

### *Oxygen Glucose Deprivation Model*

Oxygen-glucose deprivation (OGD) is a well-characterized in vitro model of ischemia (8,23). This insult involves the deprivation of oxygen and nutrients for a specific period of time followed by reoxygenation under

normal culture conditions. Medium was changed to deoxygenated Dulbecco's modified Eagle's medium without glucose [after one rinse with Dulbecco's phosphate-buffered saline (DPBS)] and plates containing neurons or slices were placed in an anaerobic chamber. The chamber was housed in a 37°C humidified incubator and was made anaerobic by flushing with 95% N<sub>2</sub>/5% CO<sub>2</sub> prior to and during the experiment. The level of O<sub>2</sub> was measured and maintained at 0.0% using a Proox sensor and controller (BioSpherix, Redfield, NY). Neurons or slices were exposed to OGD for 60 or 90 min, respectively. Parallel control cultures received normal neuronal or slice medium and were placed in the same incubator outside of the anaerobic chamber (therefore at atmospheric oxygen) for the same amount of time. Immediately after OGD or control conditions, the medium was changed again to neuronal or slice medium. At this time, neurons or slices were combined with either donor cells or donor cell-conditioned medium as described below.

#### *Coculture of Donor Cells and Neural Cells*

Following exposure to OGD, neurons or brain slices were cocultured with donor cells (either MSCs or SB623 cells). Donor cells were separated from either neurons or brain slices by a porous membrane (0.4 μm), which allowed for the exchange of molecules, but prohibited cell–cell contact. Cortical neurons were cultured in 12-well plates for 14 days *in vitro* prior to OGD. Donor cells ( $n = 11$  matched donors) were plated in 12-well inserts (polyester, 1.12 cm<sup>2</sup>; Corning, Lowell, MA) and grown to 80–100% confluence (~10,000 cells/cm<sup>2</sup>) in serum-containing growth medium. Prior to placing donor cell-containing inserts into 12-well plates containing neurons, the donor cells were rinsed one time with DPBS and medium was changed to neuronal medium, consequently removing serum from the coculture. Untreated neurons received donor cell-free (neuronal medium only) inserts. Hippocampal slices were cultured on inserts placed inside six-well plates, and donor cells ( $n = 6$  matched donors) for coculture with slices were grown in six-well plates to 80–100% confluence (~10,000 cells/cm<sup>2</sup>) in serum-containing growth medium. Prior to adding inserts containing brain slices to six-well plates containing donor cells, the donor cells were rinsed once with DPBS, and medium was changed to slice medium, thus removing serum from the coculture. Untreated slices went into cell-free (slice medium only) wells. Donor cells were combined with neural cells (neurons or slices) immediately following OGD and were cocultured for 24 or 48 (slices only) h, at which time neural cell damage was assessed.

For a subset of experiments, coculture of donor cells ( $n = 4$  matched donors) with neurons was initiated at a later time to determine if delaying the addition of the

donor cells affected recovery of neurons. At 24 h post-OGD, cell damage was assessed, the medium was changed to fresh neuronal medium, and inserts containing donor cells were added. Then cell damage was assessed after another 24 h (i.e., 48 h after the initial OGD insult).

#### *Conditioned Media*

We also studied the role of trophic support provided by donor cells by culturing post-OGD neural cells with donor cell-conditioned medium. Donor cells were grown on either 12-well (for neuron studies) or six-well (for slice studies) plates to 80–100% confluence (~10,000 cells/cm<sup>2</sup>). Then, serum-containing growth medium was removed, donor cells were rinsed one time in DPBS (to remove serum), and neuronal or slice medium was added. Donor cell-conditioned neuronal or slice medium was collected 24 h later and immediately added to wells with neurons (medium from  $n = 8$  matched donors) or slices (medium from  $n = 5$  matched donors), respectively. The donor cell-conditioned media was added immediately following OGD (or control non-OGD conditions), and cell damage was assessed 24 h later. Untreated wells received fresh neuronal or slice medium only.

To determine whether there was a dose-dependent effect, conditioned media at various dilutions were tested for their ability to rescue neurons post-OGD. Donor cell-conditioned medium ( $n = 5$  matched donors) was obtained as described above and diluted in neuronal medium at one of the following dilutions: 1:1, 1:10, 1:50, or 1:100. The diluted conditioned medium was added to neurons immediately after OGD (or control non-OGD conditions) and cell damage was assessed 24 h later. Untreated wells received fresh neuronal medium only.

#### *Assessment of Cell Damage*

At 24 and 48 (for slices only) h post-OGD, cell damage was assessed by quantifying the concentration of lactate dehydrogenase (LDH) released into the medium, which is directly proportional to cell damage/death; and normalizing to the appropriate control conditions. Neurons or slices exposed to non-OGD control conditions were also cocultured with donor cells, in conditioned medium or in neuronal/slice medium only to account for LDH release from uninjured cultures and from donor cells. Results are thus reported as the fold change in LDH release for neurons or slices that underwent OGD followed by treatment, compared to neurons or slices that underwent non-OGD control conditions followed by the same treatment. LDH was assayed using the Cytotoxicity Detection Kit from Roche Applied Science (Indianapolis, IN) according to the manufacturer's protocol, and results were read on a SpectraMax Plus plate

reader (Molecular Devices, Sunnyvale, CA). A standard curve for LDH concentration was made with serial dilutions of bovine LDH (Sigma-Aldrich). Sampling for each experiment included two to four wells/group for neurons and five to six wells/group for slices. Data from multiple wells were then averaged to obtain the value for each human donor or for "OGD only" for a specific experiment. These values for each donor or each experiment (for "OGD only") were then used for statistical analysis.

As an additional outcome measure for cell damage, propidium iodide uptake was assessed. Propidium iodide permeates cell membranes of damaged/dying cells, where it binds to DNA, resulting in greatly increased fluorescent intensity. At 24 (neurons) or 48 (slices) h post-OGD, media was changed to 5  $\mu$ M propidium iodide (Sigma-Aldrich) in PBS, incubated at 37°C for 1.5 h, and rinsed twice in PBS. Microscopy (Zeiss, Oberkochen, Germany) was performed to evaluate propidium iodide fluorescence. To further confirm the validity of using LDH as a quantitative measure of neural cell damage, the propidium iodide uptake was also quantified for neurons cocultured with MSCs or SB623 cells ( $n = 4$  matched donors) or MSC- or SB623 cell-conditioned media ( $n = 4$  matched donors) following OGD. Cells were counted in representative micrographs from four different wells per group using ImageJ software (NIH) to determine the percentage of propidium iodide-positive cells. The percentage of propidium iodide-positive cells for neurons that underwent OGD was then normalized to the neurons that underwent non-OGD control conditions followed by the same treatment. The results for cell damage were indistinguishable when assessed by propidium iodide uptake versus LDH release. Thus, the LDH assay was consistently used to obtain quantitative assessments of cell damage and is reported below.

#### *Assessment of Cytokines in Conditioned Medium*

To identify which trophic factors are secreted by MSCs and SB623 cells, the protein levels of specific candidate trophic factors in donor cell-conditioned medium were measured. To obtain conditioned medium, MSCs and SB623 cells ( $n = 5$  matched donors) were cultured separately in growth medium to ~90% confluence (~15,000 cells/cm<sup>2</sup>). The medium was then replaced with Opti-MEM® medium (~200,000 cells/ml), and the conditioned medium was collected 72 h later. A semiquantitative custom Quantibody® array (RayBiotech, Norcross, GA) was used to determine which of following 30 cytokines were detectable in the donor cell-conditioned media: bone morphogenetic proteins-4, -6, and -7 (BMP-4, -6, -7), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), Dickkopf-1 and -4 (DKK-1, -4), epidermal growth factor (EGF), erythropoietin receptor, fibroblast growth

factor-2 and -7 (FGF-2, -7), glial cell line-derived neurotrophic factor (GDNF), granulocyte colony stimulating factor (GCSF), heparin binding-epidermal growth factor like growth factor (HB-EGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), interleukin-1 $\alpha$ , -6, and -8 (IL-1 $\alpha$ , -6, -8), leukocyte inhibitory factor (LIF), matrix metalloproteinase-1 (MMP-1), monocyte chemoattractant protein-1 (MCP-1), nerve growth factor (b-NGF), neurotrophin-3 (NT-3), platelet-derived growth factor (PDGF-AA), stromal cell-derived factor (SDF-1), transforming growth factor- $\alpha$  and - $\beta$  (TGF- $\alpha$ , - $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and vascular endothelial growth factor (VEGF). Relative amounts of cytokine expression were compared to levels found in the OptiMEM® only control by comparing the normalized signal intensities. Per the recommendation of RayBiotech, if the signal intensity for a given cytokine in a given sample was over 1.5 times higher than the signal intensity for the OptiMEM® only control, then that conditioned medium sample was considered to have detectable levels of that cytokine. Notably, certain growth factors are known to contain heparin-binding domains and, therefore, although secreted by donor cells, may not become soluble in the medium because they instead bind to the endogenous heparin on the cell. To test for the production of such factors by MSCs or SB623 cells, for two matched donors, heparin (50  $\mu$ g/ml) was added to Opti-MEM® daily until conditioned medium was collected (a total of three days).

#### *Statistical Analysis*

For each experiment (which included two to four wells/group for neurons and five to six wells/group for slices), a mean value indicating the fold change in LDH release in OGD versus the matched control condition was obtained for: 1) the treatment condition for each cell type (either MSCs or SB623 cells), one value per human donor tested and 2) the untreated group (one value for each round of OGD). For statistical comparison (SigmaStat, Systat Software, San Jose, CA) each of these values was used and comparisons were made using one-way ANOVA between the following groups: 1) OGD only (untreated condition), 2) OGD + MSCs or OGD + MSC-conditioned medium, and 3) OGD + SB623 cells or OGD + SB623 cell-conditioned medium. Additional pairwise comparisons were made using Tukey's test. An alpha value of 0.05 was used to assess if the means were significantly different. Data are presented throughout the text as mean  $\pm$  SD unless indicated otherwise.

## **RESULTS**

### *MSCs and SB623 Cells Rescue Neural Cells Following OGD*

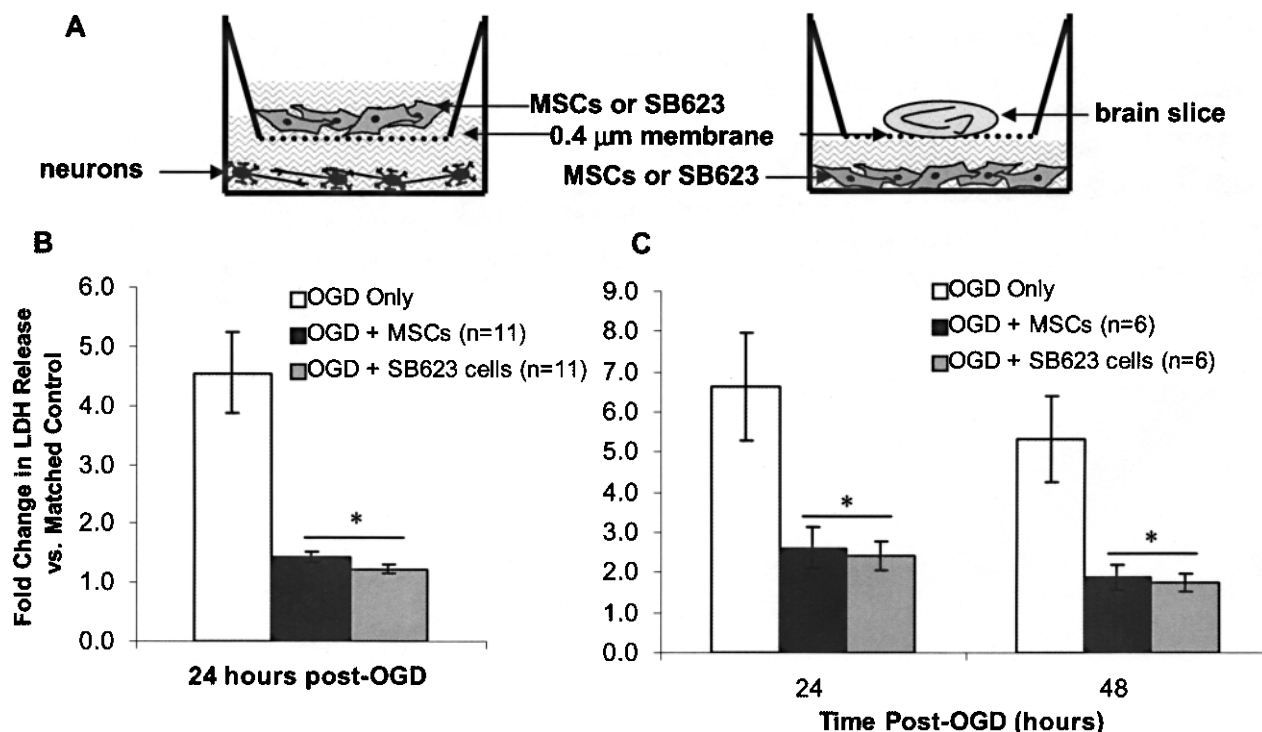
Cerebral ischemia was modeled in vitro using a well-established oxygen-glucose deprivation (OGD) protocol

applied to either primary rat cortical neurons or hippocampal brain slices. Neurons or brain slices that were exposed to 60 or 90 min of OGD, respectively, had significantly more cell death compared to uninjured controls by 24 (neurons and slices) or 48 h (slices) post-OGD ( $p < 0.05$ ), which is consistent with previous studies using these models (19,20). Immediately following exposure to OGD, neurons or brain slices were cocultured with marrow-derived donor cells (either MSCs or SB623 cells), separated by a semiporous membrane (pore size  $0.4 \mu\text{m}$ ), which allowed for the exchange of soluble molecules, but precluded cell–cell contact. There was a significant reduction in neural cell damage, measured by LDH release, when neurons/slices were cocultured with either MSCs or SB623 cells compared to untreated conditions ( $p < 0.05$ ) (Fig. 1). Importantly, the ability of the human donor cells to rescue the neural cells was consistent across multiple human donors. For neurons, injured cultures had  $4.6 \pm 2.2$  times more LDH release compared to uninjured controls at 24 h post-OGD. However, when neurons were cocultured with MSCs or SB623 cells, neuronal cell damage significantly decreased ( $p < 0.05$ ) to only  $1.4 \pm 0.3$  or  $1.2 \pm 0.2$  times more LDH release than corresponding uninjured controls, respectively. Qualitative analysis of the neuronal morphology after injury revealed that the neurites were damaged by OGD at 24 h postinjury, but the neu-

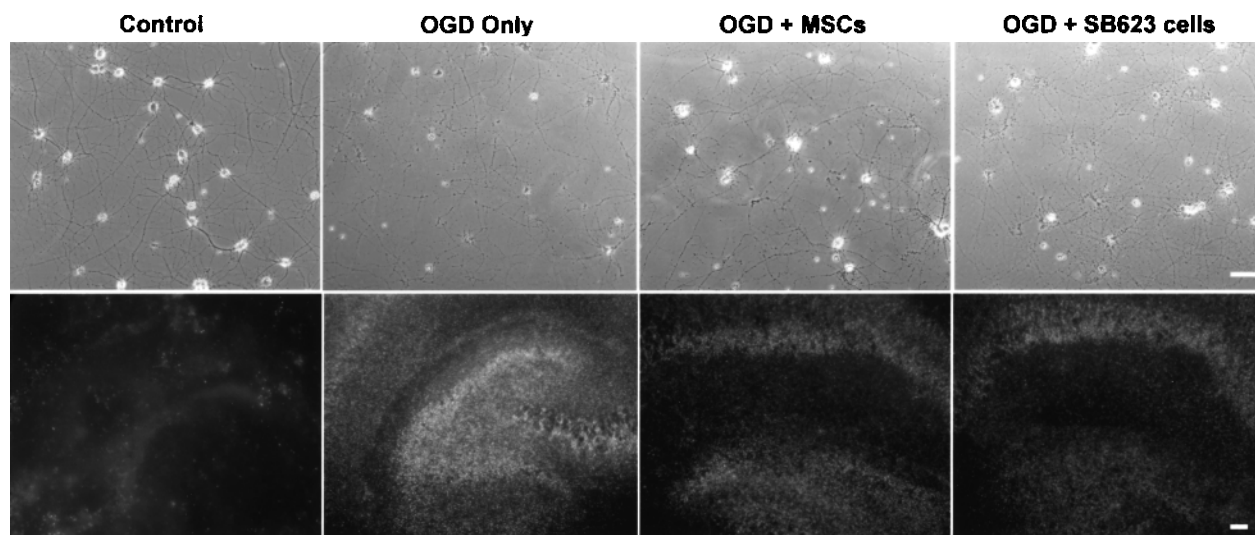
rites were partially preserved when neurons were cocultured with donor cells (Fig. 2).

The OGD insult to hippocampal slices resulted in a slightly more severe injury than neurons with  $6.6 \pm 3.3$  and  $5.3 \pm 2.6$  times more LDH release compared to uninjured controls at 24 and 48 h post-OGD, respectively. When slices were cocultured with MSCs or SB623 cells for 24 h, the amount of neural cell damage significantly decreased ( $p < 0.05$ ) to  $2.7 \pm 1.2$  or  $2.6 \pm 0.9$  times more LDH release than corresponding uninjured controls, respectively. This was further enhanced after 48 h of coculturing with MSCs or SB623 cells, resulting in only  $1.9 \pm 0.8$  or  $1.8 \pm 0.5$  times more LDH release than corresponding uninjured controls, respectively. Moreover, qualitative analysis of propidium iodide uptake in the hippocampal slices further demonstrated that coculturing with MSCs or SB623 cells reduces cell damage (Fig. 2). Note that the viability of the donor cells was not affected by the injured environment. For example, the viability (measured by trypan blue uptake) of donor cells after 48 h in coculture with injured and control slices was  $98 \pm 3\%$  and  $99 \pm 2\%$ , respectively.

When the coculturing of neurons with MSCs or SB623 cells began at 24 h (instead of immediately) post-OGD, there was still a significant reduction in neuronal cell damage 24 h later (48 h post-OGD) compared to untreated conditions at both 24 and 48 h post-OGD



**Figure 1.** Marrow-derived stromal cells rescue injured neural cells. (A) A schematic of donor cells cocultured with either neurons (left) or brain slices (right) is shown (not to scale). There is a significant reduction in cell damage when neurons (B) or brain slices (C) are cocultured with MSCs or SB623 cells following OGD. \* $p < 0.05$  versus OGD only; mean  $\pm$  SEM.

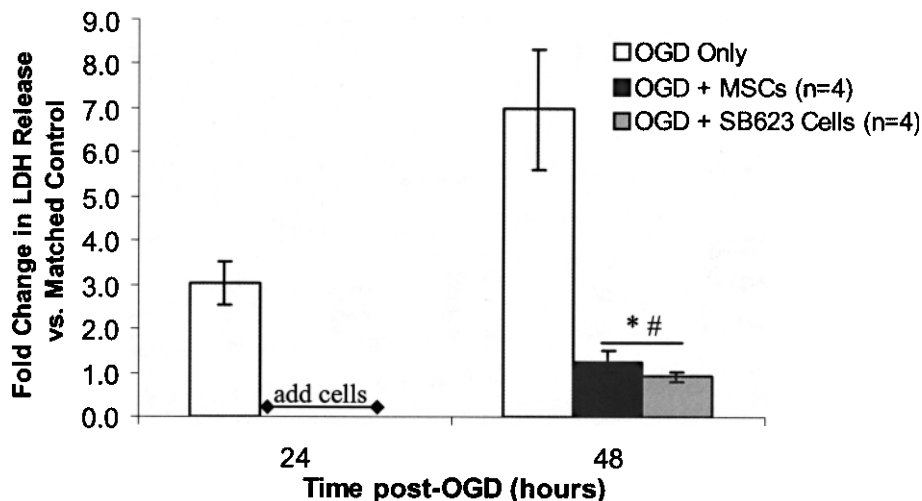


**Figure 2.** Micrographs of injured and treated neurons and slices. (Top row) Brightfield representative micrographs of cortical neurons 24 h post-OGD; (bottom row) fluorescent representative micrographs of hippocampal slices 48 h post-OGD and after 1.5-h incubation with propidium iodide. Groups from left to right are control (uninjured), OGD, OGD followed by coculture with MSCs, and OGD followed by coculture with SB623 cells. Scale bar: 100  $\mu$ m.

( $p < 0.05$ ) (Fig. 3). At 24 h post-OGD, injured neurons had  $3.0 \pm 0.7$  more LDH release than uninjured controls, which increased to  $7.0 \pm 1.8$  times more LDH release than uninjured controls by 48 h post-OGD. Coculturing neurons with MSCs or SB623 cells for the latter 24 h significantly decreased ( $p < 0.05$ ) neuronal cell damage to  $1.3 \pm 0.5$  or  $0.9 \pm 0.3$  times more LDH release, respectively, than corresponding uninjured controls. These data indicate that donor cells are able to rescue injured neural cells at multiple time points of intervention.

#### *MSC- and SB623 Cell-Conditioned Medium Rescues Neural Tissue Following OGD*

Because donor cells and neural cells were not in direct contact during coculture, the above data indicate that the donor cells likely provide beneficial cytokines and/or actively buffer the toxic environment. To further test the cytokine secretion hypothesis, we tested the ability of the donor cell-conditioned media to affect injured neural cells. Conditioned medium was obtained by cul-



**Figure 3.** Delayed timing of coculture still rescues injured neural cells. When the coculture of either MSCs or SB623 cells is initiated at 24 h post-OGD, there is a significant reduction in neuronal damage 24 h later. \* $p < 0.05$  versus OGD only at 24 h; # $p < 0.05$  versus OGD only at 48 h; mean  $\pm$  SEM.

turing MSCs or SB623 cells in either neuronal or slice medium for 24 h. Immediately following the ischemic insult, the medium in contact with neurons or brain slices was changed to donor cell-conditioned medium, and cell damage was assessed 24 h later. Neurons and hippocampal slices that were given either MSC- or SB623 cell-conditioned medium had significantly less cell damage compared to untreated conditions ( $p < 0.05$ ) (Fig. 4). At 24 h post-OGD, neurons and slices had  $3.3 \pm 1.2$  and  $5.8 \pm 3.6$  times more LDH release, respectively, than uninjured controls. Culturing neurons with MSC- or SB623 cell-conditioned media for 24 h significantly reduced ( $p < 0.05$ ) cell damage to  $1.2 \pm 0.2$  or  $1.1 \pm 0.2$  times more LDH release, respectively, than corresponding uninjured controls. Culturing slices with MSC- or SB623 cell-conditioned media for 24 h significantly reduced ( $p < 0.05$ ) cell damage to  $2.8 \pm 1.0$  or  $2.6 \pm 0.4$  times more LDH release, respectively, than corresponding uninjured controls. There were no significant differences in cell damage between neurons or brain slices treated with donor cells in coculture compared to those treated with donor cell-conditioned medium ( $p > 0.05$  in all cases). Furthermore, the observed rescue effect on injured neurons decreased when the conditioned medium was diluted, indicating a dose-dependent response to the conditioned medium (Fig. 5). These data reveal that factors secreted by MSCs and SB623 cells are beneficial to injured neural cells, and that the concentration of such factors is important.

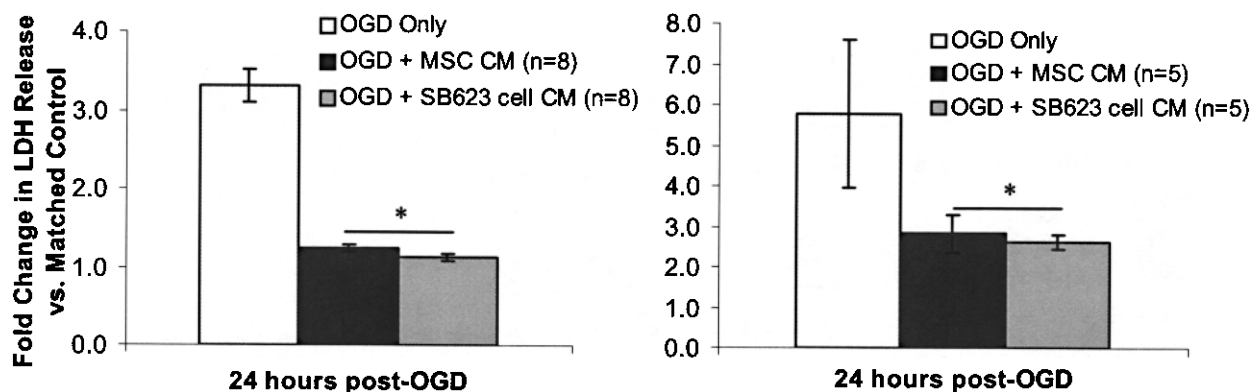
#### *MSCs and SB623 Cells Secrete Multiple Cytokines That Are Potentially Beneficial to Neural Cells*

The data presented above suggest that marrow-derived stromal cells are able to rescue injured neural cells by secreting one or more trophic factors. The presence of 30 specific candidate trophic factors in donor cell-conditioned medium was assessed using a custom

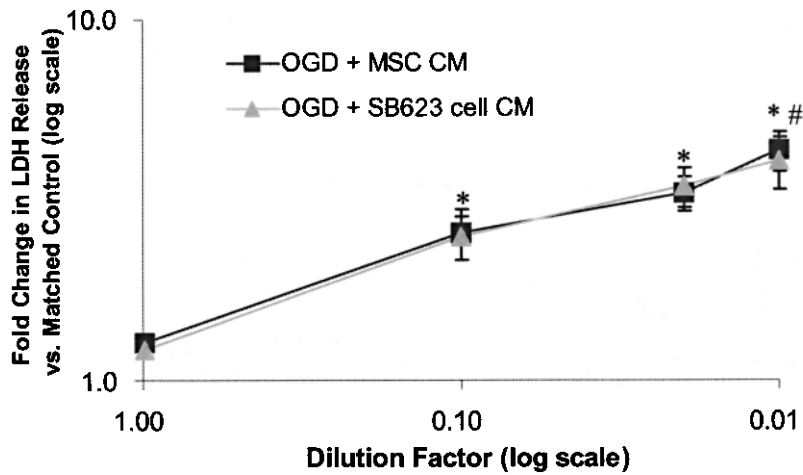
antibody array from RayBiotech. For this analysis, conditioned medium was obtained by culturing MSCs or SB623 cells in serum-free medium for 72 h ( $\sim 200,000$  cells/ml). Relative amounts of cytokine expression in conditioned medium were compared to levels found in the medium only (without cells) control to account for cytokines present in the medium. Thus, for each donor cell type tested (either MSCs or SB623 cells), the mean fold-change versus medium only is reported. Per recommendation by RayBiotech, for each cytokine, a signal with a fold-change greater than 1.5 times the “medium only” control indicates that the cytokine was secreted by the donor cells. Based on this analysis, the following 10 cytokines were detected in conditioned medium from either MSCs or SB623 cells: BMP-4, DKK-1, FGF-7, HB-EGF, IL-6, IL-8, MCP-1, MMP-1, PDGF-AA, and VEGF. A subset of cytokines had increased expression when heparin was added daily to the medium during the conditioning period. Of these, HGF was present in sufficiently high levels to be considered to be secreted by the donor cells. Thus, we found that 11 of the 30 cytokines tested were secreted by either MSCs or SB623 cells. While all 11 of these cytokines were detected in SB623 cell-conditioned medium, IL-8 and MMP-1 were not detected in MSC-conditioned medium. Furthermore, four of the cytokines tested were consistently found at increased levels in SB623 cell-conditioned medium compared to MSC-conditioned medium: DKK-1, IL-6, IL-8, and MCP-1. Results are summarized in Table 1.

#### DISCUSSION

In this study we demonstrate that both MSCs and SB623 cells are able to rescue neural cells *in vitro* through a trophic support mechanism. A well-characterized OGD model was used to apply an ischemic insult to either primary neurons or hippocampal slices. Two types of potential human donor cells were tested for



**Figure 4.** Marrow-derived stromal cell-conditioned medium (CM) rescues injured neural cells. There is a significant reduction in cell damage when neurons (left plot) or brain slices (right plot) are cultured with MSC- or SB623 cell-conditioned medium following OGD. \* $p < 0.05$  versus OGD only; mean  $\pm$  SEM.



**Figure 5.** Dose–response to conditioned medium. The ability of donor cell-conditioned media (CM) to rescue injured neurons is dose dependent. Plot is on a log–log scale. \* $p < 0.05$  versus CM 1:1; # $p < 0.05$  versus CM 1:10; mean  $\pm$  SEM.

their ability to rescue injured neural cells via trophic support: MSCs and SB623 cells, which are MSCs transiently transfected with an NICD expression vector. There was a significant increase in cell survival when primary neurons were cocultured with either MSCs or SB623 cells following ischemic insult. A previous study by Zhong et al. (45) showed that coculturing rat MSCs with hippocampal slices following OGD led to both increase in cell survival and increase in neurite outgrowth. Here we corroborate these results and find that human MSCs as well as their derivatives, SB623 cells, improve neural cell survival when they are cocultured with hippocampal slices following OGD.

Notably, a decrease in neural cell damage was observed when donor cells were added either immediately after the OGD or 24 h later, suggesting that the donor cells can rescue injured neurons at multiple time points of intervention. This is important for clinical applications, as it is preferable to have a wide treatment window, particularly with stroke patients whose early symptoms often go unrecognized. The coculture system used in these studies separated the donor cells from the neurons or slices by a semiporous membrane, which prevented direct cell–cell contact. Therefore, we infer that trophic support is playing a role. There are additional factors that could be contributing to the observed rescue effect, such as the presence of the donor cells actively buffering toxic components present after injury. Thus, in order to further isolate the effects of trophic factors, donor cell-conditioned medium was also tested for its ability to rescue injured neural cells. Both MSC- and SB623 cell-conditioned medium rescued injured neurons or slices, and this occurred in a dose-dependent manner. This suggests that the observed beneficial effects are

mediated by one or more of the soluble factors secreted by these donor cells and that the concentration of such factor(s) is critical.

Previous studies have shown that MSCs produce a variety of neurotrophic factors, including BDNF, FGF-2, GDNF, HGF, IGF-1, MCP, NGF, NT-3, PDGF, and VEGF (3,13,27,40). Here we examined MSC- and SB623 cell-conditioned medium for the presence of 30 candidate cytokines, and found that the following 11 were secreted by these cells in the specific conditions tested here: BMP-4, DKK-1, FGF-7, HB-EGF, HGF, IL-6, IL-8, MCP-1, MMP-1, PDGF-AA, and VEGF. With the exception of DKK-1, all of these proteins are involved in mechanisms that are potentially beneficial to neural tissue following an ischemic insult, such as promoting neural cell survival, migration, neurite outgrowth, neurogenesis, gliogenesis, and angiogenesis (see Table 2 for a summary of potential beneficial mechanisms).

Further studies are warranted to determine the specific combination of factors that are critical for the rescue effects observed in the study presented here. Interestingly, the SB623 cells, which are MSCs transiently transfected with an NICD expression vector, retain their ability to rescue neural cells and secrete multiple trophic factors. Moreover, the production of certain trophic factors by SB623 cells is higher compared to their parent MSCs. One of these, DKK-1, suppresses osteogenesis by inhibiting Wnt signaling (9). The remaining three factors found at higher levels in medium conditioned by SB623 cells versus MSCs were IL-6, IL-8, and MCP-1. All of these are proinflammatory molecules that have been shown to play a dual role after CNS injury (4,24). In addition, both IL-8 and MCP-1 enhance MSC migra-

tion (39). Compared to MSCs, SB623 cells may be increasing cytokine production as they approach a senescent state. Previous reports indicate that when fibroblasts become senescent, the production of cytokines involved in matrix remodeling and local inflammation increases (33). We have seen that SB623 cells display more senescent qualities (e.g., increased expression of p16Ink4A) compared to MSCs, and that this is a result of the transient expression of the NICD, and not strictly a result of a longer culture period compared to their parent MSCs (unpublished observations).

One potential advantage of using cell therapy to treat

stroke and other neurological disorders is the ability of these cells to respond to the needs of the injured tissue. Studies have shown that human MSCs increase trophic factor production when cultured with injured brain tissue extracts (5). In the study presented here, we did not detect significant differences in neural cell damage (as measured by the relative amount of LDH release) when injured neural cells were treated for 24 h with donor cell-conditioned medium compared to when donor cells were present. This implies that any communication between neural cells and donor cells that could potentially alter the observed rescue effect was not detectable using

**Table 1.** Cytokines in Marrow-Derived Stromal Cell-Conditioned Medium

	No. MSC Donors >1.5× OptiMEM®	MSCs (n = 5)		No. SB623 donors >1.5× OptiMEM®	SB623 Cells (n = 5)		No. Donors SB623 >MSC	SB623/MSC	
		Avg.	SD		Avg.	SD		Avg.	SD
BDNF	0	0.6	0.3	0	0.8	0.3	—	1.5	0.7
<b>BMP-4</b>	2	<b>1.6</b>	0.8	4	<b>2.2</b>	0.8	4	<b>1.6</b>	0.8
BMP-6	0	0.6	0.2	0	0.7	0.2	—	1.2	0.6
BMP-7	1	1.1	0.5	2	1.4	0.5	—	1.4	0.9
b-NGF	0	0.9	0.3	1	1.3	0.3	—	1.5	0.3
CNTF	0	0.8	0.2	0	0.8	0.2	—	1.1	0.2
<b>DKK-1</b>	4	<b>2.7</b>	1.1	5	<b>12.5</b>	0.5	5	<b>5.8</b>	3.4
DKK-4	0	0.5	0.2	0	0.7	0.2	—	1.6	1.2
EGF	0	0.6	0.2	0	0.9	0.3	—	1.4	0.3
Erythropoietin R	0	0.8	0.3	0	1.1	0.2	—	1.4	0.4
FGF-2	0	1.0	0.2	1	1.2	0.3	—	1.2	0.4
<b>FGF-7</b>	5	<b>3.7</b>	1.4	4	<b>2.2</b>	0.6	1	0.7	0.4
GCSF	0	0.7	0.2	0	0.9	0.3	—	1.4	0.3
GDNF	0	0.8	0.2	0	0.9	0.1	—	1.3	0.7
<b>HB-EGF</b>	2	<b>2.3</b>	2.2	2	<b>2.0</b>	1.9	2	1.0	0.6
HGF*	0	0.9	0.3	0	1.0	0.4	—	1.0	0.2
IGF-I	0	0.8	0.2	0	1.0	0.2	—	1.2	0.2
IL-1α	0	0.7	0.2	0	0.8	0.2	—	1.2	0.4
<b>IL-6</b>	5	<b>5.0</b>	2.4	5	<b>20.7</b>	24.0	5	<b>3.8</b>	2.7
<b>IL-8</b>	0	0.8	0.0	5	<b>2.8</b>	1.4	5	<b>3.7</b>	1.7
LIF	0	0.8	0.2	0	0.9	0.3	—	1.3	0.2
<b>MCP-1</b>	5	<b>17.0</b>	13.1	5	<b>50.3</b>	20.3	5	<b>4.7</b>	4.6
<b>MMP-1</b>	0	0.9	0.1	3	<b>1.9</b>	1.5	4	<b>2.3</b>	2.0
NT-3	0	0.8	0.2	0	1.1	0.3	—	1.4	0.4
<b>PDGF-AA</b>	5	<b>2.7</b>	1.0	5	<b>3.5</b>	0.5	4	1.4	0.2
SDF-1	0	0.7	0.4	0	0.7	0.4	—	1.2	0.4
TGF-α	0	0.9	0.3	0	1.2	0.3	—	1.3	0.2
TGF-β	0	1.0	0.3	0	1.2	0.3	—	1.2	0.4
TNF-α	0	0.8	0.2	1	1.0	0.2	—	1.3	0.2
<b>VEGF</b>	5	<b>35.0</b>	19.6	5	<b>45.2</b>	8.9	4	<b>1.8</b>	1.5

An ELISA assay was performed to detect cytokines present in conditioned OptiMEM® medium from five matched donors. A fold-change of 1.5 or greater indicates there is a difference between the two groups compared (indicated by bold font). Out of 30 cytokines tested, 10 cytokines were detected in MSC- or SB623 cell-conditioned medium. Four cytokines were found at higher levels in medium conditioned by SB623 cells compared to MSCs for all five of the donors tested (indicated by italic font).

\*Notably, HGF was also detected in two out of two donors when heparin was added daily to the conditioned medium (not shown).

**Table 2.** Potential Beneficial Roles of Cytokines Present in MSC- or SB623 Cell-Conditioned Medium

Cytokine	Potential Beneficial Role Post-Cerebral Ischemia
BMP-4	↑ neural cell survival (11)
FGF-7	involved in neural development (21)
HB-EGF	↑ neurogenesis, ↓ lesion size (12)
HGF	↓ glial scar, ↑ neurite outgrowth, ↑ angiogenesis, improve recovery (34)
IL-6	↓ lesion size (17), ↑ astrogenesis (35)
IL-8	↑ neuronal cell survival (2)
MCP-1	↑ neuroblast migration (42)
MMP-1	↑ angiogenesis (32)
PDGF-AA	↑ neural cell survival, promotes myelination (38)
VEGF	↑ neurogenesis (41), ↑ angiogenesis (16,41), improve recovery (16)

Ten of the 11 cytokines found in MSC- or SB623 cell-conditioned medium are involved in functions potentially beneficial to neural tissue following ischemic injury.

this system. However, conditioned medium from SB623 cells precultured with injured brain slices was more effective at rescuing neurons following OGD compared to conditioned medium from a control environment (data not shown). It is difficult to conclude from this that SB623 cells are responding to the injured environment by increasing trophic factor secretion. It could also be that cytokines found in the injured environment are beneficial to neurons. The effect that an injured environment has on the ability of MSCs and SB623 cells to rescue neural cells warrants further examination.

This study emphasizes the potential benefit of soluble cytokines for rescuing injured neural cells, which are likely to be critical in promoting repair and regeneration when cells are transplanted into the injured brain. MSCs may also be secreting insoluble matrix proteins that promote neural cell survival and regeneration. For example, fibronectin is neuroprotective following brain injury (30,37) and is known to be secreted by MSCs both in vitro and in vivo (6,44). We have previously shown that both MSCs and SB623 cells secrete an extracellular matrix in vitro that is beneficial for neural cell growth, survival, and neurite outgrowth (1). Also, transplanted cells could potentially replace brain cells lost to injury. Following transplantation into the mammalian brain, both MSCs and SB623-like cells have been shown to express neuronal and glial markers (7,14,22,25). Although donor cells can theoretically integrate into endogenous neuronal circuitry, there is decidedly a lack of evidence showing functional integration of donor neuronal-like cells (15,18,26). Donor cells may also promote repair and regeneration by replacing or mimicking the function of astrocytes or oligodendrocytes in the normal adult or developing brain. It is likely that a combination of pro-

tective mechanisms occurs, and is dependent on soluble trophic factors secreted by the donor cells. The work presented here further characterizes the trophic factor profile and rescue ability of SB623 cells and MSCs. This study underscores the importance of trophic support in mediating beneficial effects of transplanting MSCs and their derivatives into the injured brain.

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