

# Extracellular Matrix Produced by Bone Marrow Stromal Cells and by Their Derivative, SB623 Cells, Supports Neural Cell Growth

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Several studies have shown the benefits of transplanting bone marrow-derived multipotent mesenchymal stromal cells (MSC) into neurodegenerative lesions of the central nervous system, despite a low engraftment rate and the poor persistence of grafts. It is known that the extracellular matrix (ECM) modulates neuritogenesis and glial growth, but little is known about effects of MSC-derived ECM on neural cells. In this study, we demonstrate in vitro that the ECM produced by MSC can support neural cell attachment and growth. We also compare the neurosupportive properties of MSC to the MSC derivative, SB623 cells, which is being developed as a cell therapy for stroke. Embryonic rat brain cortical cells cultured for 3 weeks on human MSC- and SB623 cell-derived ECM exhibit about a 1.5 and 3 times higher metabolic activity, respectively, compared with the cultures grown on poly-D-lysine (PDL), although the initial neural cell adhesion to cell-derived ECM and PDL is similar. The MSC- and SB623 cell-derived ECM protects neural cells from nutrient and growth factor deprivation. Under the conditions used, only neurons grow on PDL. In contrast, both MSC- and SB623 cell-derived ECMs support the growth of neurons, astrocytes, and oligodendrocytes, as demonstrated by immunostaining. Morphologically, neurons on cell-derived ECM form more complex and extended neurite networks than those cultured on PDL. Together, these data indicate that the beneficial effect of MSC and SB623 cells in neurotransplantation could be explained in part by the neurosupportive properties of the ECM produced by these cells. © 2009 Wiley-Liss, Inc.

**Key words:** neuroprotection; extracellular matrix molecules; mesenchymal stem cell; astrocytes; neurite outgrowth

Bone marrow-derived, multipotent mesenchymal stromal cells—also referred to as *mesenchymal stem cells* or *marrow stromal cells* (MSC)—represent an attractive source for regenerative transplantation for neurodegenerative diseases. Recently, several animal models and clinical studies have shown that the transplantation of MSC

improves poststroke functional recovery (Chen et al., 2001; Zhao et al., 2002; Bang et al., 2005) and has beneficial effects on spinal cord injuries (Chopp et al., 2000; Hofstetter et al., 2002; Ankeny et al., 2004; Himes et al., 2006; for review see Dezawa et al., 2005; Hardy et al., 2008). The benefits of using these cells include the ease of their propagation in large quantities, their suitability for allogeneic transplantation owing to their immunosuppressive and immunoprivileged properties (Niemeyer et al., 2006; Jones and McTaggart, 2008), their ability to migrate into sites of injury (Satake et al., 2004; Ji et al., 2004), and their ability to participate in tissue repair (for review see Phinney and Prockop, 2007).

The mechanisms behind the beneficial effects of MSC in neurological applications are largely unknown. It has been suggested by some researchers that either MSC or their subpopulations can transdifferentiate into neurons and/or glial cells (Kopen et al., 1999; Zhao et al., 2002) or fuse with neurons (Bae et al., 2005), but currently these hypotheses are disputed (for review see Phinney and Prockop, 2007). Another mechanism attracting extensive research is the secretion of growth factors and cytokines by MSC. Several MSC-secreted factors have indeed been shown to exert neurotrophic and haptotactic effects on neurons (Ankeny et al., 2004; Crigler et al., 2006; Phinney, 2007; Hokari et al., 2008).

SB623 cells are derivatives of MSC that are being developed by SanBio as a potential cell therapy for stroke and other disorders of the central nervous system. SB623 cells are derived from MSC by transient transfection with a vector encoding the human Notch1 intracellular domain (NICD). Similarly generated human and rat cells treated with neurotrophic factors demonstrated superior beneficial effects in a Parkinson's disease model (Dezawa et al.,

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2004) and focal cerebral infarction models (Mimura et al., 2005) compared with untransfected MSC.

Studies of the effects of cell-derived extracellular matrix (ECM) on cell morphology, proliferation, differentiation, and growth factor dependence were pioneered by D. Gospodarowicz and colleagues (1980). It has been shown that neurite outgrowth could be supported by the ECM derived from endothelial cells, Schwann cells, or astrocytes and is dependent on intact ECM sugar moieties (Vlodavsky et al., 1982; Ard et al., 1987; Wujek and Akeson, 1987). The MSC-derived ECM has been reported to facilitate expansion and preserve stem cell properties of MSC (Chen et al., 2007). In transplantations under the renal capsule, the MSC-derived ECM reportedly remains detectable for 3 months and mediates formation of the functional hematopoietic organ (Sadovnikova et al., 1991). The effects of MSC-derived ECM on neural cell growth and differentiation have not been yet studied.

Here we show *in vitro* that the insoluble ECM produced by MSC and SB623 cells promotes growth and differentiation of neural cells and therefore could be a potential means of neurosupportive action of these cell types. We also show that the ECM produced by SB623 cells has greater neurosupportive effects than the ECM produced by the parental MSC.

## MATERIALS AND METHODS

### MSC and SB623 Cell Preparation

Human adult bone marrow aspirates were purchased from Lonza (Walkersville, MD). Cells were washed once and plated in Corning flasks (Corning, Corning, NY) in the growth medium  $\alpha$ MEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin (both from Invitrogen, Carlsbad, CA). After 3 days, unattached cells were removed; the MSC cultures were maintained in the growth medium for about 2 weeks and then subcultured with 0.25% trypsin/EDTA (Invitrogen). On the second passage, some cells were cryopreserved (MSC preparation), and others were plated for the preparation of SB623 cells.

For SB623 cell preparation, MSC were transfected with the pCI-neo expression plasmid encoding human NICD. The transfection was performed with Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. On the next day, the medium was replaced with growth medium containing 100  $\mu$ g/ml G418 (Invitrogen), and the selection continued for 7 days. Then, the selection medium was replaced with G418-free growth medium, the cultures were maintained for about 2 weeks and twice expanded by subculturing. The SB623 cells were harvested and cryopreserved. The frozen MSC and SB623 cells were stored in the vapor phase of liquid nitrogen until they were needed.

Both the MSC and SB623 cells were characterized by flow cytometry on the second passage and before cryopreservation and were found to be positive for CD29, CD90, and CD105 (>95%), and negative for CD31, CD34, and CD45

(<5%). All experiments described here were performed with MSC and SB623 cells that were cryopreserved, then thawed, grown for 5–6 days to allow recovery, and plated for the experiments.

### Plate Coating and ECM Preparation

For the preparation of wells coated with the cell-produced ECM, both MSC and SB623 cells were plated at  $3 \times 10^4$  cells/cm<sup>2</sup> or at the indicated density in the growth medium. After 4 days, the medium was changed to serum-free medium, and the cells were cultured for additional 2 days. Cells were removed from the ECM using a protocol described in Giese et al. (1994), with some modifications. Briefly, they were treated with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in water at room temperature for 30 min. Cell lysates were then carefully aspirated, and a solution of concentrated ammonium hydroxide (Sigma-Aldrich) diluted 1:100 in water was slowly added to the wells for 5–7 min. Then, the wells were carefully washed with PBS twice and used immediately or stored in the last PBS wash at 4°C. In experiments comparing the neurosupportive properties of MSC- and SB623 cell-derived ECM, aliquots of MSC and SB623 cell lysates were removed 5 min after addition of Triton solution and used in an intracellular lactate dehydrogenase (LDH) activity assay (see below) to check the relative cell numbers after culturing.

Poly-D-lysine (PDL), fibronectin, and laminin were purchased from Sigma-Aldrich and Matrigel from BD Biosciences (Bedford, MA). For PDL coating, wells were incubated with PDL at 10  $\mu$ g/ml in water for 1 hr at room temperature. The excess PDL solution was aspirated, and wells were dried and then washed once with PBS. Fibronectin coating was prepared by incubating wells with fibronectin at 5  $\mu$ g/ml in PBS for 1 hr at room temperature. The solution was then aspirated, and wells were dried and washed once with PBS. Laminin coating was prepared by incubating wells with laminin at 2  $\mu$ g/ml in PBS for 2 hr at 37°C and then washing three times with PBS. Matrigel coating was carried out according to the manufacturer's protocol, with undiluted Matrigel. Some wells were also coated with growth medium containing 10% FBS, incubated overnight at 37°C, and then washed with PBS. Before the plating of neural cells in the wells coated as described above, the PBS was replaced with neural growth medium (see below), and the plates were warmed in an incubator until the neural cell suspension was prepared.

### Preparation of Rat Embryonic Brain Cortical Dissociates

Rat embryonic (E18) brain cortex pairs were purchased from BrainBits (Springfield, IL), and a cell suspension was prepared essentially as described by Cullen and LaPlaca (2006). Briefly, cortices were incubated with 0.25% trypsin/EDTA at 37°C for 5–7 min, with occasional shaking. The trypsin was removed and the tissue washed with  $\alpha$ MEM containing 10% FBS. DNase (MP Biomedicals, Solon, OH) at 0.25 mg/ml was then added; and the tube was vortexed for 30 sec. The resulting suspension was triturated 10 times and centrifuged

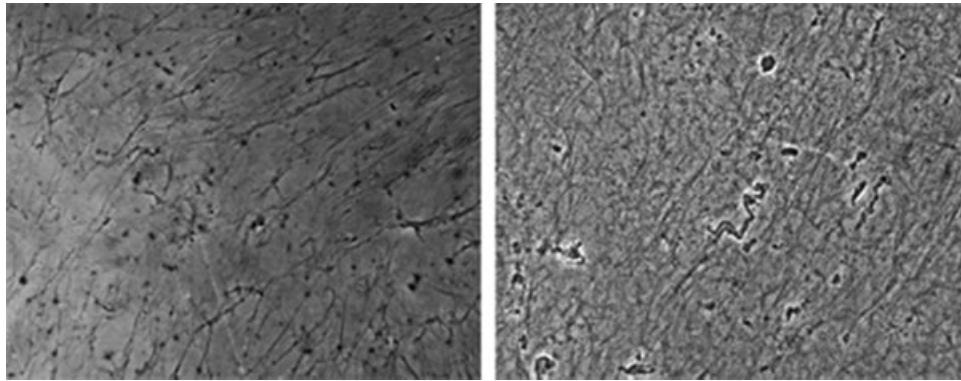


Fig. 1. Phase-contrast microphotograph of the ECM derived from MSC plated at  $2.5 \times 10^4$  cells/cm<sup>2</sup> (left) and  $5 \times 10^4$  cells/cm<sup>2</sup> (right).  $\times 400$ .

for 1 min at 200g. Cells were resuspended in a neural growth medium comprising Neurobasal medium supplemented with B-27 and 0.5 mM Glutamax (NB/B27/Glx; all from Invitrogen). They were then plated into precoated wells at  $1.5 \times 10^4$  cells/cm<sup>2</sup>. The viability of neural cells after the dissociation procedure was 92–98%, as measured by trypan blue exclusion.

#### LDH Assay

An intracellular LDH activity test was used to quantify viable cells in cultures grown in 96-well plates. Culture supernatants were aspirated, and cells were lysed in 2% Triton X-100. Standards were prepared by serial dilutions of bovine LDH (Sigma-Aldrich) in 2% Triton X-100 on each plate. The activity of intracellular LDH was immediately assayed with an LDH kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. After a few minutes, a colorimetric analysis was done with SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). The results were expressed in LDH activity units per well or as a percentage of control.

For time course experiments, different plates were set for each time point. After the aspiration of culture supernatants, plates were frozen in  $-80^\circ\text{C}$  until all time points were harvested. On the day of the LDH test, plates were thawed, cells lysed, and lysates transferred to a new plate, where the analysis of samples from all time points was done simultaneously.

#### Immunocytochemistry

Cells were cultured on glass coverslips (VWR Scientific, West Chester, PA) coated as described above. The morphology of the cultures on glass was similar to that observed on plastic. Fixation and staining procedures were conducted at room temperature. Cultures were washed once with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for 20 min, washed once with PBS, and incubated in blocking solution containing 0.3% Triton X-100 and 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS for 1 hr. The cultures were then incubated for 1.5 hr with the following primary antibodies: rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako, Glostrup,

Denmark), mouse monoclonal anti-microtubule-associated protein 2 (MAP2; Sigma-Aldrich), mouse monoclonal anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; Millipore, Billerica, MA), or control mouse or rabbit IgG (Millipore). Secondary antibodies [fluorescein (FITC)-anti-rabbit and Cy3-anti-mouse, both affinity-purified F(ab')<sub>2</sub> fragments of donkey IgG minimally cross-reactive with serum proteins of other species (Jackson ImmunoResearch)] were added for 1 hr, followed by washing and mounting with ProLong Gold antifade reagent containing DAPI (Invitrogen). Fluorescent microscopy was performed with an Axioskop microscope (Zeiss, Oberkochen, Germany).

## RESULTS

### Neural Cell Attachment to Different Matrices

The ECM was prepared from MSC or SB623 cells plated at different cell densities. Both MSC- and SB623 cell-derived ECM preparations appeared similar: they contained a network of fibers, which was denser where a higher density of cells was used to produce the ECM. The MSC-derived ECM is shown in Figure 1. (Some cell debris usually remained after cell removal.)

Rat embryonic (E18) brain cortical cells were plated onto the MSC-derived ECM in NB/B27/Glx. In parallel, the cells were also plated on PDL, a standard substrate for growing neurons from cortical dissociates in NB/B27/Glx. Within 1 hr after plating, cortical cells attached to both ECM and PDL as single cells. Several hours later, the difference in neurite outgrowth on these substrates became apparent. On the cell-derived ECM, neurites grew faster and tended to align with the matrix fibrils, whereas, on PDL, neurite outgrowth was slower and randomly directed (Fig. 2A–C). With time, a denser network of neurites was formed and more cells were growing on the ECM than on PDL (Fig. 2D–F). It was also noticeable that wells coated with the ECM produced by higher MSC densities had a more developed neurite network and/or more neural cells.

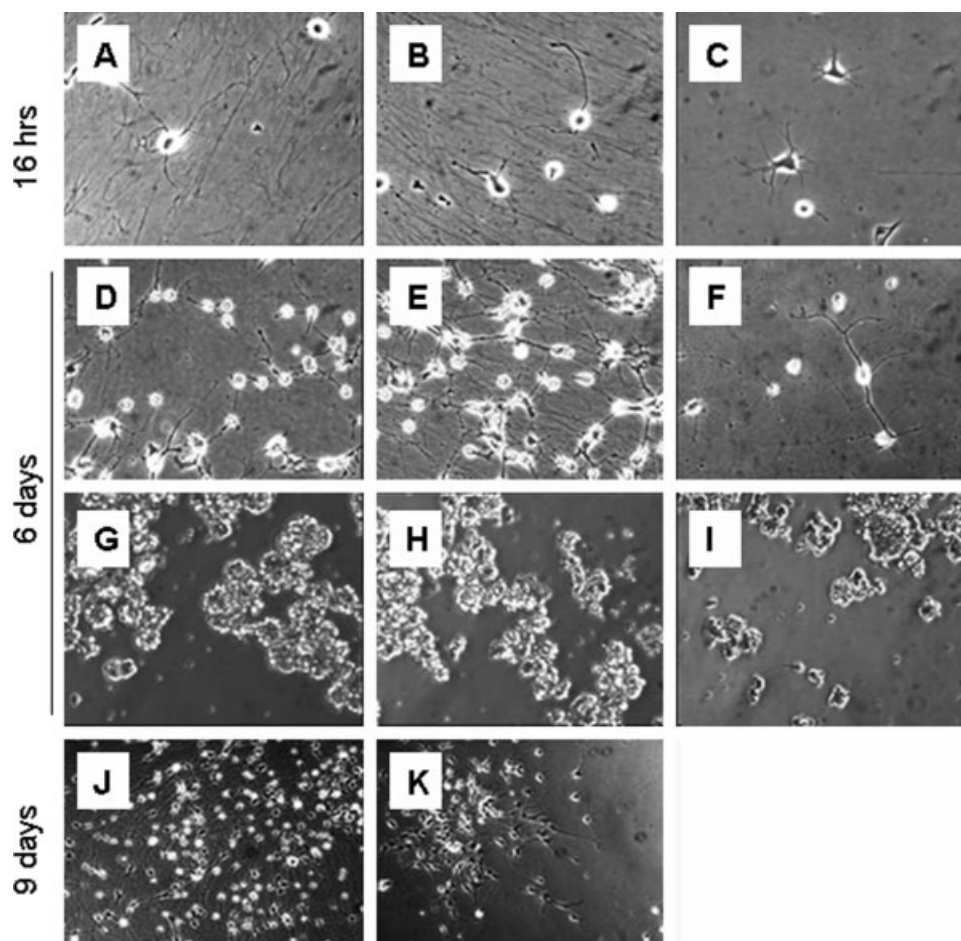


Fig. 2. Phase-contrast microphotographs of the attachment and growth of rat embryonic brain cortical cells on different matrices. Neural cells were plated on ECM produced by MSC plated either at  $2.5 \times 10^4$  cells/cm<sup>2</sup> (A,D,J) or at  $5 \times 10^4$  cells/cm<sup>2</sup> (B,E), on PDL (C,F), or on plastic coated with MSC growth medium containing 10% FBS (G), fibronectin (H), laminin (I), or Matrigel (K). Neural cells were cultured for 16 hr (A–C), 6 days (D–I), or 9 days (J,K). Note that longer cell processes aligned with the matrix fibrils on the

MSC-derived ECM, compared with the random shorter outgrowth on PDL (A,B vs. C), and the effect of more dense plating of MSC (E vs. D). Neural cells did not attach to wells coated with 10% FBS, fibronectin, or laminin. Cells grew on Matrigel, but the growth was focal, insofar as the initial formation of neurospheres preceded cell spreading. This contrasts with the even distribution of neural cells on the MSC-derived ECM (K vs. J). A–I:  $\times 400$ . J,K:  $\times 200$ .

To ensure that neural cells attached to the ECM and not to the adsorbed components of MSC growth medium that contained FBS, neural cell growth was tested in wells that were coated with the growth medium, fibronectin, or laminin. We also tested a Matrigel coating, because it has been reported to support neural tissue-like growth from cortical dissociates (Braun et al., 2006). There was no cell attachment to wells coated with fibronectin, laminin, or the growth medium when observed for as long as 6 days (Fig. 2G–I). In contrast, the cells had attached to ECM- and PDL-coated wells, where they had formed an apparent neurite network by that time (Fig. 2D–F). This result indicates that neural cells attached to MSC-produced ECM rather than to the components of the growth medium. Matrigel supported neural cell attachment; however, the nature of the cul-

ture growth was different from that observed on MSC-derived ECM or PDL. On MSC-derived ECM or PDL, neural cells initially attached to the substrate and started outgrowth as single cells. On Matrigel, homotypic aggregation preceded adhesion to the substrate, so that neurospheres formed (not shown), and outgrowth from them was noted several days later. As a result, the foci of neural cell growth were apparent 1 week after plating on Matrigel (Fig. 2K), whereas on the MSC-derived ECM and on PDL the neural cells were evenly distributed (Fig. 2J).

To test whether the soluble ECM components that accumulated in an MSC-conditioned medium can support neural cell attachment, the serum-free medium conditioned by confluent layers of cells for 2 days was coated on MaxiSorp plates (Nunc) for 2 hr. This

coating did not support neural cell attachment (not shown).

The morphology of neural cultures grown on the ECM derived from SB623 cells was very similar to that observed on the MSC-derived ECM (not shown). However, over time, the cultures grown on the SB623 cell-derived ECM had visibly more cells.

**Characterization of Neural Cell Growth on MSC- and SB623 Cell-Derived ECM**

To compare the ability of MSC-produced ECM to support neural growth with that of SB623-produced ECM, MSC and SB623 cells from the same donor were plated at decreasing cell densities in a 96-well plate. ECM-coated wells were prepared. Cortical cells were plated on the ECM- or on control PDL-coated wells in the same plate. After 5 days, the cultures were lysed, and an intracellular LDH activity test was performed on neural cell lysates. We found that the numbers of cells in neural cultures increased as increasing numbers of MSC or SB623 cells were used to produce the ECM (Fig. 3A). At a plating density of ECM-forming cells above  $1.5 \times 10^4$  cells/cm<sup>2</sup>, more neural cells were detected in ECM-based cultures than in PDL-based cultures. At higher cell plating densities, the ECM produced by SB623 cells supported cortical cell cultures better than the ECM produced by MSC. Based on these results, the optimal plating density for ECM production was determined to be  $3 \times 10^4$  cells/cm<sup>2</sup>. At higher plating densities, the stromal cell layers sometimes detached during the growth period.

Next, a time course of neural cell growth on the ECM produced by MSC and SB623 cells was established and compared with neural cell growth on PDL. MSC and SB623 cells from the same donor were plated at  $3 \times 10^4$  cells/cm<sup>2</sup>; the ECM was prepared and used as substrata for cortical cells grown for various times. The cell content of neural cultures grown on the ECM derived from either MSC or SB623 cells was higher than that of cultures grown on PDL, and the differences in cell numbers increased with time (Fig. 3B). By the end of the experiment, the neural cultures grown on the SB623 cell-derived ECM exhibited intracellular LDH activity that was two or three times higher than that of

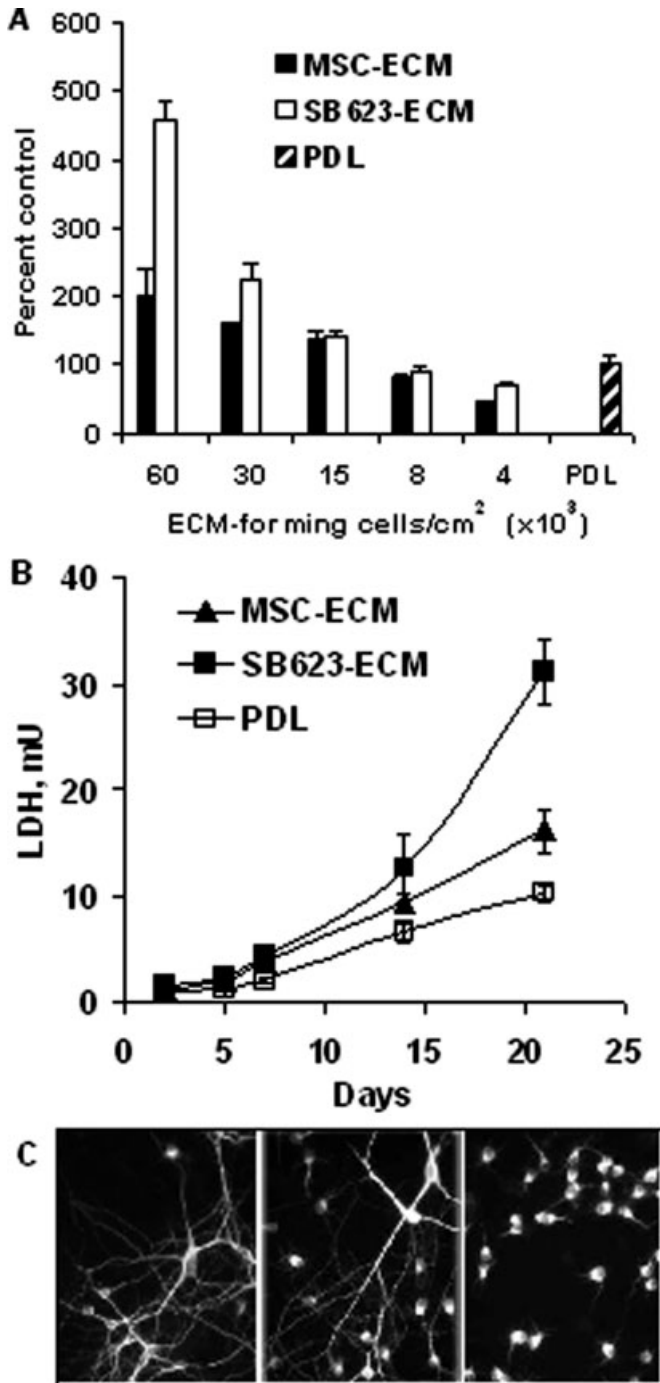


Fig. 3. Growth characteristics of the rat embryonic brain cortical cells on the MSC- and SB623 cell-derived ECM. **A:** Neural cell growth on the ECM produced by either MSC or SB623 cells plated at different densities. MSC (solid bars) and SB623 cells (open bars) from the same donor were plated in 96-well plate at decreasing cell densities. Neural cells were grown for 5 days, and intracellular LDH activity was measured and expressed as percentage control (PDL-coated wells). This is a representative result from four experiments with cells from four donors. Bars represent means of duplicate samples and SD. **B:** The time course of neural cell growth on ECM formed by MSC (triangles), by SB623 cells (solid squares) plated at  $3 \times 10^4$  cells/cm<sup>2</sup>, or on PDL (open squares). Neural cells were allowed to grow for various times, and the intracellular LDH activity was determined from five replicas/point and expressed as mean LDH activity/well  $\pm$  SD. This is a representative result from two experiments with cells from two donors. **C:** Better long-term survival of neurons on the MSC (left)- and SB623 (center)-derived ECM compared with that of the neurons on PDL (right) is demonstrated by MAP2 immunostaining on day 21. Note the prominent MAP2-positive neurite networks on the ECM from both MSC and SB623 and only residual neurite staining on PDL.  $\times 400$ .

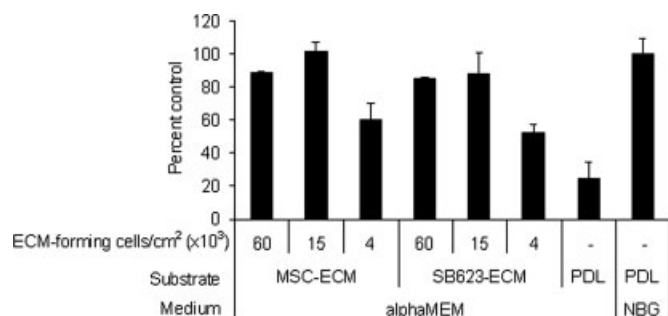


Fig. 4. MSC- and SB623 cell-derived ECM supports rat brain cortical cell survival in nonneural, supplement-free medium. Wells were coated with the ECM derived from MSC or SB623 cells plated at indicated densities or with PDL. Cortical cells were plated on ECM and PDL in  $\alpha$ MEM or, as a positive control, on PDL in NB/B27/Glx (abbreviated NBG on the graph). After 5 days, LDH activity of neural cell lysates was determined. Bars represent mean percent of LDH activity of the positive control, five replicas/point and SD. Representative results from two experiments with cells from two donors.

cultures grown on MSC-derived ECM or on PDL, respectively. By day 21 in culture, the rat brain cells on ECM continued to grow, forming a neural tissue-like layer, whereas the PDL-based cultures started to decline. The decline of the PDL culture was apparent from the decomposition of neurites, as illustrated by the disappearance of neurite-associated MAP2 staining (Fig. 3C).

We also tested whether the higher cell numbers in neural cultures on the ECM compared with those on PDL were due simply to better cell adhesion to the ECM. MSC and SB623 cells were plated at various cell densities to produce ECM. Neural cells were allowed to adhere for 1 hr to wells coated with ECM or with PDL or not coated at all. Nonadherent cells were then washed off the plate, and the remaining cells were quantified by intracellular LDH activity. No differences were found in neural cell adhesion to PDL or to either MSC- or SB623 cell-derived ECM formed by either  $1.5 \times 10^4$  cells/cm<sup>2</sup> or  $3 \times 10^4$  cells/cm<sup>2</sup>, and no LDH activity at all was detected in noncoated wells (results not shown). This indicates that the beneficial effects of the ECM for neural cell growth were not a result of better initial cell adherence but appeared at later stages of the development of neural cultures.

#### Cell-Derived ECM Supports Neural Cell Survival in a Nonneural Medium

We tested whether ECM could support neural cell growth in nonneural, supplement-free medium. When neural cells were plated in  $\alpha$ MEM on PDL, the 5-day survival was about 25% of that in NB/B27/Glx. The ECM coating produced by cells plated at various densities supported neural cell survival in  $\alpha$ MEM, and the ECM from  $1.5 \times 10^4$  or more cells per cm<sup>2</sup> increased the survival of neural cells in  $\alpha$ MEM to levels observed in cultures grown in NB/B27/Glx on PDL. The SB623

cell-derived ECM exhibited the same beneficial effect as did the MSC-derived ECM (Fig. 4).

#### Identification of Neural Cell Types in ECM-Based Cultures

To identify the neural cells growing on ECM, triple staining for neurons (anti-Map2), astrocytes (anti-GFAP), and nuclei (DAPI) was performed on cultures grown on the ECM or PDL for 12 days (Fig. 5A). Cultures grown on the ECM derived from either MSC or SB623 cells exhibited extensive GFAP staining, indicating the presence of large numbers of astrocytes. MAP2-positive neurons with prominent neurites were found growing among the astrocytes. In cultures grown on PDL, MAP2- but no GFAP-positive cells were observed, and the MAP2 staining was weaker. Later in the experiment (on days 18–21), CNPase, an early and specific oligodendrocyte marker, could be detected by immunostaining in some cells grown on ECM (Fig. 5B). At that point, PDL cultures were in decline, and no specific CNPase staining was detected (not shown).

The immunostaining of the cells on the first few days of ECM-based cultures usually did not reveal GFAP. However, GFAP-positive cells were clearly detected after 1 week in culture, indicating that at about this time astrocytes differentiated and proliferated extensively.

#### DISCUSSION

Several studies have shown the persistent beneficial effects of intracranial injections of MSC despite an observed low engraftment rate and poor persistence of grafts (Bang et al., 2005; Isakova et al., 2006). In fact, the data presented here may in part explain this phenomenon, because they demonstrate that neurosupport is provided by MSC and SB623 cells, yet it does not require living mesenchymal cells or cell-derived soluble factors. Instead, it depends solely on the cell-derived insoluble ECM left after the cells were removed. As we have shown, this ECM-mediated neurosupport is characterized by enhanced neurogenesis, growth of astroglia, oligodendrocyte differentiation, protection from nutrient and growth factor deprivation, and prolonged survival of cultures.

We have shown here that embryonic rat cortical dissociates plated at a relatively low density ( $1.5 \times 10^4$  cells/cm<sup>2</sup>) can produce well-developed three-dimensional cell structures composed of neurons and glia on a cell-derived ECM. The cell content of neural cultures grown on a cell-derived ECM is proportional to the number of ECM-forming cells and, correspondingly, to the density of the matrix. The limiting factor in effectiveness of ECM as a substrate for neural cell cultures is therefore the maximal number of ECM-forming cells that can be plated without the cell monolayer becoming detached. We observe similar numbers of neural cells adhering to the cell-derived ECM and to the PDL. After the initial adhesion, however, the neural cells grow differently on these two substrata. Rapidly growing neurites are aligned with the ECM fibrils as opposed to the slower and randomly directed neurite outgrowth on

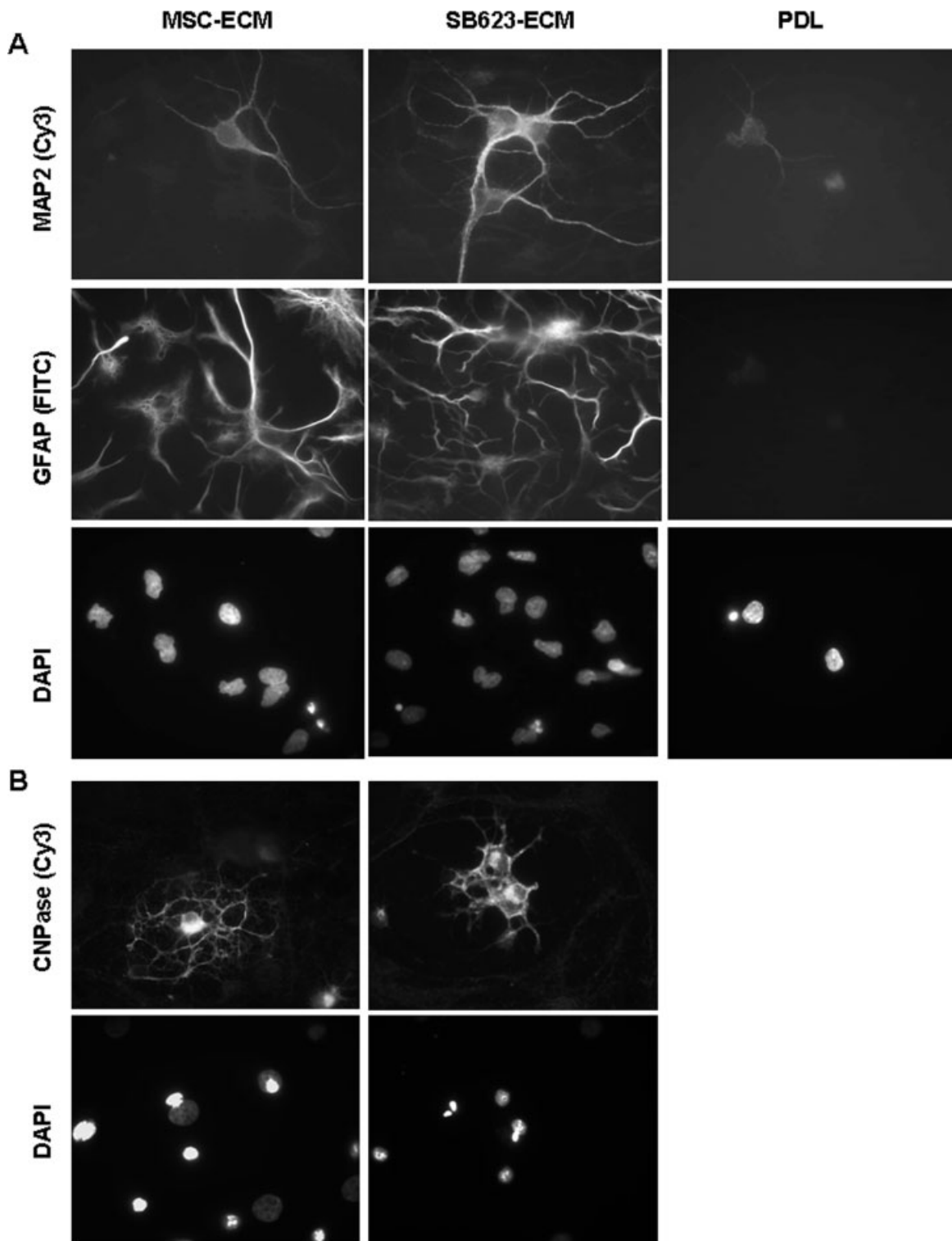


Fig. 5. Immunostaining identification of neural cell types on the cell-derived ECM. **A:** Double immunostaining of MAP2 and GFAP on day 12 of culturing of neural cells on MSC- or SB623 cell-derived ECM or on PDL. Fixed exposure times were used to photograph all MAP2 and all GFAP staining. Note that MAP2 staining is strongest on SB623 cell-derived ECM and weakest on PDL. ECM

supports growth and proliferation of GFAP-positive cells (astrocytes) and growth of MAP2-positive cells (neurons), whereas PDL supports only neuronal growth. **B:** The early marker of oligodendrocytes CNPase is detected in 21-day cultures grown on the ECM derived from MSC and SB623.  $\times 1,000$ .

PDL. Thus, after the initial adhesion, neural cell behavior indicates the presence of the ECM-associated signals that ultimately guide and promote neurite outgrowth. These signals could originate from the growth factors secreted by neural cells and preferentially adsorbed by certain ECM structures. This could result in trails of high local concentrations of growth factors and in the oriented growth of neurites. The accumulation of the neural cell-derived growth factors by elements of ECM could also explain the support of neural cell survival by cell-derived ECM in nonneural, supplement-free medium. An alternative explanation, that neurotogenic factors originate from ECM-forming cells, seems unlikely. Indeed, in their murine MSC-derived ECM preparations treated with 0.5% Triton X-100 and 20 mM NH<sub>4</sub>OH, Chen et al. (2007) were unable to detect BMP2, a cytokine known to be produced by MSC and strongly associated with the ECM. Our finding of neurotogenic properties of MSC-derived ECM is in line with a report showing that not only MSC cells but also their trails (left by migrating cells and presumably containing ECM material) can serve as bridges for neurites to cross over nerve-inhibitory substances (Wright et al., 2007).

We observe three beneficial effects of the MSC- or SB623 cell-derived ECM on neural cell cultures compared with other matrices tested. First, the cell-derived ECM supports the adhesion of neural cells, including neurons, astrocytes, and precursors of oligodendrocytes, as evident by the immunotyping of neural cells at later stages of cultures. Second, it strongly promotes neurite outgrowth immediately after neural cell plating. Third, the ECM promotes the proliferation of astrocytes in a serum-free medium, which provides further support to neurons growing on the ECM. As a result, the dynamic of the ECM-based cultures is very different from that observed on PDL (no astrocyte and oligodendrocyte growth, random neurogenesis) and on Matrigel. The cultures on Matrigel exhibit focal distribution of cells and retarded growth resulting from the initial formation of neurospheres, as described by Braun et al. (2006) and repeated in our study.

We demonstrate that the ECM derived from SB623 cells is more efficient in promoting neural cell growth than the ECM produced by their counterpart MSC. This is evident in the higher intracellular LDH activity of neural cultures grown on SB623 cell-derived ECM. More intense staining for the neuronal marker MAP2 in cultures grown on the SB623 cell-derived ECM also suggests its superior neurotogenic properties. Preliminary data, based on a microarray analysis, suggest differential expression between SB623 cells/parental MSC of multiple genes encoding ECM and enzymes remodeling ECM, including collagens, laminins, matrix metalloproteinases, tissue inhibitors of matrix metalloproteinase, tenascin, and fibulin. Further analysis should clarify whether these changes are responsible for the improved efficacy.

The production of large quantities of ECM is a hallmark of MSC (Wagner et al., 2005). In murine

MSC-derived ECM preparations purified using a protocol similar to ours, various ECM molecules have been detected in a detergent-insoluble fraction including collagen types I and III, syndecan-1, perlecan, fibronectin, laminin, and decorin, whereas collagen type V and biglycan are mostly removed (Chen et al., 2007). Obviously, the composition of the Triton X-100-insoluble fraction of ECM is much more complex, taking into account a wide variety of ECM and ECM-remodeling proteins expressed by MSC (Wagner et al., 2005). Further studies are needed to clarify the contribution of different ECM components in the observed supportive effects on different types of neural cells.

It is worth noting that the culture system described here may be helpful in distinguishing between soluble and insoluble neurosupportive factors produced by MSC and SB623. It may provide an *in vitro* assay for the efficiency of MSC- and SB623 cell-based therapy.

In summary, we have demonstrated *in vitro* that the cell-free ECM from MSC and SB623 cells supports the growth of neural cells and that the ECM produced by SB623 cells exhibits enhanced neurosupport compared with the parental MSC. We suggest that cell-derived ECM could be a mediator of neuroregenerative properties of MSC and SB623 cells observed *in vivo*. However, further studies are needed to explore the relationship among ECM composition, physical properties, and function *in vivo*.

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