Journal of Cellular Biochemistry 100:362–371 (2007)

Epigenetic Modifiers Promote Efficient Generation of Neural-Like Cells From Bone Marrow-Derived Mesenchymal Cells Grown in Neural Environment

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Abstract Understanding mechanisms that govern cell fate decisions will lead to developing techniques for induction of adult stem cell differentiation to desired cell outcomes and, thus, production of an autologos source of cells for regenerative medicine. Recently, we demonstrated that stem cells derived from adult central nervous system or bone marrow grown with other cell lineages or with more undifferentiated cells sometimes take on those characteristics. This indicates that manipulating extracellular factors may be sufficient to alter some developmental restrictions regulated by the epigenetic system. In this study, using pharmacological agents that interfere with the main components of the epigenetic program such as DNA methylation and histone deacetylation, we induce high-level expression of embryonic and neural stem cell (NSC) marker Sox2 in bone marrow-derived mesenchymal stem cells (MSCs). Exposure of these modified cells to a neural environment via juxtacrine and paracrine interactions promote efficient generation of neural stem-like cells as well as cells with neuronal and glial characteristics. We concluded that the manipulation strategy used in this study can be a useful method for efficient production of NSC-like cells from MSCs. J. Cell. Biochem. 100: 362–371, 2007.

Key words: epigenetic; bone marrow; mesenchymal stem cells; neural stem cells; neurons; glia

Adult stem cells have been shown to display a broader differentiation potential than anticipated and may contribute to tissues other than those in which they reside via processes designated as dedetermination and transdifferentiation [Bjornson et al., 1999; Brazelton et al., 2000; Theise et al., 2000; Jackson et al., 2001; Krause et al., 2001; Grant et al., 2002; Woodbury et al., 2002; Alexanian and Sieber-Blum, 2003; Camargo et al., 2003; Ianus et al., 2003; Kale et al., 2003; Weimann et al., 2003]. While some recent reports indicate that cell fusion events may account for such a high level of plasticity of somatic cells, other studies suggest that progressive restriction of cells during development is reversible and can be altered under the influence of extracellular factors [Alexanian and Nornes, 2001; Condorelli et al., 2001; Terada et al., 2002; Ying et al., 2002; Alexanian and Sieber-Blum, 2003; Cusella De Angelis et al., 2003; Spees et al., 2003; Alexanian and Kurpad, 2005]. However, these processes are inefficient, and some of the experimental claims have been difficult to reproduce. To alter the phenotype of cells in a rational way, molecular parameters that distinguish these different cell types must be modified. Epigenetic regulation of gene expression is recognized as a key mechanism governing cell determination, commitment, and differentiation as well as maintenance of those states. DNA methylation and histone deacetylation are the main components of an epigenetic program [Li, 2002; Jaenisch and Bird, 2003]. Disturbances of any of these components may shift the balance between an active and silent chromatin conformation, resulting in an altered transcriptional state. Pharmacological agents

Grant sponsor: VA Medical Research; Grant sponsor: Department of Neurosurgery at the Medical College of Wisconsin.

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Received 7 April 2006; Accepted 22 May 2006

DOI 10.1002/jcb.21029

that interfere with this system activate expression of many genes, including those required for maintenance of pluripotent or multipotent state of the cells [Kass et al., 1997; Sheikhnejad et al., 1999; Benjamin and Jost, 2001; Kohyama et al., 2001; Reik et al., 2001; Hattori et al., 2004; Milhem et al., 2004; Xu et al., 2004]. Thus, dedetermination or transdifferentiation of adult stem cells induced by an extracellular signaling system may be explained by still unknown extracellular factors that interfere (at least partly) with these diverse epigenetic mechanisms. In our recent studies, we have shown that stem cells derived from the adult central nervous system grown with other cell lineages or with more undifferentiated cells sometimes take on those characteristics, indicating that manipulating extracellular factors may be sufficient to alter some developmental restrictions regulated by the epigenetic system [Alexanian and Sieber-Blum, 2003; Alexanian and Kurpad, 2005]. Similarly, we have demonstrated that mesenchymal stem cells (MSCs) derived from mice bone marrow, grown with neural stem cells (NSCs), alive or fixed (with paraformaldehyde or methanol) generate neural stem-like cells, which eventually differentiated into cells with neuronal and glial characteristics [Alexanian, 2005]. All these studies indicated that adult NSCs and MSCs exhibit a much wider potential for differentiation and cell–cell interactions are important for such transformations.

In this study, we investigated whether manipulation of MSCs with epigenetic modifiers could enhance their efficiency to generate neural phenotypes when exposed to a neural environment via juxtacrine and paracrine interactions. The main idea underlying this experimental design was to change (in the first step) plasticity of the expanded MSCs by growing them in embryonic stem cell maintenance medium (ESCmm) supplemented with bFGF and EGF and containing epigenetic modifiers such as hypomethylating agent 5-aza-2′-deoxycytidine (5azadC) or deacetylating agent—histone deacetylase inhibitor Trichostatin A (TSA), or a combination of the two. At that stage, we studied the expression level of transcription factor Sox2, which is a marker for pluripotent embryonic stem cells, multipotent NSCs, and a critical determinant of neurogenesis [Graham et al., 2003; Ellis et al., 2004; Episkopou, 2005; Okumura-Nakanishi et al., 2005]. Second, by exposing these presumably modified cells to a NSC environment, we aimed to induce dedetermination of MSCs into neural stem-like cells. To this end, MSCs grown in neural induction medium (NIM) with or without fixed NSCs. Further, by growing these cells in Neurobasal A/B27 without mitogen bFGF, we intended to differentiate them into more mature neural phenotypes. Our results demonstrated that bone marrow-derived MSCs treated with inhibitors of methylation and deacetylation in ESCmm/bFGF/EGF exhibited a high expression of Sox2 and, in contrast to untreated MSCs, more efficiently generated neural stem-like cells and cells with neuronal and glial morphology when grown with or without fixed NSCs in NIM. Similar neural induction was achieved when MSCs were exposed simultaneously to epigenetic modifiers and NIM after expansion in ESCmm/bFGF/EGF.

MATERIALS AND METHODS

Mouse Bone Marrow-Derived MSC Culture

The method used to isolate and expand bone marrow-derived MSCs relied on differential adherence of MSCs to uncoated plastic dishes, in MesenCult Basal Media supplemented with MSC stimulatory supplements (MBMMSS) (Stem Cell Technologies, Vancouver, Canada) to eliminate most non-adherent hematopoietic cells. After sacrifice by CO_2 exposure and cervical dislocation of Balb/c and GFP transgenic mice, the femora and tibia were visualized and removed by dissecting away the muscles. Both ends of the long bones were cut with scissors so the marrow could be flushed. A 21- to 25-gauge needle (attached to a 3-ml syringe) was placed at the proximal end of the femora or tibia, and the marrow was flushed into a 15-ml sterile tube using 1–2 ml of Iscove's MDM medium containing 2% fetal bovine serum (FBS) (Stem Cell Technologies). After spinning down the marrow at 400*g*, the supernatant was discarded, and cells were washed with MBMMSS by centrifugation. The resulting pellets were resuspended in the same medium and plated in uncoated 6-well and 100-mm plastic dishes (300,000 cells/cm^2). Hematopoietic and non-adherent cells were removed by changes of medium. After 4 days, in half of the samples, the medium was replaced with ESCmm (15% FBS, 1 mM sodium pyruvate, 100 U·ml/100 μg/ml PenicillineG/Streptomycin,

2 mM glutamine, 0.1 mM non-essential amino acids, 20 ng/ml LIF, 100 μM MTG, high glucose DMEM) containing 25 ng/ml of bFGF and EGF. Next MSCs derived from GFP mice (GFP-MSCs) or from Balb/c mice (Balb/c-MSCs) were exposed to epigenetic modifiers and/or factors for neural induction in ESCmm/bFGF/EGF or MBMMSS or NIM: composed (33%/33%/33%v/v) from NS-A medium supplemented with N2 (NSA-N2), neural stem cell conditioned medium (NSCcm), and ESCmm/bFGF/EGF.

Exposing MSCs to Epigenetic Modifiers Such as 5azadC and TSA

Half of the samples in which MSCs grown in ESCmm/bFGF/EGF medium replaced with fresh ESCmm/bFGF/EGF and half with NIM containing pharmacological agents 1 μM 5azadC or 40 nM TSA or 1 μM 5azadC + 40 nM TSA. In cultures where MSCs grown in MBMMSS replaced with fresh MBMMSS containing 5azadC or TSA or 5azadC + TSA. Control samples were free from these pharmacological agents.

Analysis of Sox2 Expression by Western Blot

Preparation of nuclear extract. MSCs cultured on 10-cm plastic dishes in ESCmm/bFGF/EGF and in MBMMSS. These cultures free or containing 5azadC or TSA or 5azadC + TSA were used for preparation of nuclear extract. The cells were washed with PBS and harvested from the plate by scraping into 400 μl of cold buffer (50 mM TRIS, pH 7.5, 10 mM KCl, 0.5 mM EDTA, 1 mM EGTA, 1 mM DTT, and 10 μl/ml protease inhibitor cocktail). After incubating on ice for 15 min, 25 μl of 10% IGEPAL was added and the tubes were vigorously vortexed for 10 s. The lysed cells were centrifuged for 30 s in a microfuge (10,000*g*). The nuclear pellets were resuspended in 200 μl SDS buffer (1% SDS, 50 mM Tris-Cl pH 7.5, 5 mM DTT, 1 mM EGTA, 0.5 mM EDTA, 10 μl/ml protease inhibitor cocktail). After boiling and sonicating the nuclei, extracted protein was precipitated with chloroform/methanol [Wessel and Flugge, 1984]. The precipitate was dissolved in SDS sample buffer, and protein concentrations were determined by BCA method (kit from Pierce, Rockford, IL) and additionally, with a filter paper dye-binding assay (Analyt. Biochemistry 190, 66–70, 1990) [Minamide and Bamburg, 1990], which is not subjected to interference by common buffer components such as thiol-reducing agents, detergents, Tris, nucleic acids, and which is not subjected to variation due to pH differences.

SDS-polyacrylamide gel electrophoresis and Western blotting. Equal amounts of protein extracted from cell nuclei (30 μg) were resolved on 4–15% polyacrylamide gradient (Bio-Rad, Richmond, CA). For Western blotting, proteins were transferred to Nitrocellulose membrane using the transfer buffer of Towbin et al. [1979]. The equal loading was verified by staining the nitrocellulose membrane after protein transfer with Ponceau S stain. After blocking with 5% reconstituted dry milk in Tris-buffered saline and washing thoroughly between each step, blots were incubated with the primary antibody, mouse polyclonal Sox2 (1:1,000, Chemicon International, Inc., Temecula, CA). After incubation with HRP-conjugated secondary antibody (1:10,000, Pierce Chemical Company) the bands were detected using the chemiluminescent substrate SuperSignal West Dura or SuperSignal West Maximum Sensitivity Substrate (Pierce Chemical Company) and by capturing and digitizing the images with Kodak Image Station 2000 MMT. Quantification of bands was performed using Kodak 1D image analysis software. Similar results obtained from two different experiments, and relative levels of nuclear Sox2 were quantified from the represented image.

Isolation and Expansion of NSCs From Mouse Periventricular Subependymal Zone (SEZ)

Six- to 8-month-old mice (male or female of Balb/c) (The Jackson Laboratory, Bar Harbor, ME) were exposed to CO_2 then sacrificed by cervical dislocation. The brains were removed and placed in HibernateA/B27 medium (HibernateA, BrainBits LLC, Springfield, IL; B27, Life Technologies, Inc., Gaithersburg, MD). Two coronal cuts were made in the area between the rhinal fissure and hippocampus. The resulting tissue was laid on its posterior surface, and two parasagittal cuts were made just lateral to the lateral ventricles, and one horizontal cut was made at approximately the level of the corpus callosum. The tissue was cut into 1-mm pieces and digested for 30 min at 37°C in a solution of papain (2.5 U/ml; Worthington Biochemical Corp., Freehold, NJ) activated with cystein and EDTA, DNAase (250 U/ml, Worthington Biochemical Corp.) and natural protease (1 U/ml Dispase II,

Boehringer Mannheim, Indianapolis, IN) dissolved in HibernateA. After washing three times with HibernateA/10% FBS, slices were transferred to 15-ml tubes containing 3 ml of HibernateA/B27 at room temperature. Slices were triturated 10 times with a 1-ml polypropylene pipette tip and allowed to settle for 3 min. The resulting supernatant was transferred to another tube. The sediment was resuspended two more times in 3 ml HibernateA/B27, triturated 20 times, allowed to settle for 3 min, and the resulting supernatants were combined. The 6-ml cell suspension was then enriched and purified from debris by fractionation using a Percoll density gradient. Cells were washed twice by centrifugation in HibernateA/10% FBS and plated on poly-l-ornithine/laminin (PO/L)-coated plastic tissue culture dishes in chemically defined NS-A medium (Cedarlane Laboratories Ltd., Hornby, Canada) containing N2 supplement (Life Technologies) and 10% FBS. FGF2 (human recombinant, 20 ng/ml; Invitrogen, Carlsbad, CA) was added to the above medium. After 24 h, the medium was exchanged with NS-A medium supplemented with N2 containing 20 ng/ml bFGF. Spheres formed after 8–10 days, were harvested, collected by centrifugation (5 min, 500*g*), mechanically dissociated to a single-cell suspension, and plated on PO/L-coated dishes in NS-A/N2 + bFGF. NSCcm from these cultures was collected, filtered, and frizzed (−20°C) for further studies.

Fixation of NSCs for Fixed-NSC/MSCs Culture Studies

NSCs derived from mice SEZ were plated on PO/L-coated glass coverslips and grown for 7 days in NS-A medium supplemented with N2 and 20 ng/ml bFGF. Cells were subsequently fixed with cold methanol for 5 min. Fixed (killed) cells were washed extensively (five times, 3 min) with PBS and stored overnight with PBS at 4°C.

Protocols for Neural Induction and Immunocytochemistry

GFP-MSCs or Balb/c-MSCs that grown in ESCmm/bFGF/EGF in the absence or presence epigenetic modifiers were replated on PO/L-coated glass coverslips without or with fixed NSCs at (100,000 cells/cm^2) in NIM. To distinguish the descendents of MSCs from NSCs in culture, GFP-MSCs were cultured with fixed Balb/c-NSCs. After 1 week, cultures were fixed with 4% paraformaldehyde or replated on PO/L-coated dishes and grown for another 1 week in the same medium and an additional week in NeurobasalA/B27.

In another set of experiments, MSCs expended in ESCmm/bFGF/EGF (free from 5azadC and TSA) were replated on PO/L-coated glass coverslips and exposed to epigenetic modifiers in the same medium ESCmm/bFGF/EGF or in NIM. After 1 week, half of the samples were fixed for immunocytochemical studies and half were grown an additional week in Neurobasal/B27 before fixation.

For immunocytochemistry, cells were permeabilized (5 min with 1% BSA and 0.5% Triton X-100 in PBS) and incubated for 1 h with one of the following primary antibodies in the same solution (except for 10-fold less Triton X-100) monoclonal anti-nestin (1:500, Chemicon International, Inc.); polyclonal anti-Sox2 (1:800, Chemicon); polyclonal anti-GFAP (1:1,000, Novus Biologicals, Inc., Littleton, CO); monoclonal anti-GFAP (1:400, Sigma Aldrich, St. Louis, MO); monoclonal (mouse) anti-β-III-tubulin (B3T) (1:300; gift of A Frankfurter [Katsetos et al., 1994]); polyclonal anti-NCAM (1:500, Chemicon International, Inc.). Immunoreactive cells were visualized with AMCA-conjugated goat anti-mouse; Texas Red (TxR)-conjugated goat anti-mouse IgG or fluorescent-conjugated (FITC) goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). In some experiments, FITC-conjugated goat anti-mouse IgG and TxR-conjugated goat anti-rabbit IgG were used. Glass coverslips were then mounted in ProLong Antifade reagent (Molecular Probes) to retard fluorescence quenching and dried on microscope slides. A Nikon inverted microscope equipped with color digital camera (Spot II) was used to capture representative images. Metamorph software (Universal Imaging) was used for cell counts.

Incorporation of BrdU and Double Immunostaining With Anti-BrdU and Anti-Sox2

Proliferating cells in vitro were detected by BrdU (Sigma, B5002) incorporation after incubation with 10 μM BrdU in growth medium for 48 h. Cells on coverslips were rinsed with PBS and fixed in 4% paraformaldehyde in PBS. After washing, cell DNA was denaturated by immersing coverslips in 2 N hydrochloric acid for 30 min. The coverslips then washed

extensively in medium to restore a neutral pH. Monoclonal antibody against BrdU (1:1,000, Sigma) was used to detect incorporated BrdU. For double immunostaining the cells were incubated for 1 h at room temperature with mouse anti-BrdU and rabbit anti-Sox2. Immunoreactive cells were visualized with appropriate secondary antibodies. Glass coverslips were then mounted in ProLong Antifade reagent (Molecular Probes) to retard fluorescence quenching and dried on microscope slides.

RESULTS

To efficiently generate neural-like phenotypes from expended adherent MSCs derived from mice bone marrow, the following sequence of in vitro cellular manipulations has been conducted. First, cells derived from GFP or Balb/c mice bone marrow expended in MBMMSS in 6-well and 100-mm plastic dishes. This medium was used for expansion of mice MSCs that retain their ability to differentiate along various lineages upon the addition of the appropriate stimulatory supplements (Stem Cell Technologies, Inc.). After 4 days, in half of the samples, the medium was changed to ESCmm/bFGF/EGF. After 2 days these mediums changed with appropriate fresh mediums containing 1 μM 5azadC, or 40 nM TSA or both. Control samples were free from these pharmacological agents. After 2 days, cells grown in 100-mm plastic dishes were used for confirmation of Sox2 activation and translocation to the nucleus by quantitative Western blotting. The relative levels of nuclear Sox2 in nuclear extract of control cells grown in MBMMSS without or with 1 μM 5azadC or 40 nM TSA or 1μM 5azadC + 40 nM TSA for 48 h, were 1.0, 0.75, 1.12, and 1.2, respectively. (Fig. 1a–d). The relative level of Sox2 of cells grown in MBMMSS (without treatment) and cells grown in ESCmm for 48 h without or with 1 μM 5azadC or 1 μM 5azadC + 40 nM TSA were 1.0, 1.81, 1.97, and 3.53, respectively (Fig. 1a,e–g). These results demonstrated that the level of Sox2 expression in MSCs grown in MBMMSS was very low and epigenetic modifiers 5azadC, TSA, and 5azadC + TSA only slightly increased the expression of this transcription factor (Fig. 1a–d). MSCs grown in ESCmm/bFGF/EFG exhibited higher expression of Sox2 (in contrast to the cell grown in MBMMSS with or without epigenetic modifiers), and 5azadC or a combination of 5azadC with TSA augmented this expression (Fig. 1e–g). Therefore, further plasticity studies were conducted with the cells grown in ESCmm/bFGF/EGF with or without these pharmacological agents. To this end, MSCs grown in 6-well plastic dishes and treated for 2 days with epigenetic modifiers in ESCmm/bFGF/EGF were dissociated and replated on PLO/L-coated glass coverslips or on methanol-fixed NSCs (previously plated on PLO/L-coated glass coverslips and grown to 80% confluency) in NIM. After 1 week, MSCs which grown with methanol-fixed NSCs fixed with 4% paraformaldehyde or replated on PO/L and grown for 1 week in NIM and an additional week in NeurobasalA/B27. In the samples, where MSCs grown on PLO/L-coated glass coverslips (without fixed NSCs), also fixed with paraformaldehyde or grown for another week in Neurobasal A/B27 before paraformaldehyde fixation.

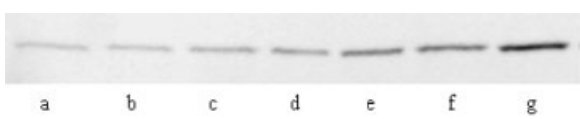

Fig. 1. Western blot analysis of Sox2 protein expression in 5azadC, TSA, and 5azadC + TSA-treated MSCs grown on plastic dishes in MBSSMM or ESCmm/bFGF/EGF. MSCs grown in MBMMSS expressed low levels of Sox2 (**a**) that increased with 5azadC (**b**), TSA (**c**), and 5azadC + TSA (**d**) treatment. MSCs grown in ESCmm/bFGF/EGF exhibited a higher expression of Sox2 in comparison to MSCs grown in MBSSMM with or without epigenetic modifiers (**e**). Treatment with 5azadC (**f**) and 5azadC + TSA (**g**) further augmented these expression.

Results demonstrated that MSCs that grown in NIM on the top of fixed NSCs produced clusters or neurosphere-like structures positive to Sox2 and nestin (Fig. 2a–d). GFP-positive cells in clusters mainly were positive to Sox2 and nestin that demonstrated with higher magnification (Fig. 2d). Counts from two experiments revealed in average nine GFP/nestin/Sox2-positive clusters (per cm^2) equal or bigger than 50 μm. However, MSCs generated 2.6 ± 0.86 and 2.7 ± 0.24 times more Sox2 and nestin-positive clusters if preliminary treated with 5azadC (Fig. 2e–h) or 5azadC + TSA (Fig. 2i–l), respectively. Interestingly, in these cultures some clusters (40%) were positive to NCAM and nestin (Fig. 2m–p), markers that co-expressed in neuronal progenitors. Incorporation of BrdU suggested that these cells undergo cell proliferation (Fig. 2q–t). After replating on PO/L-coated plastic dishes and grown in NIM for a week, these epigenetically modified and

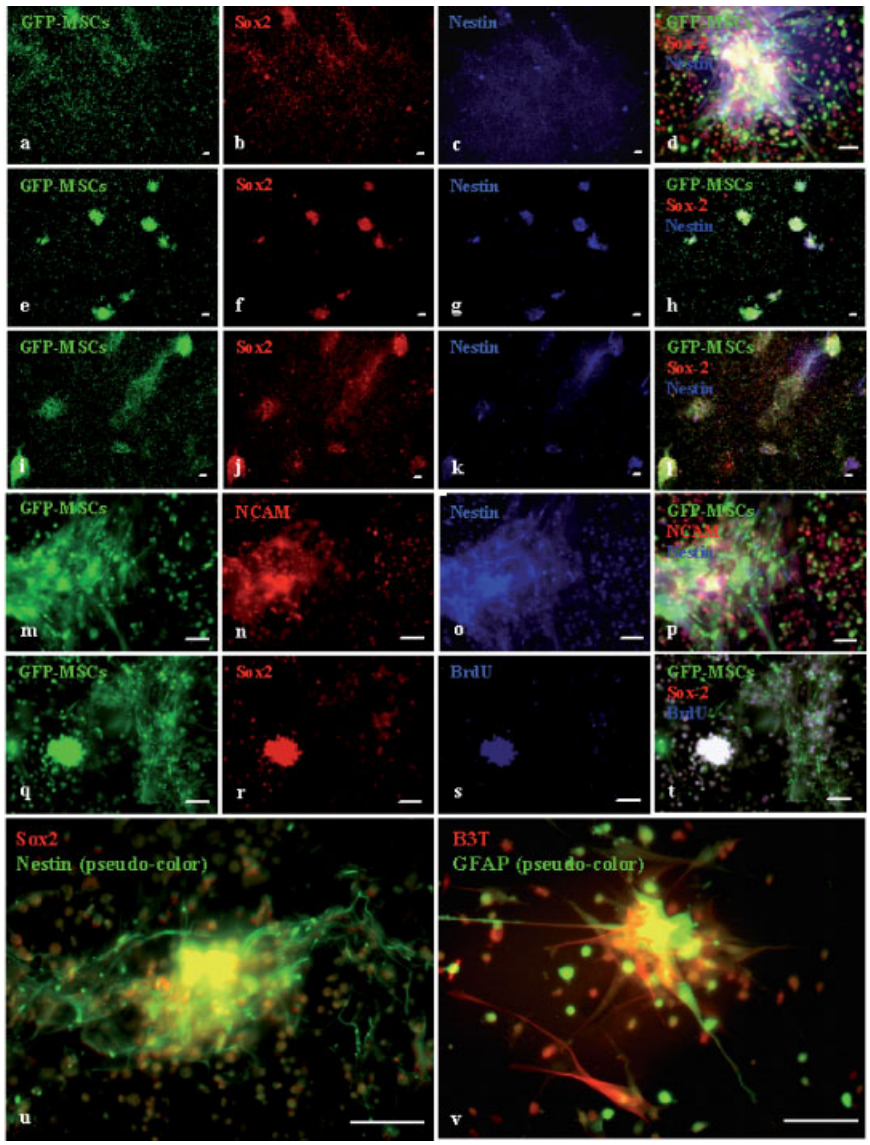

Fig. 2. Morphology and immunoreactivity of GFP-MSCs grown in NIM with methanol-fixed Balb/c-NSCs. MSCs grown in 6-well plastic dishes in ESCmm/bFGF/EGF in the absence or presence of epigenetic modifiers were dissociated and replated on methanol-fixed NSCs in NIM. To identify descendents of MSCs in co-culture, GFP mice-derived MSCs (GFP-MSCs) were co-cultured with Balb/c mice-derived fixed NSCs (Balb/c-NSCs). GFP-MSCs (untreated) grown for 1 week on glass coverslips with fixed NSCs in NIM produced Sox2, and a nestin immunopositive cluster of cells (**a–d**), that apparent with higher magnification (d). Under the same culture conditions, MSCs generated 2.0 ± 0.86 and 2.6 ± 0.24 times more Sox2 and nestin-positive clusters when preliminarily treated with 5azadC and 5azadC + TSA, respectively (**e–l**). Some clusters in these cultures co-expressed nestin and NCAM (**m–p**). Anti-BrdU immunostaining demonstrated that these Sox2-expressing clusters incorporated BrdU (**q–t**). These neurally induced MSCs generated neural stem-like cells when dissociated and replated on PO/L-coated glass coverslips in NIM (**u**). Growing these modified cells an additional week in Neurobasal A/B27 without bFGF produced neuronal and glial-like cells expressing B3T and GFAP (**v**). While nestin and GFAP immunostaining in (u,v) were visualized with a blue (AMCA-conjugated secondary antibody), green pseudo-color was used to demonstrate details in these images. Scale bars are 50 μm.

neurally induced MSCs produce similar Sox2 and nestin-positive cluster of cells (Fig. 2u) that eventually differentiated into B3T and GFAP-positive cells when grown an additional week in NeurobasalA/B27 (Fig. 2v). Epigenetically modified MSCs produce only a few (1–2 per cm^2) Sox2 and nestin-positive clusters when grown without fixed NSCs in the same culture condition. Immunostaining of MSCs (untreated or epigenetically modified) with other neural differentiation markers identified B3T and GFAP-positive cells with different morphology.

Thus, MSCs grown without fixed NSCs in NIM generated cells mainly expressing mature neural markers. In this condition, untreated MSCs exhibited fibroblast morphology, some had a compact round shape, and others had a strange morphology (Fig. 3a–c). In contrast, MSCs that preliminary treated with 5azadC or 5azadC + TSA produced B3T and GFAP immunopositive cells with some resemblance to neuronal and glial morphology (Fig. 3d–i). In 5azadC + TSA-treated cultures 70% of cells demonstrated a neural-like morphology.

In another set of experiments, MSCs expended in ESCmm/bFGF/EGF (free from 5azadC and TSA) were plated on PO/L-coated glass coverslips and exposed to epigenetic modifiers in NIM. The purpose of the experiment was to study the neural differentiation potential of MSCs when they were simultaneously subjected to epigenetic modifiers and factors for neural differentiation, such as NIM and substrate PO/L. To demonstrate the importance of NIM in this neural differentiation protocol, for some experiments, NIM was changed to ESCmm/bFGF/EGF. After 1 week, half of the samples were fixed for immunocytochemical studies and half grown an additional week in Neurobasal/B27 before fixation. MSCs that grown in ESCmm/bFGF/EGF with 5azadC generated cells with flat and multipolar morphology (Fig. 4a–c). MSCs grown in NIM with 5azadC produced B3T immunopositive cells with neuronal morphology (Fig. 4d–f). In NIM supplemented with 5azadC + TCS, MSCs differentiated into B3T and GFAP immunopositive cells with very similar neuronal and glial morphology (Fig. 4g–i). However, several cells co-expressed both markers. Cells became more differentiated, mainly expressing B3T or GFAP, when grown for another week in Neurobasal A/B27 without bFGF (Fig. 4j–l).

DISCUSSION

Strategies to manipulate stem cells for therapeutic applications are limited by our inability to efficiently control or predict stem cell fate decisions in response to exogenous stimuli. It has been suggested that epigenetic regulation plays an important role in governing stemness,

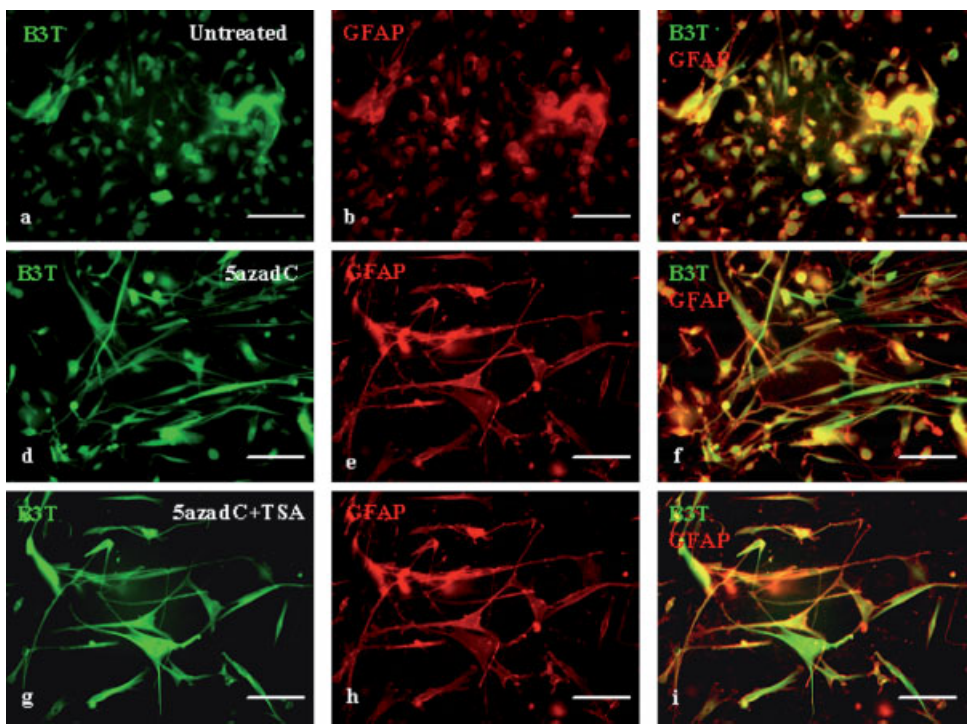

Fig. 3. Generation of neural-like cells from bone marrow-derived MSCs preliminarily treated with 5azadC or 5azadC + TSA. Balb/c-MSCs preliminarily expanded in ESCmm/bFGF/EGF (without epigenetic modifiers) grown on PO/L-coated glass coverslips in NIM gave rise B3T and GFAP-positive cells with fibroblastic and round shape morphology (**a–c**). Under the same culture conditions, MSCs produced B3T and GFAP-positive cells with neuronal and glial morphology when preliminary treated with 5azadC (**d–f**) or 5azadC + TSA (**g–i**). 5azadC + TSA-treated MSCs exhibited more differentiated morphology than cells treated with 5azadC. Scale bars are 50 μm.

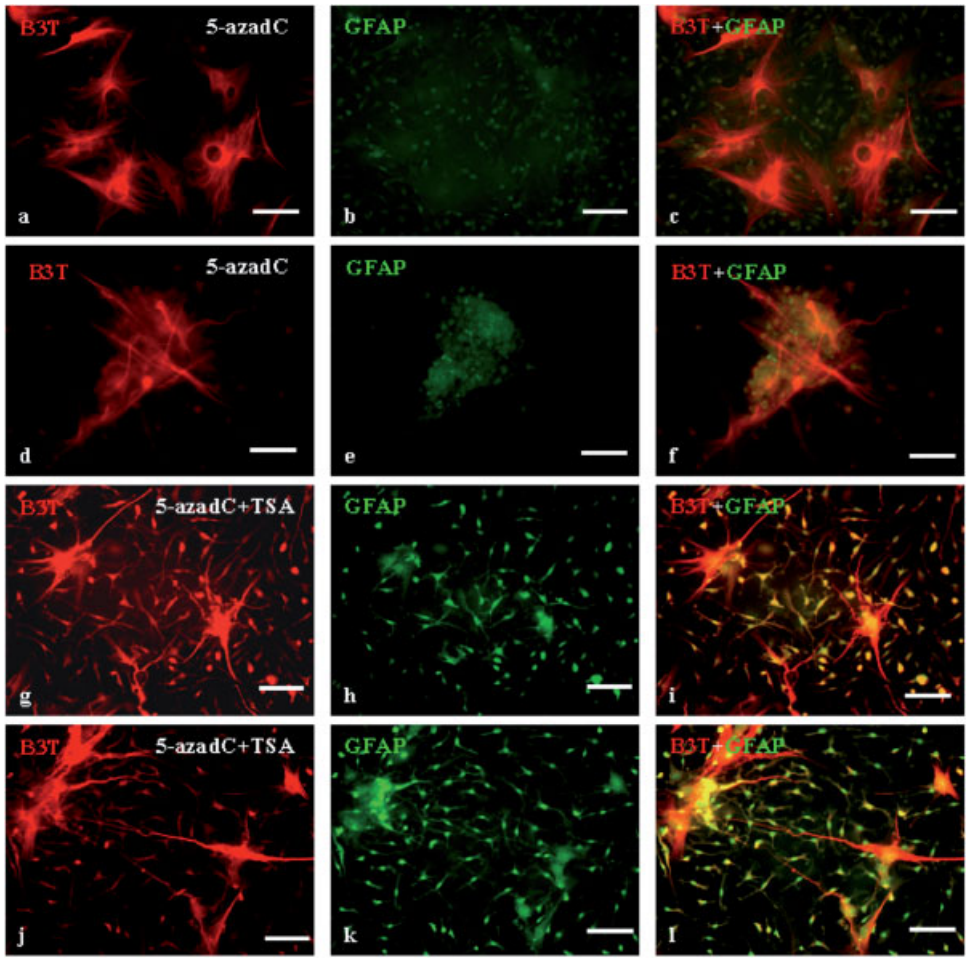

Fig. 4. Generation of cells with neuronal and glial morphology from MSCs grown with epigenetic modifiers in ESCmm/bFGF/EGF or NIM on PO/L-coated glass coverslips. Balb/c-MSCs grown on PO/L-coated glass coverslips in ESCmm/bFGF/EGF containing 5azadC produced B3T-expressing cells with flat multipolar or smooth muscle morphology (**a–c**). In NIM containing 5azadC, MSCs produce B3T immunopositive neuronal-like cells (**d–f**). In NIM that containing 5azadC + TSA, MSCs generated B3T and GFAP-positive cells with neuronal and glial morphology (**g–i**). The majority of the cells co-expressed these markers (i). Cells grown an additional week in Neurobasal A/B27 produced neuronal and glial-like cells with distinguishable B3T and GFAP expression (**j–l**). Scale bars are 50 μm.

commitment, differentiation, and maintenance of these states. DNA methylation, histone acetylation, and chromatin structure are involved in establishment of epigenetic modifications. Methylation marking system is the best understood epigenetic inheritance system. Inactive genes are often highly methylated, whereas the same genes may be transcribed if the methylation level is low. Thus, hypomethylation is the molecular mechanism by which previously silenced genes can be reactivated. Other then methylation marks, there exist other types of marks, involving DNA-associated proteins (histones) that affect gene activity and can also transmitted in cell lineages, and are maintained and reconstituted following DNA replication. In general, histone acetylation, through the action of histone acetylase, correlates with gene activation. Conversely deacetylation, via histone deacetylase correlates with gene silencing. Pharmacological agents (hypomethylating agent 5azadC and deacetylating agent TSA), which interfere with epigenetic inheritance system have dramatic effects on transcriptional regulation of many genes [Morgan et al., 2005]. These two classes of drugs in combination capable of synergistically reactivating developmentally silenced genes [Kass et al., 1997; Sheikhnejad et al., 1999; Benjamin and Jost, 2001], including those that essential for cell pluripotency [Hattori et al., 2004]. In this study, by exposing MSCs to

inhibitors of methylation and deacetylation or to combinatorial effects of these agents, we aimed to reverse the differentiated state of these cells into an immature pluripotent-like state in which they could exhibit higher differentiation potential. Our results demonstrated that treatment of MSCs with a DNA hypomethylating agent 5azadC, and with a deacetylating agent, TSA, or a combination of the two, induced an elevated expression of Sox2, transcription factor whose expression (in combination with other transcription factors) governs the fate of the primitive inner cell mass [Okumura-Nakanishi et al., 2005], embryonic neuroepithelial cells, and adult neural progenitors [Komitova and Eriksson, 2004]. Growing these preliminarily modified cells with fixed NSCs in NIM induced efficient production of neural stem-like cells (Sox2/nestin-positive) that eventually differentiated into neuronal and glial-like cells when grown in Neurobasal A/B27 without bFGF. Epigenetically modified MSCs produced cells with neuronal and glial-like characteristics when grown in NIM without fixed NSCs. Under this condition, MSCs produced only a few (1–2 cm^2) Sox2 and nestin-positive clusters. Similar results were obtained growing MSCs in NIM containing 5azadC or 5azadC + TSA. These results demonstrated that the combination of 5azadC and TSA most effectively increased the expression of Sox2 and yielded the highest numbers of neural stem-like clusters when MSCs were grown with fixed NSCs in NIM. In addition, 5azadC + TSA-treated MSCs grown without fixed NSCs generated cells that exhibited the most resemblance to neuronal and glial morphology. Thus, a high expression level of Sox2 induced by 5azadC + TSA treatment could be one of the important prerequisites for such plasticity. We concluded that the manipulation strategy used in this study can be a useful method for efficient production of NSC-like cells from MSCs. Further studies will be designed to investigate functional characteristics of these neuronal and glial-like cells in vitro and differentiation potential in vivo.

ACKNOWLEDGMENTS

This work was supported by VA Medical Research and the Department of Neurosurgery at the Medical College of Wisconsin. I thank Mara Bacon for technical assistance and Laura Roberts for critical reading.

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